IARC MONOGRAPHS

ASPARTAME, METHYLEUGENOL, AND ISOEUGENOL

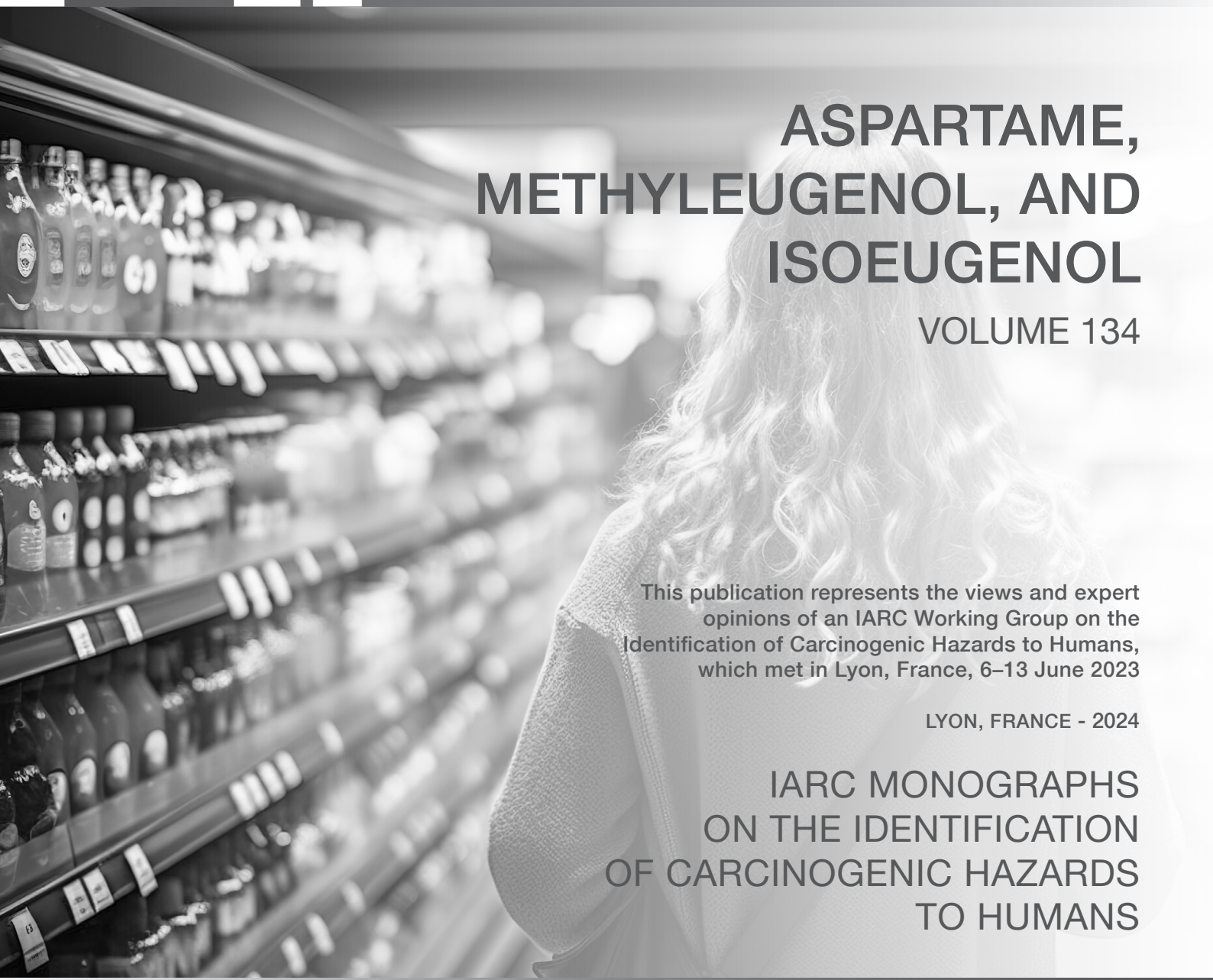
VOLUME 134

IARC MONOGRAPHS ON THE IDENTIFICATION OF CARCINOGENIC HAZARDS TO HUMANS

International Agency for Research on Cancer

World Health Organization

IARC MONOGRAPHS



ASPARTAME, METHYLEUGENOL, AND ISOEUGENOL

VOLUME 134

This publication represents the views and expert opinions of an IARC Working Group on the Identification of Carcinogenic Hazards to Humans, which met in Lyon, France, 6–13 June 2023

LYON, FRANCE - 2024

IARC MONOGRAPHS ON THE IDENTIFICATION OF CARCINOGENIC HAZARDS TO HUMANS

International Agency for Research on Cancer

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IARC MONOGRAPHS

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic hazard of chemicals to humans, involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic hazards associated with exposures to complex mixtures, lifestyle factors and biological and physical agents, as well as those in specific occupations. The objective of the programme is to elaborate and publish in the form of monographs critical reviews of data on carcinogenicity for agents to which humans are known to be exposed and on specific exposure situations; to evaluate these data in terms of cancer hazard to humans with the help of international working groups of experts in carcinogenesis and related fields; and to identify gaps in evidence. The lists of IARC evaluations are regularly updated and are available on the internet at https://monographs.iarc.who.int/.

This programme has been supported since 1982 by Cooperative Agreement U01 CA33193 with the United States National Cancer Institute, Department of Health and Human Services. Additional support has been provided since 1986 by the European Commission Directorate-General for Employment, Social Affairs, and Inclusion, initially by the Unit of Health, Safety and Hygiene at Work, and since 2014 by the European Union Programme for Employment and Social Innovation "EaSI" (for further information please consult: https://ec.europa.eu/social/easi). Support has also been provided since 1992 by the United States National Institute of Environmental Health Sciences, Department of Health and Human Services. The contents of this volume are solely the responsibility of the Working Group and do not necessarily represent the official views of the United States National Cancer Institute, the United States National Institute of Environmental Health Sciences, the United States Department of Health and Human Services, or the European Commission.

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About the cover: A wide range of beverages and food products sweetened with aspartame is available to the consumer.

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NOTE TO THE READER

The evaluations of carcinogenic hazard in the *IARC Monographs on the Identification of Carcinogenic Hazards to Humans* series are made by international working groups of independent scientists. The *IARC Monographs* classifications do not indicate the level of risk associated with a given level or circumstance of exposure. The *IARC Monographs* do not make recommendations for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic hazard of an agent to humans is encouraged to make this information available to the *IARC Monographs* programme, International Agency for Research on Cancer, 25 avenue Tony Garnier, CS 90627, 69366 Lyon Cedex 07, or via email at imo@iarc.who.int, in order that the agent may be considered for re-evaluation by a future Working Group.

Although every effort is made to prepare the monographs as accurately as possible, mistakes may occur. Readers are requested to communicate any errors to the *IARC Monographs* programme. Corrigenda are published online on the relevant webpage for the volume concerned (IARC Publications: https://publications.iarc.who.int/).

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[1] Working Group Members and Invited Specialists serve in their individual capacities as scientists and not as representatives of their government or any organization with which they are affiliated. Affiliations are provided for identification purposes only. Invited Specialists do not serve as Meeting Chair or Subgroup Chair, draft text that pertains to the description or interpretation of cancer data, or participate in the evaluations. Each participant was asked to declare potentially relevant research, employment, and financial interests that are current or that have occurred during the past 4 years. Minimal interests are not disclosed here, and include stock valued at no more than US$ 1000 overall, grants that provide no more than 5% of the research budget of the expert's organization and that do not support the expert's research or position, and consulting or speaking on matters not before a court or government agency that does not exceed 2% of total professional time or compensation. All other non-publicly funded grants that support the expert's research or position and all consulting or speaking on behalf of an interested party on matters before a court or government agency are disclosed as potentially significant conflicts of interests.

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[4] Each Observer agreed to respect the Guidelines for Observers at *IARC Monographs* meetings. Observers did not serve as Working Group members, draft any part of a monograph, or participate in the evaluations. They also agreed not to contact participants before the meeting, not to lobby them at any time, not to send them written materials, and not to offer them meals or other favours. IARC asked and reminded Working Group members to report any contact or attempt to influence that they may have encountered, either before or during the meeting.

[5] Dr Borghoff reported being a salaried employee of ToxStrategies LLC, which receives undisclosed (but presumed to be substantial) consulting fees from the American Beverage Association and the Calorie Control Council, and that the International Council of Beverages Associations sponsored her travel to and attendance at this IARC meeting.

[6] Dr Elmore reported being the owner of ElmorePathology LLC, which receives substantial consulting fees from the American Beverage Association, which also sponsored her travel to and attendance at this IARC meeting.

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[7] Dr Galligan reported being a salaried employee of the Center for Science in the Public Interest (CSPI). In this role, he has provided written comments on the safety of aspartame in response to a regulatory docket issued by the United States Department of Health and Human Services. CSPI has previously petitioned the United States Food and Drug Administration to prohibit the use of methyleugenol in food in the USA.
[8] Dr Magnuson reported being part owner of Health Science Consulting Inc. and President of BMagnuson Consulting, receiving consultancy fees from the Calorie Control Council for studies on aspartame toxicity. The Calorie Control Council sponsored her travel to and attendance at this IARC meeting.
[9] Ms Voge reported being a salaried employee and stockholder of Whole Earth Brands, which uses sweeteners being evaluated at this IARC meeting.

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PREAMBLE

The Preamble to the *IARC Monographs* describes the objective and scope of the programme, general principles and procedures, and scientific review and evaluations. The *IARC Monographs* embody principles of scientific rigour, impartial evaluation, transparency, and consistency. The Preamble should be consulted when reading a *Monograph* or a summary of a *Monograph*'s evaluations. Separate Instructions for Authors describe the operational procedures for the preparation and publication of a volume of the *Monographs*.

A. GENERAL PRINCIPLES AND PROCEDURES

1. Background

Soon after the International Agency for Research on Cancer (IARC) was established in 1965, it started to receive frequent requests for advice on the carcinogenicity of chemicals, including requests for lists of established and suspected human carcinogens. In 1970, an IARC Advisory Committee on Environmental Carcinogenesis recommended "that a compendium on carcinogenic chemicals be prepared by experts. The biological activity and evaluation of practical importance to public health should be referenced and documented." The next year, the IARC Governing Council adopted a resolution that IARC should prepare "monographs on the evaluation of carcinogenic risk of chemicals to man", which became the initial title of the series.

In succeeding years, the scope of the programme broadened as *Monographs* were developed for complex mixtures, occupational exposures, physical agents, biological organisms, pharmaceuticals, and other exposures. In 1988, "of chemicals" was dropped from the title, and in 2019, "evaluation of carcinogenic risks" became "identification of carcinogenic hazards", in line with the objective of the programme.

Identifying the causes of human cancer is the first step in cancer prevention. The identification of a cancer hazard may have broad and profound implications. National and international authorities and organizations can and do use information on causes of cancer in support of actions to reduce exposure to carcinogens in the workplace, in the environment, and elsewhere. Cancer prevention is needed as much today as it was when IARC was established, because the global burden of cancer is high and continues to increase as a result of population growth and ageing and upward trends in some exposures, especially in low- and middle-income countries (https://publications.iarc.who.int/Non-Series-Publications/World-Cancer-Reports).

IARC's process for developing *Monographs*, which has evolved over several decades, involves

the engagement of international, interdisciplinary Working Groups of expert scientists, the transparent synthesis of different streams of evidence (exposure characterization, cancer in humans, cancer in experimental animals, and mechanisms of carcinogenesis), and the integration of these streams of evidence into an overall evaluation and classification according to criteria developed and refined by IARC. Since the *Monographs* programme was established, the understanding of carcinogenesis has greatly deepened. Scientific advances are incorporated into the evaluation methodology. In particular, strong mechanistic evidence has had an increasing role in the overall evaluations since 1991.

The Preamble is primarily a statement of the general principles and procedures used in developing a *Monograph*, to promote transparency and consistency across *Monographs* evaluations. In addition, IARC provides Instructions for Authors (https://monographs.iarc.who.int/preamble-instructions-for-authors/), which specify more detailed working procedures. IARC routinely updates these Instructions for Authors to reflect advances in methods for cancer hazard identification and accumulated experience, including input from experts.

2. Objective and scope

The objective of the programme is to prepare, with the engagement of international, interdisciplinary Working Groups of experts, scientific reviews and evaluations of evidence on the carcinogenicity of a wide range of agents.

The *Monographs* assess the strength of the available evidence that an agent can cause cancer in humans, based on three streams of evidence: on cancer in humans (see Part B, Section 2), on cancer in experimental animals (see Part B, Section 3), and on mechanistic evidence (see Part B, Section 4). In addition, the exposure to each agent is characterized (see Part B, Section 1). In this Preamble, the term "agent" refers to any chemical, physical, or biological entity or exposure circumstance (e.g. occupation as a painter) for which evidence on the carcinogenicity is evaluated.

A cancer *hazard* is an agent that is capable of causing cancer, whereas a cancer *risk* is an estimate of the probability that cancer will occur given some level of exposure to a cancer hazard. The *Monographs* assess the strength of evidence that an agent is a cancer hazard. The distinction between hazard and risk is fundamental. The *Monographs* identify cancer hazards even when risks appear to be low in some exposure scenarios. This is because the exposure may be widespread at low levels, and because exposure levels in many populations are not known or documented.

Although the *Monographs* programme has focused on hazard identification, some epidemiological studies used to identify a cancer hazard are also used to estimate an exposure–response relationship within the range of the available data. However, extrapolating exposure–response relationships beyond the available data (e.g. to lower exposures, or from experimental animals to humans) is outside the scope of *Monographs* Working Groups (IARC, 2014). In addition, the *Monographs* programme does not review quantitative risk characterizations developed by other health agencies.

The identification of a cancer hazard should trigger some action to protect public health, either directly as a result of the hazard identification or through the conduct of a risk assessment. Although such actions are outside the scope of the programme, the *Monographs* are used by national and international authorities and organizations to inform risk assessments, formulate decisions about preventive measures, motivate effective cancer control programmes, and choose among options for public health decisions. *Monographs* evaluations are only one part of the body of information on which decisions to control exposure to carcinogens may be based.

Options to prevent cancer vary from one situation to another and across geographical regions and take many factors into account, including different national priorities. Therefore, no recommendations are given in the *Monographs* with regard to regulation, legislation, or other policy approaches, which are the responsibility of individual governments or organizations. The *Monographs* programme also does not make research recommendations. However, it is important to note that *Monographs* contribute significantly to the science of carcinogenesis by synthesizing and integrating streams of evidence about carcinogenicity and pointing to critical gaps in knowledge.

3. Selection of agents for review

Since 1984, about every five years IARC convenes an international, interdisciplinary Advisory Group to recommend agents for review by the *Monographs* programme. IARC selects Advisory Group members who are knowledgeable about current research on carcinogens and public health priorities. Before an Advisory Group meets, IARC solicits nominations of agents from scientists and government agencies worldwide. Since 2003, IARC also invites nominations from the public. IARC charges each Advisory Group with reviewing nominations, evaluating exposure and hazard potential, and preparing a report that documents the Advisory Group's process for these activities and its rationale for the recommendations.

For each new volume of the *Monographs*, IARC selects the agents for review from those recommended by the most recent Advisory Group, considering the availability of pertinent research studies and current public health priorities. On occasion, IARC may select other agents if there is a need to rapidly evaluate an emerging carcinogenic hazard or an urgent need to re-evaluate a previous classification. All evaluations consider the full body of available evidence, not just information published after a previous review.

A *Monograph* may review:

(a) An agent not reviewed in a previous *Monograph*, if there is potential human exposure and there is evidence for assessing its carcinogenicity. A group of related agents (e.g. metal compounds) may be reviewed together if there is evidence for assessing carcinogenicity for one or more members of the group.

(b) An agent reviewed in a previous *Monograph*, if there is new evidence of cancer in humans or in experimental animals, or mechanistic evidence to warrant re-evaluation of the classification. In the interests of efficiency, the literature searches may build on previous comprehensive searches.

(c) An agent that has been established to be carcinogenic to humans and has been reviewed in a previous *Monograph*, if there is new evidence of cancer in humans that indicates new tumour sites where there might be a causal association. In the interests of efficiency, the review may focus on these new tumour sites.

4. The Working Group and other meeting participants

Five categories of participants can be present at *Monographs* meetings:

(i) *Working Group* members are responsible for all scientific reviews and evaluations developed in the volume of the *Monographs*. The Working Group is interdisciplinary and comprises subgroups of experts in the fields of (a) exposure characterization, (b) cancer in humans, (c) cancer in experimental animals, and (d) mechanistic evidence. IARC selects Working Group members on the basis of expertise related to the subject matter and relevant methodologies, and absence

of conflicts of interest. Consideration is also given to diversity in scientific approaches and views, as well as demographic composition. Working Group members generally have published research related to the exposure or carcinogenicity of the agents being reviewed, and IARC uses literature searches to identify most experts. Since 2006, IARC also has encouraged public nominations through its Call for Experts. IARC's reliance on experts with knowledge of the subject matter and/or expertise in methodological assessment is confirmed by decades of experience documenting that there is value in specialized expertise and that the overwhelming majority of Working Group members are committed to the objective evaluation of scientific evidence and not to the narrow advancement of their own research results or a pre-determined outcome (Wild and Cogliano, 2011). Working Group members are expected to serve the public health mission of IARC, and should refrain from consulting and other activities for financial gain that are related to the agents under review, or the use of inside information from the meeting, until the full volume of the *Monographs* is published.

IARC identifies, from among Working Group members, individuals to serve as Meeting Chair and Subgroup Chairs. At the opening of the meeting, the Working Group is asked to endorse the selection of the Meeting Chair, with the opportunity to propose alternatives. The Meeting Chair and Subgroup Chairs take a leading role at all stages of the review process (see Part A, Section 7), promote open scientific discussions that involve all Working Group members in accordance with normal committee procedures, and ensure adherence to the Preamble.

(ii) *Invited Specialists* are experts who have critical knowledge and experience but who also have a conflict of interest that warrants exclusion from developing or influencing the evaluations of carcinogenicity. Invited Specialists do not draft any section of the *Monograph* that pertains to the description or interpretation of cancer data, and they do not participate in the evaluations. These experts are invited in limited numbers when necessary to assist the Working Group by contributing their unique knowledge and experience to the discussions.

(iii) *Representatives of national and international health agencies* may attend because their agencies are interested in the subject of the meeting. They do not draft any section of the *Monograph* or participate in the evaluations.

(iv) *Observers* with relevant scientific credentials may be admitted in limited numbers. Attention is given to the balance of Observers from constituencies with differing perspectives. Observers are invited to observe the meeting and should not attempt to influence it, and they agree to respect the Guidelines for Observers at *IARC Monographs* meetings. Observers do not draft any section of the *Monograph* or participate in the evaluations.

(v) The *IARC Secretariat* consists of scientists who are designated by IARC and who have relevant expertise. The IARC Secretariat coordinates and facilitates all aspects of the evaluation and ensures adherence to the Preamble throughout development of the scientific reviews and classifications (see Part A, Sections 5 and 6). The IARC Secretariat organizes and announces the meeting, identifies and recruits the Working Group members, and assesses the declared interests of all meeting participants. The IARC Secretariat supports the activities of the Working Group (see Part A, Section 7) by searching the literature and performing title and abstract screening, organizing conference calls to coordinate the development of pre-meeting

Table 1 Roles of participants at *IARC Monographs* meetings

Category of participant	Role			
	Prepare text, tables, and analyses	Participate in discussions	Participate in evaluations	Eligible to serve as Chair
Working Group members	✓	✓	✓	✓
Invited Specialists	✓[a]	✓		
Representatives of health agencies		✓[b]		
Observers		✓[b]		
IARC Secretariat	✓[c]	✓	✓[d]	

[a] Only for the section on exposure characterization.
[b] Only at times designated by the Meeting Chair and Subgroup Chairs.
[c] When needed or requested by the Meeting Chair and Subgroup Chairs.
[d] Only for clarifying or interpreting the Preamble.

drafts and discuss cross-cutting issues, and reviewing drafts before and during the meeting. Members of the IARC Secretariat serve as meeting rapporteurs, assist the Meeting Chair and Subgroup Chairs in facilitating all discussions, and may draft text or tables when designated by the Meeting Chair and Subgroup Chairs. Their participation in the evaluations is restricted to the role of clarifying or interpreting the Preamble.

All participants are listed, with their principal affiliations, in the front matter of the published volume of the *Monographs*. Working Group members and Invited Specialists serve as individual scientists and not as representatives of any organization, government, or industry (Cogliano et al., 2004).

The roles of the meeting participants are summarized in Table 1.

5. Working procedures

A separate Working Group is responsible for developing each volume of the *Monographs*. A volume contains one or more *Monographs*, which can cover either a single agent or several related agents. Approximately one year before the meeting of a Working Group, a preliminary list of agents to be reviewed, together with a Call for Data and a Call for Experts, is announced on the *Monographs* programme website (https://monographs.iarc.who.int/).

Before a meeting invitation is extended, each potential participant, including the IARC Secretariat, completes the WHO Declaration of Interests form to report financial interests, employment and consulting (including remuneration for serving as an expert witness), individual and institutional research support, and non-financial interests such as public statements and positions related to the subject of the meeting. IARC assesses the declared interests to determine whether there is a conflict that warrants any limitation on participation (see Table 2).

Approximately two months before a *Monographs* meeting, IARC publishes the names and affiliations of all meeting participants together with a summary of declared interests, in the interests of transparency and to provide an opportunity for undeclared conflicts of interest to be brought to IARC's attention. It is not acceptable for Observers or third parties to contact other participants before a meeting or to lobby them at any time. Meeting participants are asked to report all such contacts to IARC (Cogliano et al., 2005).

The Working Group meets at IARC for approximately eight days to discuss and finalize the scientific review and to develop summaries

Table 2 Public engagement during *Monographs* development

Approximate timeframe	Engagement
Every 5 years	IARC convenes an Advisory Group to recommend high-priority agents for future review
~1 year before a *Monographs* meeting	IARC selects agents for review in a new volume of the *Monographs* IARC posts on its website: Preliminary List of Agents to be reviewed Call for Data and Call for Experts Request for Observer Status WHO Declaration of Interests form
~8 months before a *Monographs* meeting	Call for Experts closes
~4 months before a *Monographs* meeting	Request for Observer Status closes
~2 months before a *Monographs* meeting	IARC posts the names of all meeting participants together with a summary of declared interests, and a statement discouraging contact of the Working Group by interested parties
~1 month before a *Monographs* meeting	Call for Data closes
~2–4 weeks after a *Monographs* meeting	IARC publishes a summary of evaluations and key supporting evidence
~9 months after a *Monographs* meeting	IARC Secretariat publishes the verified and edited master copy of plenary drafts as a *Monographs* volume

and evaluations. At the opening of the meeting, all participants update their Declaration of Interests forms, which are then reviewed by IARC. Declared interests related to the subject of the meeting are disclosed to the meeting participants during the meeting and in the published volume (Cogliano et al., 2004). The objectives of the meeting are peer review and consensus. During the first part of the meeting, subgroup sessions (covering exposure characterization, cancer in humans, cancer in experimental animals, and mechanistic evidence) review the pre-meeting drafts, develop a joint subgroup draft, and draft subgroup summaries. During the last part of the meeting, the Working Group meets in plenary session to review the subgroup drafts and summaries and to develop the consensus evaluations. As a result, the entire volume is the joint product of the Working Group, and there are no individually authored sections. After the meeting, the master copy is verified by the IARC Secretariat and is then edited and prepared for publication. The aim is to publish the volume within approximately nine months of the Working Group meeting. A summary of the evaluations and key supporting evidence is prepared for publication in a scientific journal or is made available on the *Monographs* programme website soon after the meeting.

In the interests of transparency, IARC engages with the public throughout the process, as summarized in Table 2.

6. Overview of the scientific review and evaluation process

The Working Group considers all pertinent epidemiological studies, cancer bioassays in experimental animals, and mechanistic evidence, as well as pertinent information on exposure in humans. In general, for cancer in humans, cancer in experimental animals, and mechanistic evidence, only studies that have been published or accepted for publication in the openly available scientific literature are reviewed. Under some circumstances, materials that are publicly available and whose content is final may be reviewed if there is sufficient information to permit an evaluation of the quality of the methods and results of the studies (see Step 1,

below). Such materials may include reports and databases publicly available from government agencies, as well as doctoral theses. The reliance on published and publicly available studies promotes transparency and protects against citation of premature information.

The principles of systematic review are applied to the identification, screening, synthesis, and evaluation of the evidence related to cancer in humans, cancer in experimental animals, and mechanistic evidence (as described in Part B, Sections 2–4 and as detailed in the Instructions for Authors). Each *Monograph* specifies or references information on the conduct of the literature searches, including search terms and inclusion/exclusion criteria that were used for each stream of evidence.

In brief, the steps of the review process are as follows:

Step 1. Comprehensive and transparent identification of the relevant information: The IARC Secretariat identifies relevant studies through initial comprehensive searches of literature contained in authoritative biomedical databases (e.g. PubMed, PubChem) and through a Call for Data. These literature searches, designed in consultation with a librarian and other technical experts, address whether the agent causes cancer in humans, causes cancer in experimental systems, and/or exhibits key characteristics of established human carcinogens (in humans or in experimental systems). The Working Group provides input and advice to IARC to refine the search strategies, and identifies literature through other searches (e.g. from reference lists of past *Monographs*, retrieved articles, and other authoritative reviews).

For certain types of agents (e.g. regulated pesticides and pharmaceuticals), IARC also provides an opportunity to relevant regulatory authorities, and regulated parties through such authorities, to make pertinent unpublished studies publicly available by the date specified in the Call for Data. Consideration of such studies by the Working Group is dependent on the public availability of sufficient information to permit an independent evaluation of (a) whether there has been selective reporting (e.g. on outcomes, or from a larger set of conducted studies); (b) study quality (e.g. design, methodology, and reporting of results), and (c) study results.

Step 2. Screening, selection, and organization of the studies: The IARC Secretariat screens the retrieved literature for inclusion based on title and abstract review, according to pre-defined exclusion criteria. For instance, studies may be excluded if they were not about the agent (or a metabolite of the agent), or if they reported no original data on epidemiological or toxicological end-points (e.g. review articles). The Working Group reviews the title and abstract screening done by IARC, and performs full-text review. Any reasons for exclusion are recorded, and included studies are organized according to factors pertinent to the considerations described in Part B, Sections 2–4 (e.g. design, species, and endpoint). Inclusion of a study does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results.

Step 3. Evaluation of study quality: The Working Group evaluates the quality of the included studies based on the considerations (e.g. design, methodology, and reporting of results) described in Part B, Sections 2–4. Based on these considerations, the Working Group may accord greater weight to some of the included studies. Interpretation of the results and the strengths and limitations of a study are clearly outlined in square brackets at the end of study descriptions (see Part B).

Step 4: *Report characteristics of included studies, including assessment of study*

quality: Pertinent characteristics and results of included studies are reviewed and succinctly described, as detailed in Part B, Sections 1–4. Tabulation of data may facilitate this reporting. This step may be iterative with Step 3.

Step 5: *Synthesis and evaluation of strength of evidence*: The Working Group summarizes the overall strengths and limitations of the evidence from the individual streams of evidence (cancer in humans, cancer in experimental animals, and mechanistic evidence; see Part B, Section 5). The Working Group then evaluates the strength of evidence from each stream of evidence by using the transparent methods and defined descriptive terms given in Part B, Sections 6a–c. The Working Group then develops, and describes the rationale for, the consensus classification of carcinogenicity that integrates the conclusions about the strength of evidence from studies of cancer in humans, studies of cancer in experimental animals, and mechanistic evidence (see Part B, Section 6d).

7. Responsibilities of the Working Group

The Working Group is responsible for identifying and evaluating the relevant studies and developing the scientific reviews and evaluations for a volume of the *Monographs*. The IARC Secretariat supports these activities of the Working Group (see Part A, Section 4). Briefly, the Working Group's tasks in developing the evaluation are, in sequence:

(i) Before the meeting, the Working Group ascertains that all appropriate studies have been identified and selected, and assesses the methods and quality of each individual study, as outlined above (see Part A, Section 6). The Working Group members prepare pre-meeting working drafts that present accurate tabular or textual summaries of informative studies by extracting key elements of the study design and results, and highlighting notable strengths and limitations. They participate in conference calls organized by IARC to coordinate the development of working drafts and to discuss cross-cutting issues. Pre-meeting reviews of all working drafts are generally performed by two or more subgroup members who did not participate in study identification, data extraction, or study review for the draft. Each study summary is written or reviewed by someone who is not associated with the study.

(ii) At the meeting, within subgroups, the Working Group members critically review, discuss, and revise the pre-meeting drafts and adopt the revised versions as consensus subgroup drafts. Subgroup Chairs ensure that someone who is not associated with the study leads the discussion of each study summary. A proposed classification of the strength of the evidence reviewed in the subgroup using the *IARC Monographs* criteria (see Part B, Sections 6a–c) is then developed from the consensus subgroup drafts of the evidence summaries (see Part B, Section 5).

(iii) During the plenary session, each subgroup presents its drafts for scientific review and discussion to the other Working Group members, who did not participate in study identification, data extraction, or study review for the drafts. Subgroup Chairs ensure that someone who is not associated with the study leads the discussion of each study summary. After review, discussion, and revisions as needed, the subgroup drafts are adopted as a consensus Working Group product. The summaries and classifications of the strength of the evidence, developed in the subgroup in line with the *IARC Monographs* criteria

(see Part B, Sections 6a–c), are considered, revised as needed, and adopted by the full Working Group. The Meeting Chair proposes an overall evaluation using the guidance provided in Part B, Section 6d.

The Working Group strives to achieve consensus evaluations. Consensus reflects broad agreement among the Working Group, but not necessarily unanimity. The Meeting Chair may poll the Working Group to determine the diversity of scientific opinion on issues where consensus is not apparent.

Only the final product of the plenary session represents the views and expert opinions of the Working Group. The entire *Monographs* volume is the joint product of the Working Group and represents an extensive and thorough peer review of the body of evidence (individual studies, synthesis, and evaluation) by an interdisciplinary expert group. Initial working papers and subsequent revisions are not released, because they would give an incomplete and possibly misleading impression of the consensus developed by the Working Group over a full week of deliberation.

B. SCIENTIFIC REVIEW AND EVALUATION

This part of the Preamble discusses the types of evidence that are considered and summarized in each section of a *Monograph*, followed by the scientific criteria that guide the evaluations. In addition, a section of General Remarks at the front of the volume discusses the reasons the agents were scheduled for evaluation and any key issues encountered during the meeting.

1. Exposure characterization

This section identifies the agent and describes its occurrence, main uses, and production locations and volumes, where relevant. It also summarizes the prevalence, concentrations in relevant studies, and relevant routes of exposure in humans worldwide. Methods of exposure measurement and analysis are described, and methods of exposure assessment used in key epidemiological studies reviewed by the Working Group are described and evaluated.

Over the course of the *Monographs* programme, concepts of exposure and dose have evolved substantially with deepening understanding of the interactions of agents and biological systems. The concept of exposure has broadened and become more holistic, extending beyond chemical, physical, and biological agents to stressors as construed generally, including psychosocial stressors (National Research Council, 2012; National Academies of Sciences, Engineering, and Medicine, 2017). Overall, this broader conceptualization supports greater integration between exposure characterization and other sections of the *Monographs*. Concepts of absorption, distribution, metabolism, and excretion are considered in the first subsection of mechanistic evidence (see Part B, Section 4a), whereas validated biomarkers of internal exposure or metabolites that are routinely used for exposure assessment are reported on in this section (see Part B, Section 1b).

(a) Identification of the agent

The agent being evaluated is unambiguously identified. Details will vary depending on the type of agent but will generally include physical and chemical properties relevant to the agent's identification, occurrence, and biological activity. If the material that has been tested in experimental animals or in vitro systems is different from that to which humans are exposed, these differences are noted.

For chemical agents, the Chemical Abstracts Service Registry Number is provided, as well as the latest primary name and other names in common use, including important trade names,

along with available information on the composition of common mixtures or products containing the agent, and potentially toxic and/or carcinogenic impurities. Physical properties relevant to understanding the potential for human exposure and measures of exposure used in studies in humans are summarized. These might include physical state, volatility, aqueous and fat solubility, and half-life in the environment and/or in human tissues.

For biological agents, taxonomy and structure are described. Mode of replication, life-cycle, target cells, persistence, latency, and host responses, including morbidity and mortality through pathologies other than cancer, are also presented.

For foreign bodies, fibres and particles, composition, size range, relative dimensions, and accumulation, persistence, and clearance in target organs are summarized. Physical agents that are forms of radiation are described in terms of frequency spectrum and energy transmission.

Exposures may result from, or be influenced by, a diverse range of social and environmental factors, including components of diet, sleep, and physical activity patterns. In these instances, this section will include a description of the agent, its variability across human populations, and its composition or characteristics relevant to understanding its potential carcinogenic hazard to humans and to evaluating exposure assessments in epidemiological studies.

(b) Detection and analysis

Key methods of detection and quantification of the agent are presented, with an emphasis on those used most widely in surveillance, regulation, and epidemiological studies. Measurement methods for sample matrices that are deemed important sources of human exposure (e.g. air, drinking-water, food, residential dust) and for validated exposure biomarkers (e.g. the agent or its metabolites in human blood, urine, or saliva) are described. Information on detection and quantification limits is provided when it is available and is useful for interpreting studies in humans and in experimental animals. This is not an exhaustive treatise but is meant to help readers understand the strengths and limitations of the available exposure data and of the epidemiological studies that rely on these measurements.

(c) Production and use

Historical and geographical patterns and trends in production and use are included when they are available, to help readers understand the contexts in which exposures may occur, both within key epidemiological studies reviewed by the Working Group and in human populations generally. Industries that produce, use, or dispose of the agent are described, including their global distribution, when available. National or international listing as a high-production-volume chemical or similar classification may be included. Production processes with significant potential for occupational exposure or environmental pollution are indicated. Trends in global production volumes, technologies, and other data relevant to understanding exposure potential are summarized. Minor or historical uses with significant exposure potential or with particular relevance to key epidemiological studies are included. Particular effort may be directed towards finding data on production in low- and middle-income countries, where rapid economic development may lead to higher exposures than those in high-income countries.

(d) Exposure

A concise overview of quantitative information on sources, prevalence, and levels of exposure in humans is provided. Representative data from research studies, government reports and websites, online databases, and other citable, publicly available sources are tabulated. Data

from low- and middle-income countries are sought and included to the extent feasible; information gaps for key regions are noted. Naturally occurring sources of exposure, if any, are noted. Primary exposure routes (e.g. inhalation, ingestion, skin uptake) and other considerations relevant to understanding the potential for cancer hazard from exposure to the agent are reported.

For occupational settings, information on exposure prevalence and levels (e.g. in air or human tissues) is reported by industry, occupation, region, and other characteristics (e.g. process, task) where feasible. Information on historical exposure trends, protection measures to limit exposure, and potential co-exposures to other carcinogenic agents in workplaces is provided when available.

For non-occupational settings, the occurrence of the agent is described with environmental monitoring or surveillance data. Information on exposure prevalence and levels (e.g. concentrations in human tissues) as well as exposure from and/or concentrations in food and beverages, consumer products, consumption practices, and personal microenvironments is reported by region and other relevant characteristics. Particular importance is placed on describing exposures in life stages or in states of disease or nutrition that may involve greater exposure or susceptibility.

Current exposures are of primary interest; however, information on historical exposure trends is provided when available. Historical exposures may be relevant for interpreting epidemiological studies, and when agents are persistent or have long-term effects. Information gaps for important time periods are noted. Exposure data that are not deemed to have high relevance to human exposure are generally not considered.

(e) Regulations and guidelines

Regulations or guidelines that have been established for the agent (e.g. occupational exposure limits, maximum permitted levels in foods and water, pesticide registrations) are described in brief to provide context about government efforts to limit exposure; these may be tabulated if they are informative for the interpretation of existing or historical exposure levels. Information on applicable populations, specific agents concerned, basis for regulation (e.g. human health risk, environmental considerations), and timing of implementation may be noted. National and international bans on production, use, and trade are also indicated.

This section aims to include major or illustrative regulations and may not be comprehensive, because of the complexity and range of regulatory processes worldwide. An absence of information on regulatory status should not be taken to imply that a given country or region lacks exposure to, or regulations on exposure to, the agent.

(f) Critical review of exposure assessment in key epidemiological studies

Epidemiological studies evaluate cancer hazard by comparing outcomes across differently exposed groups. Therefore, the type and quality of the exposure assessment methods used are key considerations when interpreting study findings for hazard identification. This section summarizes and critically reviews the exposure assessment methods used in the individual epidemiological studies that contribute data relevant to the *Monographs* evaluation.

Although there is no standard set of criteria for evaluating the quality of exposure assessment methods across all possible agents, some concepts are universally relevant. Regardless of the agent, all exposures have two principal dimensions: intensity (sometimes defined as concentration or dose) and time. Time considerations include

duration (time from first to last exposure), pattern or frequency (whether continuous or intermittent), and windows of susceptibility. This section considers how each of the key epidemiological studies characterizes these dimensions. Interpretation of exposure information may also be informed by consideration of mechanistic evidence (e.g. as described in Part B, Section 4a), including the processes of absorption, distribution, metabolism, and excretion.

Exposure intensity and time in epidemiological studies can be characterized by using environmental or biological monitoring data, records from workplaces or other sources, expert assessments, modelled exposures, job-exposure matrices, and subject or proxy reports via questionnaires or interviews. Investigators use these data sources and methods individually or in combination to assign levels or values of an exposure metric (which may be quantitative, semi-quantitative, or qualitative) to members of the population under study.

In collaboration with the Working Group members reviewing human studies (of cancer and of mechanisms), key epidemiological studies are identified. For each selected study, the exposure assessment approach, along with its strengths and limitations, is summarized using text and tables. Working Group members identify concerns about exposure assessment methods and their impacts on overall quality for each study reviewed (see Part B, Sections 2d and 4d). In situations where the information provided in the study is inadequate to properly consider the exposure assessment, this is indicated. When adequate information is available, the likely direction of bias due to error in exposure measurement, including misclassification (overestimated effects, underestimated effects, or unknown) is discussed.

2. Studies of cancer in humans

This section includes all pertinent epidemiological studies (see Part B, Section 2b) that include cancer as an outcome. These studies encompass certain types of biomarker studies, for example, studies with biomarkers as exposure metrics (see Part B, Section 2) or those evaluating histological or tumour subtypes and molecular signatures in tumours consistent with a given exposure (Alexandrov et al., 2016). Studies that evaluate early biological effect biomarkers are reviewed in Part B, Section 4.

(a) Types of study considered

Several types of epidemiological studies contribute to the assessment of carcinogenicity in humans; they typically include cohort studies (including variants such as case–cohort and nested case–control studies), case–control studies, ecological studies, and intervention studies. Rarely, results from randomized trials may be available. Exceptionally, case reports and case series of cancer in humans may also be reviewed. In addition to these designs, innovations in epidemiology allow for many other variants that may be considered in any given *Monographs* evaluation.

Cohort and case–control studies typically have the capacity to relate individual exposures under study to the occurrence of cancer in individuals, and provide an estimate of effect (such as relative risk) as the main measure of association. Well-conducted cohort and case–control studies provide most of the evidence of cancer in humans evaluated by Working Groups. Intervention studies are much less common, but when available can provide strong evidence for making causal inferences.

In ecological studies, the units of investigation are usually whole populations (e.g. in particular geographical areas or at particular times), and cancer frequency is related to a summary

measure of the exposure in the population under study. In ecological studies, data on individual exposure and outcome are not available, which renders this type of study more prone to confounding and exposure misclassification. In some circumstances, however, ecological studies may be informative, especially when the unit of exposure is most accurately measured at the population level (see, for example, the *Monograph* on arsenic in drinking-water; IARC, 2004).

Exceptionally, case reports and case series may provide compelling evidence about the carcinogenicity of an agent. In fact, many of the early discoveries of occupational cancer hazards came about because of observations by workers and their clinicians, who noted a high frequency of cancer in workers who share a common occupation or exposure. Such observations may be the starting point for more structured investigations, but in exceptional circumstances, when the risk is high enough, the case series may in itself provide compelling evidence. This would be especially warranted in situations where the exposure circumstance is fairly unusual, as it was in the example of plants containing aristolochic acid (IARC, 2012a).

The uncertainties that surround the interpretation of case reports, case series, and ecological studies typically make them inadequate, except in rare instances as described above, to form the sole basis for inferring a causal relationship. However, when considered together with cohort and case–control studies, these types of study may support the judgement that a causal relationship exists.

Epidemiological studies of benign neoplasms, pre-neoplastic lesions, malignant precursors, and other end-points are also reviewed when they relate to the agents reviewed. On occasion they can strengthen inferences drawn from studies of cancer itself. For example, benign brain tumours may share common risk factors with those that are malignant, and benign neoplasms (or those of uncertain behaviour) may be part of the causal path to malignancies (e.g. myelodysplastic syndromes, which may progress to acute myeloid leukaemia).

(b) Identification of eligible studies of cancer in humans

Relevant studies of cancer in humans are identified by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below. Eligible studies include all studies in humans of exposure to the agent of interest with cancer as an outcome. Multiple publications on the same study population are identified so that the number of independent studies is accurately represented. Multiple publications may result, for example, from successive follow-ups of a single cohort, from analyses focused on different aspects of an exposure–disease association, or from inclusion of overlapping populations. Usually in such situations, only the most recent, most comprehensive, or most informative report is reviewed in detail.

(c) Assessment of study quality and informativeness

Epidemiological studies are potentially susceptible to several different sources of error, summarized briefly below. Qualities of individual studies that address these issues are also described below.

Study quality is assessed as part of the structured expert review process undertaken by the Working Group. A key aspect of quality assessment is consideration of the possible roles of chance and bias in the interpretation of epidemiological studies. Chance, which is also called random variation, can produce misleading study results. This variability in study results is strongly influenced by the sample size: smaller studies are more likely than larger studies to have effect estimates that are imprecise. Confidence intervals

around a study's point estimate of effect are used routinely to indicate the range of values of the estimate that could easily be produced by chance alone.

Bias is the effect of factors in study design or conduct that lead an association to erroneously appear stronger or weaker than the association that really exists between the agent and the disease. Biases that require consideration are varied but are usually categorized as selection bias, information bias (e.g. error in measurement of exposure and diseases), and confounding (or confounding bias) (Rothman et al., 2008). Selection bias in an epidemiological study occurs when inclusion of participants from the eligible population or their follow-up in the study is influenced by their exposure or their outcome (usually disease occurrence). Under these conditions, the measure of association found in the study will not accurately reflect the association that would otherwise have been found in the eligible population (Hernán et al., 2004). Information bias results from inaccuracy in exposure or outcome measurement. Both can cause an association between hypothesized cause and effect to appear stronger or weaker than it really is. Confounding is a mixing of extraneous effects with the effects of interest (Rothman et al., 2008). An association between the purported causal factor and another factor that is associated with an increase or decrease in incidence of disease can lead to a spurious association or absence of a real association of the presumed causal factor with the disease. When either of these occurs, confounding is present.

In assessing study quality, the Working Group consistently considers the following aspects:

- **Study description:** Clarity in describing the study design and its implementation, and the completeness of reporting of all other key information about the study and its results.
- **Study population:** Whether the study population was appropriate for evaluating the association between the agent and cancer. Whether the study was designed and carried out to minimize selection bias. Cancer cases in the study population must have been identified in a way that was independent of the exposure of interest, and exposure assessed in a way that was not related to disease (outcome) status. In these respects, completeness of recruitment into the study from the population of interest and completeness of follow-up for the outcome are essential measures.
- **Outcome measurement:** The appropriateness of the cancer outcome measure (e.g. mortality vs incidence) for the agent and cancer type under consideration, outcome ascertainment methodology, and the extent to which outcome misclassification may have led to bias in the measure(s) of association.
- **Exposure measurement:** The adequacy of the methods used to assess exposure to the agent, and the likelihood (and direction) of bias in the measure(s) of association due to error in exposure measurement, including misclassification (as described in Part B, Section 1f).
- **Assessment of potential confounding:** To what extent the authors took into account in the study design and analysis other variables (including co-exposures, as described in Part B, Section 1d) that can influence the risk of disease and may have been related to the exposure of interest. Important sources of potential confounding by such variables should have been addressed either in the design of the study, such as by matching or restriction, or in the analysis, by statistical adjustment. In some instances, where direct information on confounders is unavailable, use of indirect methods to evaluate the potential impact of confounding on exposure–disease associations is appropriate (e.g. Axelson and Steenland, 1988; Richardson et al., 2014).

- **Other potential sources of bias:** Each epidemiological study is unique in its study population, its design, its data collection, and, consequently, its potential biases. All possible sources of bias are considered for their possible impact on the results. The possibility of reporting bias (i.e. selective reporting of some results and the suppression of others) should be explored.
- **Statistical methodology:** Adequacy of the statistical methods used and their ability to obtain unbiased estimates of exposure–outcome associations, confidence intervals, and test statistics for the significance of measures of association. Appropriateness of methods used to investigate confounding, including adjusting for matching when necessary and avoiding treatment of probable mediating variables as confounders. Detailed analyses of cancer risks in relation to summary measures of exposure such as cumulative exposure, or temporal variables such as age at first exposure or time since first exposure, are reviewed and summarized when available.

For the sake of economy and simplicity, in this Preamble the list of possible sources of error is referred to with the phrase "chance, bias, and confounding", but it should be recognized that this phrase encompasses a comprehensive set of concerns pertaining to study quality.

These sources of error do not constitute and should not be used as a formal checklist of indicators of study quality. The judgement of experienced experts is critical in determining how much weight to assign to different issues in considering how all of these potential sources of error should be integrated and how to rate the potential for error related to each of these considerations.

The informativeness of a study is its ability to show a true association, if there is one, between the agent and cancer, and the lack of an association, if no association exists. Key determinants of informativeness include: having a study population of sufficient size to obtain precise estimates of effect; sufficient elapsed time from exposure to measurement of outcome for an effect, if present, to be observable; presence of an adequate exposure contrast (intensity, frequency, and/or duration); biologically relevant definitions of exposure; and relevant and well-defined time windows for exposure and outcome.

(d) Meta-analyses and pooled analyses

Independent epidemiological studies of the same agent may lead to inconsistent results that are difficult to interpret or reconcile. Combined analyses of data from multiple studies may be conducted as a means to address this ambiguity. There are two types of combined analysis. The first involves combining summary statistics such as relative risks from individual studies (meta-analysis), and the second involves a pooled analysis of the raw data from the individual studies (pooled analysis) (Greenland and O'Rourke, 2008).

The strengths of combined analyses are increased precision because of increased sample size and, in the case of pooled analyses, the opportunity to better control for potential confounders and to explore in more detail interactions and modifying effects that may explain heterogeneity among studies. A disadvantage of combined analyses is the possible lack of comparability of data from various studies, because of differences in population characteristics, subject recruitment, procedures of data collection, methods of measurement, and effects of unmeasured covariates that may differ among studies. These differences in study methods and quality can influence results of either meta-analyses or pooled analyses. If published meta-analyses are to be considered by the Working Group, their adequacy needs to be carefully evaluated, including the methods used to identify eligible studies

and the accuracy of data extracted from the individual studies.

The Working Group may conduct ad hoc meta-analyses during the course of a *Monographs* meeting, when there are sufficient studies of an exposure–outcome association to contribute to the Working Group's assessment of the association. The results of such unpublished original calculations, which would be specified in the text by presentation in square brackets, might involve updates of previously conducted analyses that incorporate the results of more recent studies, or de novo analyses.

Irrespective of the source of data for the meta-analyses and pooled analyses, the following key considerations apply: the same criteria for data quality must be applied as for individual studies; sources of heterogeneity among studies must be carefully considered; and the possibility of publication bias should be explored.

(e) Considerations in assessing the body of epidemiological evidence

The ability of the body of epidemiological evidence to inform the Working Group about the carcinogenicity of the agent is related to both the quantity and the quality of the evidence. There is no formulaic answer to the question of how many studies of cancer in humans are needed from which to draw inferences about causality, although more than a single study in a single population will almost always be needed. The number will depend on the considerations relating to evidence described below.

After the quality of individual epidemiological studies of cancer has been assessed and the informativeness of the various studies on the association between the agent and cancer has been evaluated, a judgement is made about the strength of evidence that the agent in question is carcinogenic to humans. In making its judgement, the Working Group considers several aspects of the body of evidence (e.g. Hill, 1965; Rothman et al., 2008; Vandenbroucke et al., 2016).

A strong association (e.g. a large relative risk) is more likely to indicate causality than is a weak association, because it is more difficult for confounding to falsely create a strong association. However, it is recognized that estimates of effect of small magnitude do not imply lack of causality and may have impact on public health if the disease or exposure is common. Estimates of effect of small magnitude could also contribute useful information to the assessment of causality if level of risk is commensurate with level of exposure when compared with risk estimates from populations with higher exposure (e.g. as seen in residential radon studies compared with studies of radon from uranium mining).

Associations that are consistently observed in several studies of the same design, or in studies that use different epidemiological approaches, or under different circumstances of exposure are more likely to indicate a causal relationship than are isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (e.g. differences in study informativeness because of latency, exposure levels, or assessment methods). Results of studies that are judged to be of high quality and informativeness are given more weight than those of studies judged to be methodologically less sound or less informative.

Temporality of the association is an essential consideration: that is, the exposure must precede the outcome.

An observation that cancer risk increases with increasing exposure is considered to be a strong indication of causality, although the absence of a graded response is not necessarily evidence against a causal relationship, and there are several reasons why the shape of the exposure–response association may be non-monotonic (e.g. Stayner et al., 2003). The demonstration of a decline in risk after cessation of or reduction in exposure

in individuals or in whole populations also supports a causal interpretation of the findings.

Confidence in a causal interpretation of the evidence from studies of cancer in humans is enhanced if it is coherent with physiological and biological knowledge, including information about exposure to the target organ, latency and timing of the exposure, and characteristics of tumour subtypes.

The Working Group considers whether there are subpopulations with increased susceptibility to cancer from the agent. For example, molecular epidemiology studies that identify associations between genetic polymorphisms and inter-individual differences in cancer susceptibility to the agent(s) being evaluated may contribute to the identification of carcinogenic hazards to humans. Such studies may be particularly informative if polymorphisms are found to be modifiers of the exposure–response association, because evaluation of polymorphisms may increase the ability to detect an effect in susceptible subpopulations.

When, in the process of evaluating the studies of cancer in humans, the Working Group identifies several high-quality, informative epidemiological studies that clearly show either no positive association or an inverse association between an exposure and a specific type of cancer, a judgement may be made that, in the aggregate, they suggest evidence of lack of carcinogenicity for that cancer type. Such a judgement requires, first, that the studies strictly meet the standards of design and analysis described above. Specifically, the possibility that bias, confounding, or misclassification of exposure or outcome could explain the observed results should be considered and ruled out with reasonable confidence. In addition, all studies that are judged to be methodologically sound should (a) be consistent with an estimate of relative effect of unity (or below unity) for any observed level of exposure, (b) when considered together, provide a combined estimate of relative risk that is at or below unity, and (c) have a narrow confidence interval. Moreover, neither any individual well-designed and well-conducted study nor the pooled results of all the studies should show any consistent tendency that the relative risk of cancer increases with increasing level of exposure. It must be noted that evidence of lack of carcinogenicity obtained from several epidemiological studies can apply only to the type(s) of cancer studied, to the exposure levels reported and the timing and route of exposure studied, to the intervals between first exposure and disease onset observed in these studies, and to the general population(s) studied (i.e. there may be susceptible subpopulations or life stages). Experience from studies of cancer in humans indicates that the period from first exposure to the development of clinical cancer is sometimes longer than 20 years; therefore, latency periods substantially shorter than about 30 years cannot provide evidence of lack of carcinogenicity. Furthermore, there may be critical windows of exposure, for example, as with diethylstilboestrol and clear cell adenocarcinoma of the cervix and vagina (IARC, 2012a).

3. Studies of cancer in experimental animals

Most human carcinogens that have been studied adequately for carcinogenicity in experimental animals have produced positive results in one or more animal species. For some agents, carcinogenicity in experimental animals was demonstrated before epidemiological studies identified their carcinogenicity in humans. Although this observation cannot establish that all agents that cause cancer in experimental animals also cause cancer in humans, it is biologically plausible that agents for which there is *sufficient evidence of carcinogenicity* in experimental animals (see Part B, Section 6b) present a carcinogenic hazard to humans. Accordingly, in the absence of additional scientific information, such as strong evidence that a given agent causes cancer in

experimental animals through a species-specific mechanism that does not operate in humans (see Part B, Sections 4 and 6; Capen et al., 1999; IARC, 2003), these agents are considered to pose a potential carcinogenic hazard to humans. The inference of potential carcinogenic hazard to humans does not imply tumour site concordance across species (Baan et al., 2019).

(a) Types of studies considered

Relevant studies of cancer in experimental animals are identified by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below. Consideration is given to all available long-term studies of cancer in experimental animals with the agent under review (or possibly metabolites or derivatives of the agent) (see Part A, Section 7) after a thorough evaluation of the study features (see Part B, Section 3b). Those studies that are judged to be irrelevant to the evaluation or judged to be inadequate (e.g. too short a duration, too few animals, poor survival; see below) may be omitted. Guidelines for conducting long-term carcinogenicity experiments have been published (e.g. OECD, 2018).

In addition to conventional long-term bioassays, alternative studies (e.g. in genetically engineered mouse models) may be considered in assessing carcinogenicity in experimental animals, also after a critical evaluation of the study features. For studies of certain exposures, such as viruses that typically only infect humans, use of such specialized experimental animal models may be particularly important; models include genetically engineered mice with targeted expression of viral genes to tissues from which human cancers arise, as well as humanized mice implanted with the human cells usually infected by the virus.

Other types of studies can provide supportive evidence. These include: experiments in which the agent was administered in the presence of factors that modify carcinogenic effects (e.g. initiation–promotion studies); studies in which the end-point was not cancer but a defined precancerous lesion; and studies of cancer in non-laboratory animals (e.g. companion animals) exposed to the agent.

(b) Study evaluation

Considerations of importance in the interpretation and evaluation of a particular study include: (i) whether the agent was clearly characterized, including the nature and extent of impurities and contaminants and the stability of the agent, and, in the case of mixtures, whether the sample characterization was adequately reported; (ii) whether the dose was monitored adequately, particularly in inhalation experiments; (iii) whether the doses, duration and frequency of treatment, duration of observation, and route of exposure were appropriate; (iv) whether appropriate experimental animal species and strains were evaluated; (v) whether there were adequate numbers of animals per group; (vi) whether animals were allocated randomly to groups; (vii) whether the body weight, food and water consumption, and survival of treated animals were affected by any factors other than the test agent; (viii) whether the histopathology review was adequate; and (ix) whether the data were reported and analysed adequately.

(c) Outcomes and statistical analyses

An assessment of findings of carcinogenicity in experimental animals involves consideration of (i) study features such as route, doses, schedule and duration of exposure, species, strain (including genetic background where applicable), sex, age, and duration of follow-up; (ii) the spectrum of neoplastic response, from pre-neoplastic lesions and benign tumours to malignant neoplasms; (iii) the incidence, latency, severity, and multiplicity of neoplasms and pre-neoplastic

lesions; (iv) the consistency of the results for a specific target organ or organs across studies of similar design; and (v) the possible role of modifying factors (e.g. diet, infection, stress).

Key factors for statistical analysis include: (i) number of animals studied and number examined histologically, (ii) number of animals with a given tumour type or lesion, and (iii) duration of survival.

Benign tumours may be combined with malignant tumours in the assessment of tumour incidence when (a) they occur together with and originate from the same cell type as malignant tumours in an organ or tissue in a particular study and (b) they appear to represent a stage in the progression to malignancy (Huff et al., 1989). The occurrence of lesions presumed to be preneoplastic may in certain instances aid in assessing the biological plausibility of any neoplastic response observed.

Evidence of an increased incidence of neoplasms with increasing level of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms. The form of the dose–response relationship can vary widely, including non-linearity, depending on the particular agent under study and the target organ. The dose–response relationship can also be affected by differences in survival among the treatment groups.

The statistical methods used should be clearly stated and should be the generally accepted techniques refined for this purpose (Peto et al., 1980; Gart et al., 1986; Portier and Bailer, 1989; Bieler and Williams, 1993). The choice of the most appropriate statistical method requires consideration of whether there are differences in survival among the treatment groups; for example, reduced survival because of non-tumour-related mortality can preclude the occurrence of tumours later in life and a survival-adjusted analysis would be warranted. When detailed information on survival is not available, comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time that the first tumour was discovered) can be useful when significant differences in survival occur before tumours appear. The lethality of the tumour also requires consideration: for rapidly fatal tumours, the time of death provides an indication of the time of tumour onset and can be assessed using life-table methods; non-fatal or incidental tumours that do not affect survival can be assessed using methods such as the Mantel–Haenszel test for changes in tumour prevalence. Because tumour lethality is often difficult to determine, methods such as the poly-*k* test that do not require such information can also be used. When results are available on the number and size of tumours seen in experimental animals (e.g. papillomas on mouse skin, liver tumours observed through nuclear magnetic resonance tomography), other, more complicated statistical procedures may be needed (Sherman et al., 1994; Dunson et al., 2003).

The concurrent control group is generally the most appropriate comparison group for statistical analysis; however, for uncommon tumours, the analysis may be improved by considering historical control data, particularly when between-study variability is low. Historical controls should be selected to resemble the concurrent controls as closely as possible with respect to species, sex, and strain, as well as other factors, such as basal diet and general laboratory environment, which may affect tumour response rates in control animals (Haseman et al., 1984; Fung et al., 1996; Greim et al., 2003). It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls.

Meta-analyses and pooled analyses may be appropriate when the experimental protocols are sufficiently similar.

4. Mechanistic evidence

Mechanistic data may provide evidence of carcinogenicity and may also help in assessing the relevance and importance of findings of cancer in experimental animals and in humans (Guyton et al., 2009; Parkkinen et al., 2018) (see Part B, Section 6). Mechanistic studies have gained in prominence, increasing in their volume, diversity, and relevance to cancer hazard evaluation, whereas studies pertinent to other streams of evidence evaluated in the *Monographs* (i.e. studies of cancer in humans and lifetime cancer bioassays in rodents) may only be available for a fraction of agents to which humans are currently exposed (Guyton et al., 2009, 2018). Mechanistic studies and data are identified, screened, and evaluated for quality and importance to the evaluation by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below.

The Working Group's synthesis reflects the extent of available evidence, summarizing groups of included studies with an emphasis on characterizing consistencies or differences in results within and across experimental designs. Greater emphasis is given to informative mechanistic evidence from human-related studies than to that from other experimental test systems, and gaps are identified. Tabulation of data may facilitate this review. The specific topics addressed in the evidence synthesis are described below.

(a) Absorption, distribution, metabolism, and excretion

Studies of absorption, distribution, metabolism, and excretion in mammalian species are addressed in a summary fashion; exposure characterization is addressed in Part B, Section 1. The Working Group describes the metabolic fate of the agent in mammalian species, noting the metabolites that have been identified and their chemical reactivity. A metabolic schema may indicate the relevant metabolic pathways and products and whether supporting evidence is from studies in humans and/or studies in experimental animals. Evidence on other adverse effects that indirectly confirm absorption, distribution, and/or metabolism at tumour sites is briefly summarized when direct evidence is sparse.

(b) Evidence relevant to key characteristics of carcinogens

A review of Group 1 human carcinogens classified up to and including *IARC Monographs* Volume 100 revealed several issues relevant to improving the evaluation of mechanistic evidence for cancer hazard identification (Smith et al., 2016). First, it was noted that human carcinogens often share one or more characteristics that are related to the multiple mechanisms by which agents cause cancer. Second, different human carcinogens may exhibit a different spectrum of these key characteristics and operate through distinct mechanisms. Third, for many carcinogens evaluated before Volume 100, few data were available on some mechanisms of recognized importance in carcinogenesis, such as epigenetic alterations (Herceg et al., 2013). Fourth, there was no widely accepted method to search systematically for relevant mechanistic evidence, resulting in a lack of uniformity in the scope of mechanistic topics addressed across *IARC Monographs* evaluations.

To address these challenges, the key characteristics of human carcinogens were introduced to facilitate systematic consideration of mechanistic evidence in *IARC Monographs* evaluations (Smith et al., 2016; Guyton et al., 2018). The key characteristics described by Smith et al. (2016) (see Table 3), such as "is genotoxic", "is immunosuppressive", or "modulates receptor-mediated effects", are based on empirical observations of the chemical and biological properties associated with the human carcinogens identified by

Table 3 The key characteristics of carcinogens

Ten key characteristics of carcinogens	
1.	Is electrophilic or can be metabolically activated to an electrophile
2.	Is genotoxic
3.	Alters DNA repair or causes genomic instability
4.	Induces epigenetic alterations
5.	Induces oxidative stress
6.	Induces chronic inflammation
7.	Is immunosuppressive
8.	Modulates receptor-mediated effects
9.	Causes immortalization
10.	Alters cell proliferation, cell death, or nutrient supply

From Smith et al. (2016).

the *IARC Monographs* programme up to and including Volume 100. The list of key characteristics and associated end-points may evolve, based on the experience of their application and as new human carcinogens are identified. Key characteristics are distinct from the "hallmarks of cancer", which relate to the properties of cancer cells (Hanahan and Weinberg, 2000, 2011). Key characteristics are also distinct from hypothesized mechanistic pathways, which describe a sequence of biological events postulated to occur during carcinogenesis. As such, the evaluation approach based on key characteristics, outlined below, "avoids a narrow focus on specific pathways and hypotheses and provides for a broad, holistic consideration of the mechanistic evidence" (National Academies of Sciences, Engineering, and Medicine, 2017).

Studies in exposed humans and in human primary cells or tissues that incorporate end-points relevant to key characteristics of carcinogens are emphasized when available. For each key characteristic with adequate evidence for evaluation, studies are grouped according to whether they involve (a) humans or human primary cells or tissues or (b) experimental systems; further organization (as appropriate) is by end-point (e.g. DNA damage), duration, species, sex, strain, and target organ as well as strength of study design. Studies investigating susceptibility related to key characteristics of carcinogens (e.g. of genetic polymorphisms, or in genetically engineered animals) can be highlighted and may provide additional support for conclusions on the strength of evidence. Findings relevant to a specific tumour type may be noted.

(c) Other relevant evidence

Other informative evidence may be described when it is judged by the Working Group to be relevant to an evaluation of carcinogenicity and to be of sufficient importance to affect the overall evaluation. Quantitative structure–activity information, such as on specific chemical and/or biological features or activities (e.g. electrophilicity, molecular docking with receptors), may be informative. In addition, evidence that falls outside of the recognized key characteristics of carcinogens, reflecting emerging knowledge or important novel scientific developments on carcinogen mechanisms, may also be included. Available evidence relevant to criteria provided in authoritative publications (e.g. Capen et al., 1999; IARC, 2003) on thyroid, kidney, urinary bladder, or other tumours in experimental animals induced by mechanisms that do not operate in humans is also described.

(d) Study quality and importance to the evaluation

Based on formal considerations of the quality of the studies (e.g. design, methodology, and reporting of results), the Working Group may give greater weight to some included studies.

For observational and other studies in humans, the quality of study design, exposure assessment, and assay accuracy and precision are considered, in collaboration with the Working Group members reviewing exposure characterization and studies of cancer in humans, as are other important factors, including those described above for evaluation of epidemiological evidence (García-Closas et al., 2006, 2011; Vermeulen et al., 2018) (Part B, Sections 1 and 2).

In general, in experimental systems, studies of repeated doses and of chronic exposures are accorded greater importance than are studies of a single dose or time-point. Consideration is also given to factors such as the suitability of the dosing range, the extent of concurrent toxicity observed, and the completeness of reporting of the study (e.g. the source and purity of the agent, the analytical methods, and the results). Route of exposure is generally considered to be a less important factor in the evaluation of experimental studies, recognizing that the exposures and target tissues may vary across experimental models and in exposed human populations. Non-mammalian studies can be synthetically summarized when they are considered to be supportive of evidence in humans or higher organisms.

In vitro test systems can provide mechanistic insights, but important considerations include the limitations of the test system (e.g. in metabolic capabilities) as well as the suitability of a particular test article (i.e. because of physical and chemical characteristics) (Hopkins et al., 2004). For studies on some end-points, such as for traditional studies of mutations in bacteria and in mammalian cells, formal guidelines, including those from the Organisation for Economic Co-operation and Development, may be informative in conducting the quality review (OECD, 1997, 2016a, b). However, existing guidelines will not generally cover all relevant assays, even for genotoxicity. Possible considerations when evaluating the quality of in vitro studies encompass the methodology and design (e.g. the end-point and test method, the number of replicate samples, the suitability of the concentration range, the inclusion of positive and negative controls, and the assessment of cytotoxicity) as well as reporting (e.g. of the source and purity of the agent, and of the analytical methods and results). High-content and high-throughput in vitro data can serve as an additional or supportive source of mechanistic evidence (Chiu et al., 2018; Guyton et al., 2018), although large-scale screening programmes measuring a variety of end-points were designed to evaluate large chemical libraries in order to prioritize chemicals for additional toxicity testing rather than to identify the hazard of a specific chemical or chemical group.

The synthesis is focused on the evidence that is most informative for the overall evaluation. In this regard, it is of note that some human carcinogens exhibit a single or primary key characteristic, evidence of which has been influential in their cancer hazard classifications. For instance, ethylene oxide is genotoxic (IARC, 1994), 2,3,7,8-tetrachlorodibenzo-*para*-dioxin modulates receptor-mediated effects (IARC, 1997), and etoposide alters DNA repair (IARC, 2012a). Similarly, oncogenic viruses cause immortalization, and certain drugs are, by design, immunosuppressive (IARC, 2012a, b). Because non-carcinogens can also induce oxidative stress, this key characteristic should be interpreted with caution unless it is found in combination with other key characteristics (Guyton et al., 2018). Evidence for a group of key characteristics can strengthen mechanistic conclusions (e.g. "induces oxidative stress" together with "is electrophilic or can be metabolically activated to an

electrophile", "induces chronic inflammation", and "is immunosuppressive"); see, for example, 1-bromopropane (IARC, 2018).

5. Summary of data reported

(a) Exposure characterization

Exposure data are summarized to identify the agent and describe its production, use, and occurrence. Information on exposure prevalence and intensity in different settings, including geographical patterns and time trends, may be included. Exposure assessment methods used in key epidemiological studies reviewed by the Working Group are described and evaluated.

(b) Cancer in humans

Results of epidemiological studies pertinent to an evaluation of carcinogenicity in humans are summarized. The overall strengths and limitations of the epidemiological evidence base are highlighted to indicate how the evaluation was reached. The target organ(s) or tissue(s) in which a positive association between the agent and cancer was observed are identified. Exposure–response and other quantitative data may be summarized when available. When the available epidemiological studies pertain to a mixed exposure, process, occupation, or industry, the Working Group seeks to identify the specific agent considered to be most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data permit.

(c) Cancer in experimental animals

Results pertinent to an evaluation of carcinogenicity in experimental animals are summarized to indicate how the evaluation was reached. For each animal species, study design, and route of administration, there is a statement about whether an increased incidence, reduced latency, or increased severity or multiplicity of neoplasms or pre-neoplastic lesions was observed, and the tumour sites are indicated. Special conditions resulting in tumours, such as prenatal exposure or single-dose experiments, are mentioned. Negative findings, inverse relationships, dose–response patterns, and other quantitative data are also summarized.

(d) Mechanistic evidence

Results pertinent to an evaluation of the mechanistic evidence on carcinogenicity are summarized to indicate how the evaluation was reached. The summary encompasses the informative studies on absorption, distribution, metabolism, and excretion; on the key characteristics with adequate evidence for evaluation; and on any other aspects of sufficient importance to affect the overall evaluation, including on whether the agent belongs to a class of agents for which one or more members have been classified as carcinogenic or probably carcinogenic to humans, and on criteria with respect to tumours in experimental animals induced by mechanisms that do not operate in humans. For each topic addressed, the main supporting findings are highlighted from exposed humans, human cells or tissues, experimental animals, or in vitro systems. When mechanistic studies are available in exposed humans, the tumour type or target tissue studied may be specified. Gaps in the evidence are indicated (i.e. if no studies were available in exposed humans, in in vivo systems, etc.). Consistency or differences of effects across different experimental systems are emphasized.

6. Evaluation and rationale

Consensus evaluations of the strength of the evidence of cancer in humans, the evidence of cancer in experimental animals, and the mechanistic evidence are made using transparent criteria and defined descriptive terms. The Working Group then develops a consensus overall evaluation of the strength of the evidence of carcinogenicity for each agent under review.

An evaluation of the strength of the evidence is limited to the agents under review. When multiple agents being evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single and unified evaluation of the strength of the evidence.

The framework for these evaluations, described below, may not encompass all factors relevant to a particular evaluation of carcinogenicity. After considering all relevant scientific findings, the Working Group may exceptionally assign the agent to a different category than a strict application of the framework would indicate, while providing a clear rationale for the overall evaluation.

When there are substantial differences of scientific interpretation among the Working Group members, the overall evaluation will be based on the consensus of the Working Group. A summary of the alternative interpretations may be provided, together with their scientific rationale and an indication of the relative degree of support for each alternative.

The categories of the classification refer to the strength of the evidence that an exposure is carcinogenic and not to the risk of cancer from particular exposures. The terms *probably carcinogenic* and *possibly carcinogenic* have no quantitative significance and are used as descriptors of different strengths of evidence of carcinogenicity in humans; *probably carcinogenic* signifies a greater strength of evidence than *possibly carcinogenic*.

(a) Carcinogenicity in humans

Based on the principles outlined in Part B, Section 2, the evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** A causal association between exposure to the agent and human cancer has been established. That is, a positive association has been observed in the body of evidence on exposure to the agent and cancer in studies in which chance, bias, and confounding were ruled out with reasonable confidence.

***Limited evidence of carcinogenicity*:** A causal interpretation of the positive association observed in the body of evidence on exposure to the agent and cancer is credible, but chance, bias, or confounding could not be ruled out with reasonable confidence.

***Inadequate evidence regarding carcinogenicity*:** The available studies are of insufficient quality, consistency, or statistical precision to permit a conclusion to be drawn about the presence or the absence of a causal association between exposure and cancer, or no data on cancer in humans are available. Common findings that lead to a determination of inadequate evidence of carcinogenicity include: (a) there are no data available in humans; (b) there are data available in humans, but they are of poor quality or informativeness; and (c) there are studies of sufficient quality available in humans, but their results are inconsistent or otherwise inconclusive.

***Evidence suggesting lack of carcinogenicity*:** There are several high-quality studies covering the full range of levels of exposure that humans are known to encounter, which are mutually consistent in not showing a positive association between exposure to the agent and the studied cancers at any observed level of exposure. The results from these studies

alone or combined should have narrow confidence intervals with an upper limit below or close to the null value (e.g. a relative risk of unity). Bias and confounding were ruled out with reasonable confidence, and the studies were considered informative. A conclusion of *evidence suggesting lack of carcinogenicity* is limited to the cancer sites, populations and life stages, conditions and levels of exposure, and length of observation covered by the available studies. In addition, the possibility of a very small risk at the levels of exposure studied can never be excluded.

When there is *sufficient evidence*, a separate sentence identifies the target organ(s) or tissue(s) for which a causal interpretation has been established. When there is *limited evidence*, a separate sentence identifies the target organ(s) or tissue(s) for which a positive association between exposure to the agent and the cancer(s) was observed in humans. When there is *evidence suggesting lack of carcinogenicity*, a separate sentence identifies the target organ(s) or tissue(s) where evidence of lack of carcinogenicity was observed in humans. Identification of a specific target organ or tissue as having *sufficient evidence* or *limited evidence* or *evidence suggesting lack of carcinogenicity* does not preclude the possibility that the agent may cause cancer at other sites.

(b) Carcinogenicity in experimental animals

The evidence relevant to carcinogenicity from studies in experimental animals is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** A causal relationship has been established between exposure to the agent and cancer in experimental animals based on an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories and/or under different protocols. An increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices (GLP), can also provide *sufficient evidence*.

Exceptionally, a single study in one species and sex may be considered to provide *sufficient evidence of carcinogenicity* when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour, or age at onset, or when there are marked findings of tumours at multiple sites.

***Limited evidence of carcinogenicity*:** The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, for example, (a) the evidence of carcinogenicity is restricted to a single experiment and does not meet the criteria for *sufficient evidence*; (b) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; (c) the agent increases tumour multiplicity or decreases tumour latency but does not increase tumour incidence; (d) the evidence of carcinogenicity is restricted to initiation–promotion studies; (e) the evidence of carcinogenicity is restricted to observational studies in non-laboratory animals (e.g. companion animals); or (f) there are unresolved questions about the adequacy of the design, conduct, or interpretation of the available studies.

***Inadequate evidence regarding carcinogenicity*:** The studies cannot be interpreted as showing either the presence or the absence of a carcinogenic effect because of major

qualitative or quantitative limitations, or no data are available on cancer in experimental animals.

***Evidence suggesting lack of carcinogenicity*:** Well-conducted studies (e.g. conducted under GLP) involving both sexes of at least two species are available showing that, within the limits of the tests used, the agent was not carcinogenic. The conclusion of *evidence suggesting lack of carcinogenicity* is limited to the species, tumour sites, age at exposure, and conditions and levels of exposure covered by the available studies.

(c) Mechanistic evidence

Based on the principles outlined in Part B, Section 4, the mechanistic evidence is classified into one of the following categories:

***Strong mechanistic evidence*:** Results in several different experimental systems are consistent, and the overall mechanistic database is coherent. Further support can be provided by studies that demonstrate experimentally that the suppression of key mechanistic processes leads to the suppression of tumour development. Typically, a substantial number of studies on a range of relevant end-points are available in one or more mammalian species. Quantitative structure–activity considerations, in vitro tests in non-human mammalian cells, and experiments in non-mammalian species may provide corroborating evidence but typically do not in themselves provide strong evidence. However, consistent findings across a number of different test systems in different species may provide strong evidence.

Of note, "strong" relates not to potency but to strength of evidence. The classification applies to three distinct topics:

(a) Strong evidence that the agent belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified as carcinogenic or probably carcinogenic to humans. The considerations can go beyond quantitative structure–activity relationships to incorporate similarities in biological activity relevant to common key characteristics across dissimilar chemicals (e.g. based on molecular docking, –omics data).

(b) Strong evidence that the agent exhibits key characteristics of carcinogens. In this case, three descriptors are possible:

1. The strong evidence is in exposed humans. Findings relevant to a specific tumour type may be informative in this determination.
2. The strong evidence is in human primary cells or tissues. Specifically, the strong findings are from biological specimens obtained from humans (e.g. ex vivo exposure), from human primary cells, and/or, in some cases, from other humanized systems (e.g. a human receptor or enzyme).
3. The strong evidence is in experimental systems. This may include one or a few studies in human primary cells and tissues.

(c) Strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans. Certain results in experimental animals (see Part B, Section 6b) would be discounted, according to relevant criteria and considerations in authoritative publications (e.g. Capen et al., 1999; IARC, 2003). Typically, this classification would not apply when there is strong mechanistic evidence that the agent exhibits key characteristics of carcinogens.

***Limited mechanistic evidence*:** The evidence is suggestive, but, for example, (a) the studies cover a narrow range of experiments, relevant end-points, and/or species; (b) there are unexplained inconsistencies in the studies of similar design; and/or (c) there is unexplained incoherence across studies of different end-points or in different experimental systems.

***Inadequate mechanistic evidence*:** Common findings that lead to a determination of inadequate mechanistic evidence include: (a) few or no data are available; (b) there are unresolved questions about the adequacy of the design, conduct, or interpretation of the studies; (c) the available results are negative.

(d) Overall evaluation

Finally, the bodies of evidence included within each stream of evidence are considered as a whole, in order to reach an overall evaluation of the carcinogenicity of the agent to humans. The three streams of evidence are integrated and the agent is classified into one of the following categories (see Table 4), indicating that the Working Group has established that:

The agent is carcinogenic to humans (Group 1)

This category applies whenever there is *sufficient evidence of carcinogenicity* in humans.

In addition, this category may apply when there is both *strong evidence in exposed humans that the agent exhibits key characteristics of carcinogens* and *sufficient evidence of carcinogenicity* in experimental animals.

The agent is probably carcinogenic to humans (Group 2A)

This category generally applies when the Working Group has made at least *two of the following* evaluations, *including at least one* that involves either exposed humans or human cells or tissues:

- *Limited evidence of carcinogenicity* in humans,
- *Sufficient evidence of carcinogenicity* in experimental animals,
- *Strong evidence that the agent exhibits key characteristics of carcinogens.*

If there is *inadequate evidence regarding carcinogenicity* in humans, there should be *strong evidence in human cells or tissues that the agent exhibits key characteristics of carcinogens*. If there is *limited evidence of carcinogenicity in humans*, then the second individual evaluation may be from experimental systems (i.e. *sufficient evidence of carcinogenicity* in experimental animals or *strong evidence in experimental systems that the agent exhibits key characteristics of carcinogens).*

Additional considerations apply when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites. Specifically, the remaining tumour sites should still support an evaluation of *sufficient evidence in experimental animals* in order for this evaluation to be used to support an overall classification in Group 2A.

Separately, this category generally applies if there is *strong evidence that the agent belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified in Group 1 or Group 2A*.

The agent is possibly carcinogenic to humans (Group 2B)

This category generally applies when only one of the following evaluations has been made by the Working Group:

- *Limited evidence of carcinogenicity* in humans,
- *Sufficient evidence of carcinogenicity* in experimental animals,

Table 4 Integration of streams of evidence in reaching overall classifications (the evidence in *bold italic* represents the basis of the overall evaluation)

Stream of evidence			Classification based on strength of evidence
Evidence of cancer in humans[a]	**Evidence of cancer in experimental animals**	**Mechanistic evidence**	
Sufficient	Not necessary	Not necessary	**Carcinogenic to humans (Group 1)**
Limited or Inadequate	***Sufficient***	***Strong (b)(1) (exposed humans)***	
Limited	***Sufficient***	Strong (b)(2–3), Limited, or Inadequate	**Probably carcinogenic to humans (Group 2A)**
Inadequate	***Sufficient***	***Strong (b)(2) (human cells or tissues)***	
Limited	Less than Sufficient	***Strong (b)(1–3)***	
Limited or Inadequate	Not necessary	***Strong (a) (mechanistic class)***	
Limited	Less than Sufficient	Limited or Inadequate	**Possibly carcinogenic to humans (Group 2B)**
Inadequate	***Sufficient***	Strong (b)(3), Limited, or Inadequate	
Inadequate	Less than Sufficient	***Strong (b)(1–3)***	
Limited	***Sufficient***	***Strong (c) (does not operate in humans)***[b]	
Inadequate	***Sufficient***	***Strong (c) (does not operate in humans)***[b]	**Not classifiable as to its carcinogenicity to humans (Group 3)**
All other situations not listed above			

[a] Human cancer(s) with highest evaluation.

[b] The *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* must specifically be for the tumour sites supporting the classification of *sufficient evidence in experimental animals*.

• *Strong evidence that the agent exhibits key characteristics of carcinogens.*

Because this category can be based on evidence from studies in experimental animals alone, there is **no** requirement that the strong mechanistic evidence be in exposed humans or in human cells or tissues. This category may be based on *strong evidence in experimental systems that the agent exhibits key characteristics of carcinogens.*

As with Group 2A, additional considerations apply when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites. Specifically, the remaining tumour sites should still support an evaluation of *sufficient evidence in experimental animals* in order for this evaluation to be used to support an overall classification in Group 2B.

The agent is not classifiable as to its carcinogenicity to humans (Group 3)

Agents that do not fall into any other group are generally placed in this category.

This includes the case when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites in experimental animals, the remaining tumour sites do not support an evaluation of *sufficient evidence in experimental animals*, and other categories are not supported by data from studies in humans and mechanistic studies.

An evaluation in Group 3 is not a determination of non-carcinogenicity or overall safety. It often means that the agent is of unknown carcinogenic potential and that there are significant gaps in research.

If the evidence suggests that the agent exhibits no carcinogenic activity, either through *evidence suggesting lack of carcinogenicity* in both humans and experimental animals, or through *evidence suggesting lack of carcinogenicity* in

experimental animals complemented by strong negative mechanistic evidence in assays relevant to human cancer, then the Working Group may add a sentence to the evaluation to characterize the agent as well-studied and without evidence of carcinogenic activity.

(e) Rationale

The reasoning that the Working Group used to reach its evaluation is summarized so that the basis for the evaluation offered is transparent. This section integrates the major findings from studies of cancer in humans, cancer in experimental animals, and mechanistic evidence. It includes concise statements of the principal line(s) of argument that emerged in the deliberations of the Working Group, the conclusions of the Working Group on the strength of the evidence for each stream of evidence, an indication of the body of evidence that was pivotal to these conclusions, and an explanation of the reasoning of the Working Group in making its evaluation.

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GENERAL REMARKS[a]

This one-hundred-and-thirty-fourth volume of the *IARC Monographs* contains evaluations of the carcinogenic hazard to humans of aspartame, methyleugenol, and isoeugenol.

Methyleugenol was considered previously by the *IARC Monographs* programme in 2011 (IARC, 2013), when it was evaluated as *possibly carcinogenic to humans* (Group 2B). Aspartame and isoeugenol have not been evaluated previously by the *IARC Monographs* programme.

The Advisory Group to Recommend Priorities for the *IARC Monographs* that met in 2019 recommended that all three agents be evaluated with high priority (IARC, 2019a; Marques et al., 2019). A summary of the findings of this volume appears in *The Lancet Oncology* (Riboli et al., 2023).

Coordination between the *IARC Monographs* programme and JECFA for the evaluation of aspartame

The monograph on aspartame is the result of a highly coordinated effort undertaken within WHO. First, IARC evaluated the carcinogenic hazard of aspartame. Subsequently, JECFA, the Joint FAO/WHO Expert Committee on Food Additives, conducted a risk assessment for cancer and other noncommunicable diseases, including reviewing and updating the acceptable daily intake (ADI) and dietary exposure assessment for aspartame. The monograph reports the results of the IARC evaluation of aspartame for cancer hazard identification; the results of the JECFA review of aspartame for dietary exposure and risk assessment have been published separately (WHO, 2023, 2024).

In line with the procedures established for communication and collaboration between the *IARC Monographs* programme and other WHO programmes, the *IARC Monographs* Meeting 134 on 6–13 June was followed closely by the JECFA Ninety-sixth Meeting on 27 June to 6 July. Aspartame was evaluated for the first time by IARC and for the third time by JECFA. The two bodies conducted independent but complementary reviews of all the available scientific literature. To ensure continuity and exchange of relevant information, three WHO scientists from the JECFA programme (Drs Sanaa and Montez and Mr Petersen) joined the IARC/WHO Secretariat for the *IARC Monographs* meeting, and two scientists from the *IARC Monographs* programme (Drs Madia and Benbrahim-Tallaa) joined the WHO Secretariat for the JECFA meeting.

[a] The previously posted "Preliminary General Remarks" relevant to the monograph on aspartame (published in advance in April 2024) were updated to include general remarks relevant to the full volume.

Furthermore, three of the seven Observers attending the IARC meeting (Drs Agudo, Barlow, and Wu) also served as members of the expert committee at the JECFA Ninety-sixth Meeting, and relevant literature search results were shared between the two programmes as permitted by any confidentiality requirements.

Exposure data for aspartame

The occurrence of aspartame in food, beverages, and consumer products and human exposure levels have been poorly documented over the years, despite the fact that this sweetener has been a commonly used food additive for several decades. The Working Group noted that few databases were available (see Sections 1.2 and 1.4 of the monograph on aspartame in the present volume) that reported comprehensive information on the presence of aspartame in various food categories, including beverages. In several databases, the Working Group also noted the lack of information on maximum permitted levels. Furthermore, information on dietary exposures in populations from low- and middle-income countries was lacking, as were data on occupational exposure during the manufacture or use of aspartame. Likewise, it was observed that precise quantification of aspartame exposure across various dietary sources in large-scale prospective cohorts has been performed only rarely (e.g. in the NutriNet-Santé cohort study by Debras et al., 2022).

Evaluation of aspartame metabolites

In the available literature investigating the absorption, distribution, metabolism, and excretion of aspartame, it was reported that once absorbed, this sweetener undergoes hydrolysis to form mainly its constituents: aspartic acid, phenylalanine, and methanol (see Section 4.1 of the monograph on aspartame in the present volume). The three hydrolytes undergo absorption from the intestinal lumen and reach the systemic circulation, in a similar manner to endogenous and exogenous amino acids and methanol obtained from other dietary sources. The homeostasis of the amino acids and methanol seems not to be influenced by the consumption of aspartame. In primates, the amino acid phenylalanine was reported to be retained in the body at higher levels than those of aspartic acid or methanol. Regarding specifically methanol, which can enter the portal circulation and is oxidized by hepatic alcohol dehydrogenase to formaldehyde (classified by IARC as *carcinogenic to humans*, Group 1; IARC, 2012) and finally to formic acid and then carbon dioxide, the Working Group noted that there was no evidence that the overall amount of formaldehyde formed as a result of aspartame consumption (up to the levels of the ADI of 40 mg/kg per day) would significantly alter normal endogenous formaldehyde concentrations. This is also valid for endogenous levels of aspartic acid and phenylalanine. For this reason, in the evaluation of the carcinogenic hazard of aspartame, the Working Group did not assess each individual metabolite separately with regard to evidence of cancer in experimental animals and mechanistic evidence.

Research gaps identified during the evaluation of aspartame

Glucose imbalance, insulin resistance, and altered lipid metabolism have been associated with increased risk of obesity, diabetes, and cancer. The Working Group reported on an increasing number of studies published over the past two decades that have investigated the effects of various non-nutritive sweeteners,

including aspartame, after single or repeated dosing in experimental systems in vivo and showed consistent alterations in insulin levels (see Section 4.3 of the monograph on aspartame in the present volume). Additionally, emerging literature has suggested associations with microbiome alterations and potential effects of aspartame on metabolism and cell growth mediated by sweet taste receptors, including a potential role of its metabolite phenylalanine. The interactions of aspartame with sweet taste receptors, which have been implicated in the signalling cascade that activates metabolism in the body and with the gut microbiota, were identified by the Working Group as notable research gaps. Likewise, studies of effects on end-points related to alterations of metabolism in humans, both those who are healthy and those with various health conditions (e.g. people with obesity or diabetes or who are pregnant), provided unclear results. A number of interventional or cross-sectional epidemiological studies presented several limitations associated with the small size of the study populations, with inadequate control for confounding variables in observational studies, or with the high complexity of the different study designs and protocols. In many studies, aspartame exposure was not precisely assessed, and the sweetener was considered as the reference positive control to be compared with other sweeteners, thus missing information on an appropriate background (unexposed) control.

The Working Group identified several major gaps in the literature: robust investigations using up-to-date methodologies on associations between precisely quantified aspartame exposure across various dietary sources and end-points related to metabolic alterations, including gut microbiome composition and function in large-scale studies in humans, were missing. There were no high-quality studies investigating mechanistic end-points associated with the key characteristics of carcinogens "induces oxidative stress" and "induces chronic inflammation" (which were observed in experimental systems) in exposed humans. Additional gaps included elucidation of the potential effects of aspartame on metabolism and metabolic outcomes (e.g. metabolic syndrome, type 2 diabetes, obesity, etc.) and on cancer risk. New research would support a better understanding of positive signals for liver cancer observed in both experimental animals and in epidemiological studies (i.e. hepatocellular carcinomas seen in the three available cohorts; Stepien et al., 2016; Jones et al., 2022; McCullough et al., 2022) and isolated signals for cancer of the mammary gland or breast (NutriNet-Santé cohort) (Debras et al., 2022). Similar research gaps have been also identified by the JECFA Committee in its review of aspartame for dietary exposure and risk assessment (IARC and JECFA, 2023).

Relevance of DNA adduct formation induced by exposure to methyleugenol

The formation of agent-specific DNA adducts can be considered to be a relevant marker of exposure and effect. DNA adducts represent an important end-point for the key characteristic of carcinogens "is electrophilic or can be metabolically activated to an electrophile" (IARC, 2019b). The relevance of the end-point and the strength of the evidence are evaluated with consideration of the specificity of the adducts and information on the evidence for mutations (key characteristic of "is genotoxic"). In a previous monograph (Volume 128; IARC, 2021), the available evidence on DNA adduct formation in exposed humans after exposure to either acrolein or crotonaldehyde was not considered to provide *strong* evidence of the key characteristics of carcinogens in exposed humans; the Working Group for Volume 134 agreed that similar considerations would also apply to methyleugenol.

However, in its evaluation of the carcinogenicity of methyleugenol, the Working Group considered that the widespread presence of agent-specific adducts in the human liver, together with the knowledge that those adducts were mutagenic in experimental systems, was central to the rationale for the IARC Group 2A classification of methyleugenol, even in the absence of direct evidence of mutations in exposed humans. In addition, the suggestion that the same mechanism would occur in exposed humans was corroborated by the study of Auerbach et al. (2018), which revealed that the mutational signature of methyleugenol in mouse liver tumours (determined by exome sequencing) closely resembled that of COSMIC (Catalogue Of Somatic Mutations In Cancer) signatures 4 and 24. The former signature is very similar to that produced by benzo[*a*]pyrene and other dietary carcinogens (e.g. PhIP) and the latter is similar to that of aflatoxin B1. The Working Group identified the study of mutagenesis in humans as a research gap, which could have been addressed by measuring genotoxicity end-points in the urine. On the basis of information from the study by Schecter et al. (2004), which demonstrated an increase in serum concentrations of methyleugenol in humans after the consumption of gingersnap cookies, urine could be evaluated for individuals who have consumed a defined amount of methyleugenol in one of the many commonly consumed foods containing methyleugenol in significant amounts. There are well-characterized methods for biomonitoring of human exposure to mutagens by looking for micronuclei in the bladder epithelial cells normally found in urine samples. Measuring methyleugenol metabolites in urine would help to establish a link (or lack thereof) by providing more detailed information about the amount and timing of any formation of the presumptive pro-mutagen after routine dietary exposures.

Similar considerations would be appropriate for isoeugenol, for which there are almost no phamacokinetic data in any system; finding evidence of the formation of micronuclei, adducts, or perhaps even metabolites formed via the quinoline methide in human urine after dietary exposure might contribute to a reclassification of isoeugenol.

Scope of the systematic review

Standardized searches of the PubMed database (NCBI, 2023) were conducted for each agent for each outcome (cancer in humans, cancer in experimental animals, and mechanistic evidence, including the key characteristics of carcinogens). For cancer in humans, searches were also conducted in the Web of Science (Clarivate, 2023) and Embase (Elsevier, 2023) databases. The literature trees for aspartame, methyleugenol, and isoeugenol, including the full set of search terms for the agent name and each outcome type, are available online.[a]

As described in the current Preamble to the *IARC Monographs* (last revised in 2019; see pages 14–15 in the present volume; IARC, 2019b), the Working Group reviews publicly available scientific data, such as peer-reviewed papers in the scientific literature, and may also review unpublished reports, if made available in their final form by governmental agencies and if they contain enough detail for critical review. In the case of aspartame, the Working Group was able to consult and review literature derived from the Call for Data in 2011 for the European Food Safety Authority (EFSA) risk assessment, which was made available and accessible on the EFSA

[a] The literature trees for the monographs in the present volume are available at: https://hawcproject.iarc.who.int/assessment/680/ (aspartame); https://hawcproject.iarc.who.int/assessment/688/ (methyleugenol); and https://hawcproject.iarc.who.int/assessment/689/ (isoeugenol).

website (EFSA, 2011). In addition, IARC opened a public Call for Data on its website 1 year ahead of the meeting for Volume 134. Eligible studies are only those published or accepted for publication in the openly available scientific literature by the time of the Working Group meeting.

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ASPARTAME

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 22839-47-0 (Chemical Abstracts Service, 2022a)

EC/List No.: 245-261-3 (ECHA, 2022)

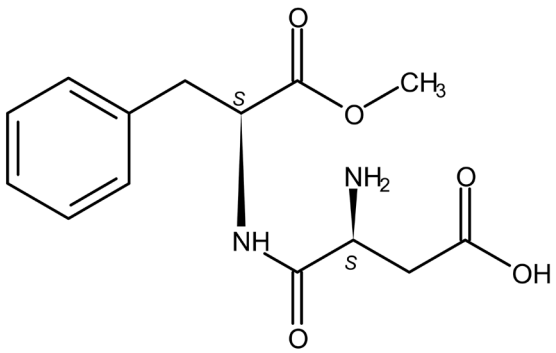
Chem. Abstr. Serv. name: aspartame (Chemical Abstracts Service, 2022a)

IUPAC systematic name: (3*S*)-3-amino-4-[[(2*S*)-1-methoxy-1-oxo-3-phenylpropan-2-yl]amino]-4-oxobutanoic acid (NCBI, 2022)

Synonyms: L-phenylalanine, L-α-aspartyl-, 2-methyl ester; succinamic acid, 3-amino-*N*-(α-carboxyphenethyl)-, *N*-methyl ester, stereoisomer; L-phenylalanine, *N*-L-α-aspartyl-, 1-methyl ester; L-aspartyl-L-phenylalanine methyl ester; aspartylphenylalanine methyl ester; methyl aspartylphenylalanine; methyl ester, aspartylphenylalanine; SC-18862; Asp-Phe-OMe (Chemical Abstracts Service, 2022a; ECHA, 2022; NCBI, 2022) [The Working Group noted that aspartame has been historically sold under several brand names (NCBI, 2022). Some of these brands have discontinued the use of aspartame in their products.]

1.1.2 Structural and molecular information

Relative molecular mass: 294.30



Molecular formula: $C_{14}H_{18}N_2O_5$ (Chemical Abstracts Service, 2022a).

1.1.3 Chemical and physical properties

Description: white, crystalline powder; aspartame has no odour, but an intense sweet taste (about 160–200 times as sweet as sucrose) (Burdock, 2010)

Taste threshold: 6.59 mg/L in water (Dietrich et al., 2021)

Boiling-point: not available

Melting-point: not well defined, often described as having a double melting-point

at 190 °C and 246–247 °C (Prankerd, 2002; von Rymon Lipinski, 2015)

Density: 1.46–1.49 g/cm^3 (Prankerd, 2002)

Solubility: sparingly soluble in water; slightly soluble in alcohol (Burdock, 2010)

Dissociation constants: pK_{a1} = 3.19; pK_{a2} = 7.87 (NCBI, 2022)

Stability and reactivity: The dry stability of aspartame is good, with < 5% conversion to its diketopiperazine at 105 °C during 100-hour storage (Homler, 1984). The stability of aspartame is affected by moisture, pH, and temperature (Magnuson et al., 2007; Burdock, 2010). Under moist conditions, the dipeptide aspartylphenylalanine and methanol may be formed by hydrolysis. Alternatively, methanol can be eliminated by the cyclization of aspartame to form its diketopiperazine, 5-benzyl-3,6-dioxo-2-piperazineacetic acid, which in turn can be hydrolysed to its individual amino acids, aspartic acid and phenylalanine. When aspartame is decomposed to these compounds, a loss of sweetness is perceived (Homler, 1984). In solution, the half-life at pH 4.3 (maximum stability) is about 260 days at 25 °C (Prankerd, 2002). Temperature and pH-dependent racemization of aspartame, or formation of its breakdown products such as the diketopiperazine, aspartic acid, and phenylalanine may occur (Boehm and Bada, 1984). In addition to these reactions, aspartame can undergo rearrangement at acidic pH to β-aspartame (Magnuson et al., 2007). The various pathways for the decomposition of aspartame are summarized in Fig. 1.1. Aspartame in aqueous solutions can be stabilized to some extent by the addition of cyclodextrin, modified cyclodextrins, or polyethylene glycol 400 (Prankerd, 2002).

1.1.4 Commercial products and impurities

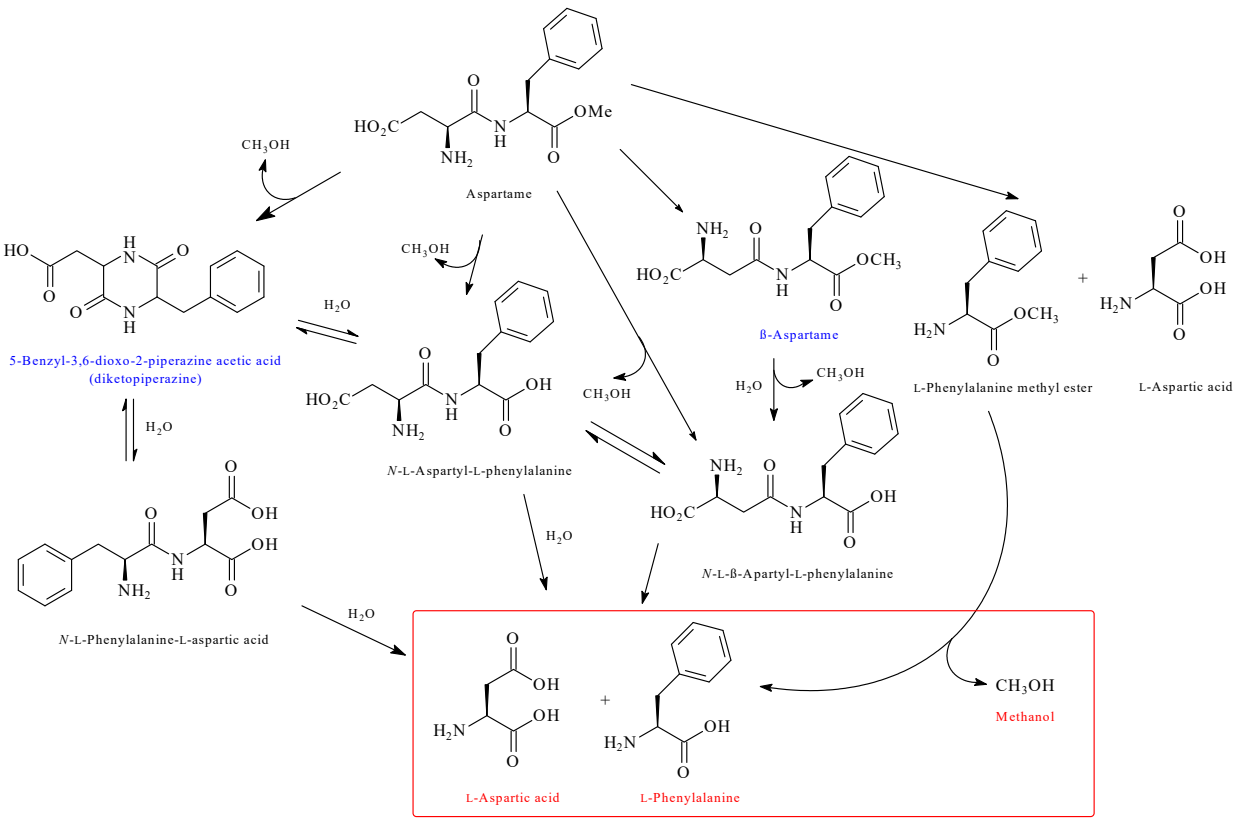
Commercial qualities with purities in the range of 90–94%, 95–98%, and ≥ 99% are available (Chemical Abstracts Service, 2022b). Typical specifications for food use include a purity of not less than 98% and maximum limits for 5-benzyl-3,6-dioxo-2-piperazineacetic acid (not more than 1.5% expressed on dry weight basis), other related substances (not more than 2%), residue on ignition (not more than 0.2%), loss on drying (not more than 4.5% at 105 °C, 4 hours); sulfated ash (not more than 0.2% expressed on dry weight basis), transmittance (not less than 0.95, equivalent to an absorbance of not more than approximately 0.022); heavy metals (not more than 10 mg/kg), arsenic (not more than 3 mg/kg expressed on dry weight basis), and lead (not more than 1 mg/kg expressed on dry weight basis) (Burdock, 2010; European Commission, 2012).

1.2 Production and use

1.2.1 Production process

Although the compound was previously known, the sweet taste of aspartame was accidentally discovered in 1965 by James M. Schlatter during the synthesis of oligopeptides related to the hormone gastrin (Mazur et al., 1969; Mazur and Ripper, 1979; von Rymon Lipinski, 2015). Aspartame can be synthesized by the coupling of the amino acids L-phenylalanine and L-aspartic acid, and the esterification of the carboxyl group of the phenylalanine moiety to produce the methyl ester. This esterification can occur before or after coupling (Burdock, 2010). Numerous enzymatic methods are available to couple aspartic acid with phenylalanine methyl ester (Prankerd, 2002). Some proteinases, such as thermolysin, can catalyse peptide bond formation (von Rymon Lipinski, 2015). Enzymatic methods applying fermentation were preferred

Fig. 1.1 Degradation pathways for aspartame



Blue text indicates impurities and red text indicates the three major metabolites.
[The Working Group noted that the diketopiperazine of aspartame is 5-benzyl-3,6-dioxo-2-piperazine acetic acid. The reaction between the diketopiperazine and *N*-L-aspartyl-L-phenylalanine might not be reversible under storage conditions.]
From EFSA (2013), with permission from John Wiley & Sons, from: Scientific opinion on the re-evaluation of aspartame (E 951) as a food additive, EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), *EFSA Journal*, Volume 11, issue 12, 2013; permission conveyed through Copyright Clearance Center, Inc.

for economic commercial production (Prankerd, 2002; Lee, 2006). A combination of an enzymatic process using α-amino acid ester acyltransferase followed by a chemical transformation reaction was recently put into industrial practice (Yokozeki and Abe, 2021).

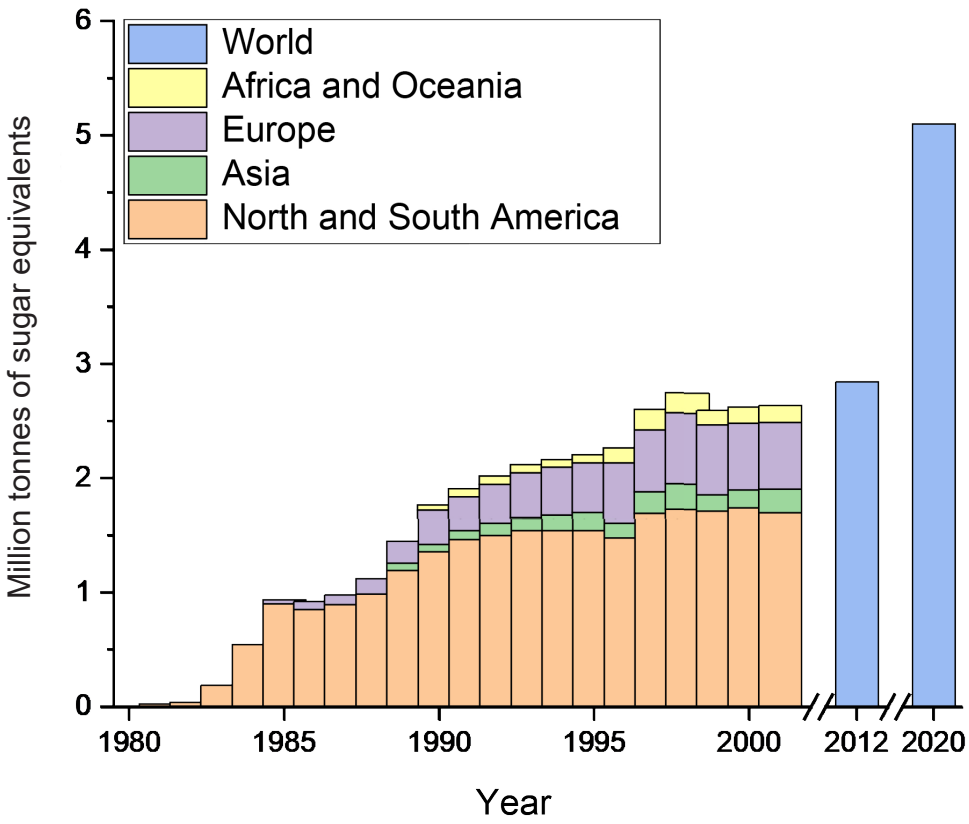
1.2.2 Production volume

(a) Worldwide

Aspartame's large-scale production began in 1981 (Hollie, 1983). The consumption of aspartame by world regions in tonnes of sugar equivalents is presented in Fig. 1.2. Consumption increased from approximately 5000 tonnes in 1985 to 13 000 tonnes in 2000.

According to encyclopaedia entries published in 2000 and 2006, aspartame was the most widely used non-nutritive sweetener worldwide at that time (Lee, 2000, 2006). In 2001, the consumption of aspartame in the world was 13 185 tonnes (2.64 million tonnes sugar equivalent), of which 1000 tonnes were consumed in Asia, 8500 tonnes in the Americas (North, South, Central, and Caribbean), 735 tonnes in Africa and Oceania, and 2950 tonnes in Europe (IASR, 2004). [More recent data including annual consumption per world region were not available to the Working

Fig. 1.2 Consumption of aspartame in 1980–2020, by world region



Created by the Working Group with data extracted from figures using WebPlotDigitizer (version 4.6, September, 2022; author, Ankit Rohatgi, Pacifica, California, USA; available from https://automeris.io/WebPlotDigitizer). Sources were Fry (1999) for data for 1980–1983, IASR (2004) for data for 1984–2001; and numerical data from Lewis and Tzilivakis (2021) for 2012 and ECHEMI (2020) for 2020 (sugar equivalents were calculated with a factor of 200 for aspartame). No data for the years 2002–2011 or 2013–2019 were available. No regional data were available for 2012 and 2020. [The Working Group noted that original data on consumption of aspartame are not publicly available and verifiable. The figure was compiled from various secondary sources, and this information may not be consistent between sources. The data for 1980–2012 were considered plausible, showing an increase leading to a plateau. The difference with the data for 2020, suggesting a considerable increase, was not consistent with other sources (see Section 1.4), which indicated a decreasing prevalence of aspartame use over the last decade, and probably cannot be explained by above-average growth rates that had been expected for Central and South America, China, Asia, Africa, and the Middle East (Lewis and Tzilivakis, 2021).]

Group.] The initial increase in production was followed by a period of saturation or even decline beginning in the mid-1990s (Fig. 1.2) (Fry, 1999; IASR, 2004). At that time, drinks that had formerly been sweetened entirely with aspartame began to be sweetened in some countries by blending aspartame with various other sweeteners, particularly acesulfame potassium (acesulfame-K); and the loss of aspartame sales was compounded by the potency gains and synergies from blending (Fry, 1999; IASR, 2004). By increasing competitiveness, the lower prices of the post-patent era (the patent expired in 1992; Reisch, 2014) were helping stimulate aspartame sales around the world (IASR, 2004). In 2016, aspartame was produced in the European Union, China, Japan, and the Republic of Korea (European Commission, 2016). In 2020, the global production of aspartame was about 25 500 tonnes, and the annual output of aspartame from China was about 19 200 tonnes (ECHEMI, 2020). Present Chinese production capacity was estimated to

be in the range of 30 000 to 35 000 tonnes. The total exports from China amounted to about 16 000 tonnes in 1 year (2020–2021) (European Commission, 2022b).

(b) USA

According to a marketing report, annual sales of aspartame in the USA increased steadily from 3300 tonnes in 1985 to 10 100 tonnes in 2002 (Bizarre et al., 2006; as cited in Magnuson et al., 2007). In 1992, about 8040 tonnes of aspartame were used in the USA (approximately 83% of world consumption) (Lee, 2000). Of this amount, approximately 85% was used for beverages (Lee, 2000). According to data for 1992 from the United States Department of Agriculture, aspartame accounted for 67.4% of the low-calorie sweeteners consumed in the country (Best and Nelson, 1993). Demand in the USA also dominated the increasing global sales of aspartame (IASR, 2004). It was estimated that about half of the global production in 2006 was consumed in the USA (Magnuson et al., 2007). The sole producer of aspartame in the USA withdrew from the market in 2014 (European Commission, 2016).

(c) Europe

In the European Union, the surge in aspartame sales emerged later and more steadily than in the USA. In the mid-1980s, aspartame accounted for a little more than 2% of the European market for intense sweeteners but had risen to 20% in volume terms by 1996 (IASR, 2004). The United Kingdom (UK) was the biggest consumer of intense sweetener in the European Union; in 1987, 31% of the general population used aspartame regularly (i.e. weekly) (IASR, 2004). In 2018–2020, the only aspartame manufacturer in the European Union (France) produced 1181–2921 tonnes of aspartame annually (European Commission, 2022b). In 2018, production volume in the European Union was 1181–1750 tonnes, in 2019 it was 1379–2043 tonnes, and in 2020 it was 1971–2921 tonnes. Total production in the European Union during 1 year (2020–2021) was in the range of 1963–2909 tonnes (European Commission, 2022b). In Sweden in 2000–2020, annual use of aspartame varied between 3 and 33 tonnes (SPIN, 2023).

1.2.3 Uses

Aspartame is used as a food additive (sweetener) to provide a sugar-like sweetness, both as an ingredient in various foods and beverages and as a tabletop sweetener (Homler, 1984). It is also used for sweetening purposes in various consumer products, such as medicines, cosmetics, tobacco products, and edible cannabis products (see Section 1.4). Soon after its introduction to the market (see Section 1.5), a major use of aspartame in the USA was in artificially sweetened beverages (ASBs), and by the mid-1980s most producers had switched to using aspartame as the only sweetener in these beverages (USDA, 1986). According to industry data supplied to the government of the UK in 1990, [84%] of aspartame use was for soft drinks, followed by tabletop sweeteners [9%], and dry mixed products [4%] (Food Advisory Committee, 1991). Less than 1% was used in pharmaceuticals. A United States Department of Agriculture report published in 2012 stated that between 85% and 90% of aspartame was used as a sweetening agent in beverages, mainly diet carbonated soft drinks (USDA, 2012). Chewing gums and tabletop sweeteners each accounted for 5% of aspartame use.

Aspartame exhibits synergy with carbohydrates and other high-potency sweeteners and may be used in combination (Homler, 1984). Aspartame also has flavour-enhancing and flavour-extending (by up to four times) properties, especially for acid fruit flavours (Abegaz et al., 2012). According to estimates by Abegaz et al. (2012), aspartame is used in approximately 6000 different products worldwide (see Section 1.4).

In biochemical research, aspartame has been used as a model compound to study structure–activity relationships of the sweet taste receptor (Morini et al., 2005).

1.3 Detection and quantification

Aspartame has been analysed in a variety of sample types, but most methods have focused on the application for foods and beverages and usually involve separation by liquid chromatography (LC) followed by different detection methods. Table 1.1 shows a selection of methods used for the detection of aspartame in various matrices.

1.3.1 Environmental samples

Most studies reporting quantification of aspartame in environmental samples have analysed surface water (Baena-Nogueras et al., 2018), wastewater (Kokotou and Thomaidis, 2013; Watanabe et al., 2016), or sewage sludge (Subedi et al., 2014) (see Table 1.1). Analysis of air (NIOSH, 1994; Gan et al., 2013, 2014), dust, and soil (Gan et al., 2014) samples has also been reported. Most methods include filtration before solid-phase extraction and separation by LC. National Institute for Occupational Safety and Health (NIOSH) method 5031 (NIOSH, 1994) describes the measurement of aspartame in air using LC coupled to an ultraviolet (UV) detector with a detection limit of 2 µg per sample of 70–1200 L of air sampled. Detection and quantification in samples with low concentrations of aspartame, e.g. environmental samples (see Section 1.4.1), are usually carried out using mass spectrometry (MS). Methods report varying limits of detection in aqueous samples, ranging from micrograms per litre (e.g. Kokotou and Thomaidis, 2013, 1.6 µg/L) to less than nanograms per litre (e.g. Baena-Nogueras et al., 2018, 0.34 ng/L).

1.3.2 Food

Many methods have described the analysis of aspartame in food and beverages. The analysis of cloudy liquids and semi-solid and solid foods usually involves homogenization and filtration and/or precipitation of particles, which might be followed by solid-phase extraction, whereas clear beverages require little sample preparation (Table 1.1). Separation is usually performed by LC. Aspartame concentrations in foods and beverages are usually higher than in environmental samples, and less sensitive detectors, such as UV detectors or evaporative light-scattering detectors, are sufficient for the analysis of these sample types in a regulatory or food safety context (reviewed in Oktavirina et al., 2021) considering the maximum limits (see Section 1.5). Recently, more sensitive MS-based approaches that allow simultaneous detection of several sweeteners have been reported (Shah and de Jager, 2017). Of the three standardized validated methods in the European Union, two use high-performance liquid chromatography-ultraviolet radiation (HPLC-UV) methodology (CEN, 1996, 1999, 2009) and one uses liquid chromatography-evaporative light-scattering detection (LC-ELSD) (CEN, 2010). One method determines aspartame content in tabletop sweeteners (CEN, 1996), whereas the other two methods allow the measurement of aspartame in a broader range of foods and beverages. Wasik et al. reported an in-house validated procedure to measure aspartame content in soft drinks, canned foods, and yogurts (Wasik et al., 2007). Maes et al. reported a nuclear magnetic resonance (NMR)-based method for the measurement of aspartame in soft drinks that involves only minimal sample preparation, including degassing and pH adjustment (Maes et al., 2012).

Table 1.1 Selected analytical methods for the measurement of aspartame

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Environmental samples				
Coastal water	Filtration, acidification, SPE	UPLC-MS/MS (0.34 ng/L)		Baena-Nogueras et al. (2018)
Sewage sludge	Freeze-drying, MeOH/water extraction, SPE	HPLC-MS/MS (LOQ, 5 ng/g dw)		Subedi et al. (2014)
Wastewater	Filtration, acidification, SPE	LC-MS/MS (1.6 µg/L)		Kokotou and Thomaidis (2013)
Wastewater	Filtration, acidification, SPE	LC-MS (≤ 23 ng/L*)	*LOD for aspartame was not reported. LODs for five artificial sweeteners including aspartame and five iodinated contrast media were 0.015–23 ng/L.	Watanabe et al. (2016)
Dust and soil	Water extraction, SPE	LC/MS (0.3 ng/L)		Gan et al. (2014)
Air	Quartz fibre filter, extraction with MeOH	LC-MS/MS (0.003 pg/m^3)		Gan et al. (2013)
Air	Polytetrafluoroethylene filter	HPLC-UV (2 µg per sample)	NIOSH method 5031, 70–1200 L per sample.	NIOSH (1994)
Foods				
Food and beverages	Clear liquids and beverages: dilution with water, filtration Cloudy liquids, powders, semi-solid and solid foods: dilution, precipitation with Carrez solution or dilution in NaCl/HCl, filtration	HPLC-UV (NA)	CEN/TS 15606:2009. Validated from 97 mg/kg to 610 mg/L in water-based drink, fruit-based drink, cheesecake with biscuit base, canned soup, and instant chocolate drink.	CEN (2009)
Tabletop sweeteners	Grinding, dissolution, filtration	HPLC-UV (NA)	EN 1378:1996.	CEN (1996)
Beverages, canned fruit	Degassing by ultrasound; homogenization (fruit only), SPE	HPLC-ELSD (NR)	EN 15911:2010. Validated from 38.1 mg/L to 702 mg/L in beverages and from 37.2 mg/kg to 1120.2 mg/kg) in canned fruits.	CEN (2010)
Soft drinks, canned food, yogurt	Homogenization, SPE	HPLC-ELSD (energy drinks, 14 µg/g; yogurt, canned fruit, 13 µg/g)	In-house validated procedure.	Wasik et al. (2007)
Soft drinks	Ultrasound degassing, acidification	NMR (zero cola sample, 2.9 mg/L; light cola sample, 4.6 mg/L)	In-house validated procedure.	Maes et al. (2012)

Table 1.1 (continued)

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Consumer products				
Alternative tobacco products Lozenges	Acetonitrile/water extraction, filtration	LC-MS (NR)		Miao et al. (2016)

dw, dry weight; HCl, hydrochloric acid; HPLC, high-performance liquid chromatography; HPLC-ELSD, high-performance liquid chromatography-evaporative light scattering detection; LC-MS, liquid chromatography-mass spectrometry; LC-UV, liquid chromatography with ultraviolet detection; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MS/MS, tandem mass spectrometry; NA, not available to the Working Group; NaCl, sodium chloride; NMR, nuclear magnetic resonance; NR, not reported; SPE, solid-phase extraction; UPLC, ultra-performance liquid chromatography.

1.3.3 Drugs and consumer products

An LC-MS based method (Miao et al., 2016) for the analysis of aspartame in lozenges and alternative tobacco products (snus) including solvent extraction has been reported.

1.3.4 Biological specimens

Details on the metabolism of aspartame in humans are described in Section 4.1. Because of the hydrolysis of aspartame to its major constituents – aspartic acid, phenylalanine, and methanol – which are also endogenous metabolites, no validated biomarkers are available for the biomonitoring of aspartame exposure. One study reported the measurement of aspartame in serum using an ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) based method (Liu et al., 2022a). [The Working Group noted that the study by Liu et al. (2022a) lacked some detail in the description of the analytical method, since the analytical conditions seemed to be based on a method for the detection of aspartame in beverages (Kubica et al., 2016), but no data on the performance of the method validation for the analysis of serum were provided. The quality of this work was uncertain, since the method was also described to be based on a method that did not aim to detect aspartame (Logue et al., 2017).]

1.4 Occurrence and exposure

1.4.1 Environmental occurrence

Aspartame is not found naturally in the environment, but it can be produced and released into the environment through various industrial processes, such as the manufacture of food and beverage products that contain aspartame. Aspartame can also enter the environment through wastewater emissions from households and industries that use products containing aspartame. See Table 1.2 for measurements of aspartame in different environmental samples.

(a) Air

Volatilization from dry and moist soil and water surfaces is not expected to be substantial (Lambert et al., 2010). Gan et al. (2014) examined air samples in China for the presence of artificial sweeteners, including aspartame. Aspartame was not detected in any air samples during the summer, but in winter it was detected at concentrations up to 5.39 pg/m^3 in 5 out of the 21 particulate-phase samples (Gan et al., 2014). This was in line with the assumption, based on physicochemical data, that aspartame would be expected to exist solely in the particulate phase (EFSA, 2021). Aspartame does not contain chromophores that absorb at wavelengths > 290 nm and therefore is not expected to be susceptible to direct photolysis by sunlight (EFSA, 2021).

(b) Water

Aspartame appears to be more persistent in freshwater than in saltwater systems. Abiotic degradation half-life (DT_{50}) in water at pH 3 was estimated as being approximately 300 days, and degradation results mainly from hydrolysis of the ester and amide bonds (Lambert et al., 2010; EFSA, 2021). Cyclization to the diketopiperazine (see Section 1.1) increases at higher pHs, and the DT_{50} at pH 5, 6, and 7 has been estimated as 245, 120, and 1 day(s), respectively (Crosby and Furia, 1980). The normal pH range for surface water and groundwater systems is usually pH 6.0–8.5; should aspartame reach surface or groundwaters, it may therefore be considered persistent under some conditions (EFSA, 2021).

Table 1.2 lists examples of studies that have aimed to measure aspartame in drinking-water, groundwater, surface water, or wastewater. In drinking-water and tap water in a study from Mexico, aspartame concentrations ranged from below the limit of detection (120 µg/L) to 760 µg/L (Medrano et al., 2019). In a study

Table 1.2 Occurrence of aspartame in environmental samples

Sample type	Location and collection date	No. of samples	Mean (range)	Median (IQR)	Analytical method (LOD)	Comments	Reference
Surface water Urban wastewater	São Paulo state, Brazil, 2019	21 24	ND ND	ND ND	Online SPE-UPLC-MS/MS (0.2 µg/L)	Surface water was sampled from a river at 7 locations, 25 km apart. Wastewater was sampled at 5 WWTPs.	Alves et al. (2021)
Surface water, wastewater, sludge	Shenzhen city, China, 2019	Wastewater, 16 SPM, 8 Dewatered sludge, 4 Surface water, 12 Offshore SPM, 12			UPLC-MS/MS (LOQ, 5 ng/g dw)	Wastewater was sampled at 4 WWTPs. Aspartame was mainly distributed in SPM and sludge, and the fractions in offshore water exceeded 45%.	Guo et al. (2021)
	West coast	4	Influent, 0.7 µg/L	NR			
		4	Effluent, 0.2 µg/L	NR			
		2	Sludge, 0.2 µg/g	NR			
		12	Offshore water, 0.2 µg/L; offshore SPM, (0.1–2.3 µg/g)	NR			
	East coast	22	ND for influent, effluent, sludge, offshore water	NR			
Wastewater	Australia, 2016	Influent, 124	16 g ± 38 (SD) g/day Mean population-weighted per capita loads, 0.12 ± 0.14 mg/day	NR	HPLC-QTrap MS/MS with ESI (0.08 µg/L)	Nationwide study of 69 WWTPs. Aspartame was detected in 91% of influent samples; 100% removal of aspartame was observed during wastewater treatment.	Li et al. (2020)
		Effluent, 35	ND in effluent	NR			
Wastewater	Lake Ontario, Canada, 2014	55	Raw sewage, 1200 (61–8007) ng/L	Raw sewage: 676 ng/L	Online SPE-LC-APCI-MS/MS (75 ng/L)	The concentration of aspartame was below the LOD in 4 (out of 55) raw sewage samples and in 18 (out of 18) treated effluents.	Tolouei et al. (2019)

Table 1.2 (continued)

Sample type	Location and collection date	No. of samples	Mean (range)	Median (IQR)	Analytical method (LOD)	Comments	Reference
Wastewater, surface water	Cadiz Bay, Spain, February to July 2015	24	WWTP, 1–4 Influent, NR (< 0.01–0.1) µg/L Effluent, NR (< 0.01–0.09) µg/L	NR	SPE-UPLC-QqQ-MS/MS (0.34 ng/L)	4 WWTPs, 13 coastal water sampling sites. Values are ranges during the sampling period. Aspartame detection frequency, depending on the sampling site, varied between 33% and 100% and it was always detected at < 0.1 µg/L.	Baena-Nogueras et al. (2018)
		24	Surface waters, 1–4 Receiving waters: NR (< 0.01–0.04) µg/L	NR NR			
		28	Coastal waters, [18.2] (7–37) ng/L	[19.5] ng/L			
Wastewater, surface water, groundwater	Hanoi, Viet Nam, 2013	7	*WWTP* Influent, NR (570–3100 ng/L)	2100 ng/L NR	LC-MS with ESI (NR)		Watanabe et al. (2016)
		7	Effluent, ND	ND			
			Surface water				
		5	Hong River, ND	ND			
		1	Canal, ND	ND			
		4	Lu River, NR (1700–2600 ng/L)	2100 ng/L			
		2	Pond, ND	ND			
			Groundwater				
		4	Urban, ND	ND			
		2	Suburb, ND	ND			
		3	Rural, ND	ND			
	Haiphong Viet Nam, 2013	6	*Surface water* Two rivers, ND	ND			
	Halong, Viet Nam, 2013	3	*Groundwater:* ND	ND			
	Manila, Philippines, 2015	5	*Surface water*: ND	ND			
	Pathein, Myanmar, 2014	4	*Surface water*: ND	ND			
	Yangon, Myanmar, 2014	4	*Surface water*: ND	ND			

Table 1.2 (continued)

Sample type	Location and collection date	No. of samples	Mean (range)	Median (IQR)	Analytical method (LOD)	Comments	Reference
Wastewater, sludge	Albany, New York, USA, 2013	11	Influent, 0.13 (0.01–0.44) µg/L Primary effluent, 0.49 (0.03–1.30) µg/L Effluent, 0.11 (0.02–0.22) µg/L Sludge, 0.08 (ND–695) µg/g dw	NR	HPLC-MS/MS (NR)	Wastewater and sludge were sampled in 2 WWTPs. Aspartame detection frequency in influent, primary effluent, and effluent was 100%; in sludge, it was [91%].	Subedi and Kannan (2014)
Sewage sludge	Republic of Korea, 2011	WWTP, 40			HPLC-ESI-MS/MS (LOQ, 5.0 ng/g)	Nationwide study.	Subedi et al. (2014)
		Industrial, 15	129 (36.3–266) ng/g dw	140 ng/g dw			
		Mixed, 9	1010 (28.4–2400) ng/g dw	321 ng/g dw			
		Domestic, 16	746 (55.9–5220) ng/g dw	311 ng/g dw			
Wastewater, surface water, soil	Eggenstein-Leopoldshafen and Karlsruhe, Germany, 2009	NR	ND	ND	HPLC-ESI-MS/MS) (LOQ, 5 ng/L)		Scheurer et al. (2009)
Wastewater	Hanoi, Viet Nam, 2018 to 2020	184	0.17 ± 0.18 (SD) µg/L (ND–2.2 µg/L)	0.12 µg/L	LC-QTrap MS (LOQ, 0.05 µg/L)	Samples collected in urban sewer canal with domestic wastewater.	Li et al. (2023)
Wastewater, surface water, tap water, coastal water, tap water, precipitation, air	Tianjin, China, 2011	Air gas phase, 4	[1.0] (0.5–1.6) pg/m^3	[1.0] pg/m^3	LC-MS/MS (tap water and seawater, 0.1 ng/L; river water, 0.3 ng/L)	Water samples collected from 2 WWTPs; 18 sites in Haihe river; 8 sites in Dagu drainage canal; 10 sites in Bohai Sea coast; 7 sites in Dahuangpu wetlands; tap water in city centre; groundwater (well water) in suburbs.	Gan et al. (2013)
		Air particulate phase, 4	[0.7] (0.2–1.4) pg/m^3	[0.6] pg/m^3			
		Air TSP, 4	[0.10] (0.07–0.12) pg/m^3	[0.11] pg/m^3			
		Wastewater influent, 6	[48.5] (NR) ng/L	NR			
		Surface water, 54	[33.9] (29–36) ng/L	[33.5] ng/L			
		Wetlands, 21	[22.9] (20–28) ng/L	[22.0] ng/L			
		Drainage canal, 24	[92.5] (53–210) ng/L	[58.5] ng/L			
		Sea coast, 30	[16.4] (7.7–37) ng/L	[15.0] ng/L			
		Groundwater, 15	ND	NR			
		Tap water, 15	ND	NR			

Table 1.2 (continued)

Sample type	Location and collection date	No. of samples	Mean (range)	Median (IQR)	Analytical method (LOD)	Comments	Reference
Air, surface water, outdoor dust, soil	Tianjin, China December 2012 (winter); September 2013 (summer)	Air gas phase, 42	NR (ND–0.63) pg/m^3	NR	LC-MS/MS Air (0.003 pg/m^3); Precipitation (rain or snow) (0.3 ng/L); River water (0.4 ng/L); Wastewater (0.5 ng/L); Dust (dry precipitate) (0.3 ng/g); Soil (0.3 ng/g)	Air and water samples collected once per 2 days, and 7 samples were collected at each of the 3 sampling sites in Tianjin. Precipitation samples were collected in Tianjin. Outdoor dust and soil samples were collected nationwide.	Gan et al. (2014)
		Air particulate phase, 42	NR (ND–5.39) pg/m^3	NR			
		Water (river water and wastewater), 42	[0.15] (ND–6.21) ng/L	NR			
		Outdoor dust, 98	[0.28] (ND–23.0) ng/g	NR			
		Soil, 98	ND	NR			
		Dry precipitate, 3	ND	NR			
		Rainfall, 6	NR (ND to < 1.1) ng/L	NR			
		Snow, 3	[9.4] (< 1.1–14.7) ng/L	NR			
Domestic sewage	Two cities in Zhejiang province, China, 2017–2018	NR	NR (ND–7.57) µg/L	NR		Detection rate of aspartame in sewage water was 33.3%.	Ma et al. (2020)
Source water		NR	NR (ND–0.73) µg/L	NR		In source water it was 18.2%.	
Drinking-water, tap water	Pachuca City, Mexico, NR	Bottled water, 7	[0.25] (ND–0.75) mg/L	NR	SPE-LVSS-CE (0.12 mg/L)	Aspartame was found in 3 bottled water samples, 2 tap water samples, 2 distilled water samples, and 1 spring water sample.	Medrano et al. (2019)
		Tap water, 7	[0.14] (ND–0.76) mg/L				
		Drinking-water, 4	[0.13] (ND–0.33) mg/L				
		Spring water, 2	[0.25] (ND–0.49) mg/L				

Table 1.2 (continued)

Sample type	Location and collection date	No. of samples	Mean (range)	Median (IQR)	Analytical method (LOD)	Comments	Reference
River water, groundwater (drinking-water)	Ganges river basin, India, 2014	River water, 14 Groundwater, 14	River water, < LOD Groundwater, < LOD	NR	UPLC-MS (0.2 ng/mL)	Groundwater was collected from handpumps – from which it was used as drinking-water.	Sharma et al. (2019)
Biosolids	Australia, 2016	71 WWTP	GM, 2.8 ng/g (< 1.6–73 ng/g)	0.81 ng/g	LC-MS/MS (LOQ, 1.6 ng/g)	Nationwide study.	Li et al. (2021b)

APCI, atmospheric pressure chemical ionization; dw, dry weight; effluent, treated wastewater; ESI, electrospray ionization; GM, geometric mean; HPLC, high-performance liquid chromatography; influent, raw wastewater; IQR, interquartile range; LC, liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; MS/MS, tandem mass spectrometry ND, not detected; NR, not reported; QTrap, quadrupole ion trap; SD, standard deviation; SPE-LVSS-CE, solid-phase extraction-large-volume sample stacking-capillary electrophoresis; SPE-QqQ-MS/MS, solid-phase extraction triple quadrupole-tandem mass spectrometry; SPE-UPLC-MS/MS, solid-phase extraction-ultra-high-performance liquid chromatography-tandem mass spectrometry; SPM, suspended particulate matter; TSP, total suspended particulates; UPLC-MS, ultra-performance liquid chromatography-mass spectrometry; wt, weight; WWTP, wastewater treatment plant.

from Tianjin, China, aspartame was found in all samples of surface waters at concentrations up to 0.21 µg/L but was not found in groundwater or tap water (Gan et al., 2013). In India, water from the Ganges River and groundwater in the river basin were sampled in several locations and aspartame was not detected (limit of detection, 0.2 µg/L) (Sharma et al., 2019).

The occurrence of aspartame in wastewater has been monitored mainly in wastewater treatment plants (WWTPs) since the late 2000s in various locations, namely Australia, Brazil, Canada, China, Germany, the Republic of Korea, Spain, the USA, and Viet Nam (Table 1.2). Most of the studies either did not detect aspartame or detected it only at low concentrations. In four WWTPs in the Bay of Cadiz, Spain, the highest measured influent aspartame concentrations varied between 0.07 and 0.10 µg/L between February and July 2015 (Baena-Nogueras et al., 2018), and a median concentration of 2.1 µg/L was measured in a WWTP in Hanoi, Viet Nam, between October and December 2013 (Watanabe et al., 2016). In another study of wastewater in Hanoi, Viet Nam, in 2018–2020, aspartame was found in 97% of 184 samples; the median concentration was 0.12 µg/L and the maximum was 2.2 µg/L (Li et al., 2023). Aspartame was detected in 33% of wastewater samples collected in Zhejiang Province, China; the highest concentration measured was 7.57 µg/L (Ma et al., 2020). In 2014, the median value in the influent of a WWTP located in Lake Ontario, Canada, was 0.68 µg/L and the aspartame detection rate was [93%] (Tolouei et al., 2019).

In addition to WWTP effluent discharges, the sludge generated can also contain aspartame. In a nationwide survey in 40 WWTPs in the Republic of Korea, aspartame concentrations in sludge ranged from 0.03 to 5.22 µg/g dry weight (dw) (Subedi et al., 2014). In the Albany area of New York state, USA, sludge samples collected from two WWTPs contained aspartame at a mean concentration of 0.08 µg/g dw (Subedi and Kannan, 2014).

WWTPs are considered to be a major source of environmental emissions of artificial sweeteners (Subedi et al., 2014). When aspartame reaches a WWTP, whether the sweetener is then emitted into the aquatic environment will depend upon the efficiency of treatment facilities to remove it from wastewater (EFSA, 2021). The average removal efficiency was about 70% in one study (Subedi and Kannan, 2014) and up to 100% in two other studies (Tolouei et al., 2019; Li et al., 2020).

[The Working Group noted that, because of the complete metabolism of aspartame in mammals, aspartame in wastewater most probably derives from food and beverage residues discarded into the sewage system rather than from human or animal excretions (see Section 4.1 for details on aspartame metabolism).]

(c) Soil

Aspartame is expected to rapidly degrade in soil because of hydrolysis by microbial activity (EFSA, 2021). Aspartame may degrade in moist soil at pH 7 or higher, with a hydrolysis half-life of about 1 day (Lambert et al., 2010). This suggests that aspartame would not be persistent in soil systems (EFSA, 2021). Aspartame would be expected to be highly mobile and not readily absorb to soils, sewage sludge or sediments (EFSA, 2021). The partition coefficient (log *P*) for aspartame suggests an affinity for the aqueous phase rather than for lipids and, considering that the predicted bioconcentration factor for aspartame is 3.2, this would imply that the bioaccumulation risk is low (EFSA, 2021). [The Working Group noted that there were limited data available to support or refute theoretical assumptions about the fate of the compound in soil.]

(d) Consumer products

Aspartame occurs commonly as an excipient in oral pharmaceutical preparations, such as chewable tablets and liquid formulations, to improve the flavour of medicines and mask unpleasant taste characteristics (Al Humaid, 2018; NCBI, 2022). For example, the taste of pioglitazone can be effectively masked using aspartame, making orally disintegrating tablets palatable to patients (Nakano et al., 2013). The aspartame content of medicinal products is usually < 100 mg per dosage unit. Examples of these products include effervescent tablets containing amoxicillin or ibuprofen, orodispersible tablets containing paracetamol, oral solutions containing penicillin, or bacterial suspensions to treat diarrhoea in infants. Aspartame concentrations of 1–100 mg per dosage unit may be found in medicinal products indicated for short-term use, such as paracetamol or laxatives in adults or children aged > 12 years (Duodecim Drug Database, 2023). The use of some medicinal products may result in daily aspartame doses of > 2000 mg (EMA, 2017).

According to the European Commission database for information on cosmetic substances and ingredients, CosIng (European Commission, 2022a), aspartame can occur as a flavouring agent in cosmetics, e.g. to improve the taste of oral hygiene products such as toothpaste (Welss, 2015). The INCI Beauty database (INCI Beauty, 2022) suggested that aspartame is present in 0.01% of cosmetics, examples including lip products, whitening tooth powders, intimacy moisturizing fluids, and anti-cellulite gels. The Consumer Product Information Database (CPID) lists the use of aspartame in one personal care product (DeLima Associates, 2022). [The Working Group noted that these databases might have different scopes and might not be exhaustive.]

Aspartame can also occur as a sweetener in alternative tobacco products such as snus (for example, 0.008–0.013% in 3 out of 4 analysed samples in one study; Miao et al., 2016) and in edible cannabis products (Health Canada, 2022).

(e) Food and beverages

This section contains available information on the presence of aspartame in foods and beverages that was obtained from composition and labelling databases, audits, and consumer panels, in addition to published use (concentration) levels of aspartame in retail products.

[The Working Group noted that most data on the occurrence of aspartame were from the USA, Europe, and Oceania, and that there was a lack of studies from Africa and some regions in Asia.]

(i) Presence of aspartame in food

Occurrence data from composition and labelling databases, audits, and consumer panels were available from published studies in Australia, Brazil, France, New Zealand, Slovenia, and the USA (see Table S1.1, Annex 1, Supplementary material for Section 1, Exposure Characterization, online only, available from: https://publications.iarc.who.int/627).

Recent tracking information from Australia and New Zealand suggested a reduction in occurrence over time. For example, audit data from four supermarket stores in New Zealand in 2013 and in 2019 indicated that by 2019 aspartame was no longer among the top three most commonly available non-nutritive sweeteners (Nunn et al., 2021). Aspartame was present in only 14% of products in 2019 versus 21% in 2015 in a similar Australian audit (Dunford et al., 2022). Beverages, confectionery, snack foods, and sugars and related products were the main food sources of aspartame in these countries (Nunn et al., 2021; Dunford et al., 2022). Data for France indicated that tabletop sweeteners were a key contributing source of aspartame, in addition to ASBs (Chazelas et al., 2021; Debras et al., 2022a). In addition, tracking data for more than 1000 food products during 2017–2020 in Slovenia showed that of 333 beverages that contained

an artificial sweetener, aspartame was present in 102 (31%) (Hafner and Pravst, 2021). It was present only in combination with other sweeteners, primarily acesulfame-K (42 products) (Hafner and Pravst, 2021). Supermarket audit data from Brazil (to support an exposure assessment) revealed that aspartame was one of the top three most commonly used sweeteners, often found in combination with acesulfame-K and in food groups such as "diet/light concentrates and industrialized juices" and "other sweets" (Takehara et al., 2022).

[The Working Group noted that trends in occurrence have been poorly documented in the publicly available literature. A combination of scientific references and other literature sources (e.g. government reports, marketing and industry data and news articles) (see Section 1.2.3, and Annex 2, Scientific and other publicly available data on aspartame use in artificially sweetened beverages, also available from: https://publications.iarc.who.int/627) suggested that although major diet soft drink brands in the USA from the mid-1980s until the late 1990s were sweetened entirely with aspartame, this was probably no longer the case by the mid-2000s. For example, the share of aspartame among high-intensity sweeteners in beverages was more than 80% in 2002, but dropped to about 70% in 2009 and 2014. In the same time frame, the share of aspartame decreased from about 30% to < 15% in tabletop sweeteners, and from 55% to < 20% in other foods (USDA, 2012). Trends for beverages in Europe were likely to be similar, but with probable differences in the dates of introduction of different sweeteners across European countries, generally with an earlier switch from aspartame only to mixed sweetener preparations than in the USA.] It was reported that many diet soft drink bottlers in the European Union, Canada, and the USA switched from 100% aspartame to blends of aspartame and acesulfame-K (IASR, 2004). It has also been suggested that aspartame has become a less popular low-calorie sweetener because sucralose is listed as being more commonly used (Sylvetsky and Rother, 2016).

Databases such as Open Food Facts (Open Food Facts, 2023), Mintel Global New Products Database (Mintel Group Ltd, 2023), and OQALI (data from France; INRAE, ANSES, 2023) cover many countries and also provide information on the occurrence of aspartame in food and beverages. [The Working Group noted the value of such databases, which can also provide information on the presence of aspartame in foods. However, the degree of completeness of the database may vary by country.]

(ii) Use levels of aspartame in food

Table 1.3 provides data on use levels (concentrations) of aspartame in foods as reported in published scientific papers and by the European Food Safety Authority (EFSA) (EFSA, 2013). All included studies were from European regions except for three studies from Asia (Republic of Korea; Lee et al., 2017; and China; Chang and Yeh, 2014; Li et al., 2021a). [The Working Group noted that data from the USA were not publicly available, although this has been the largest market for aspartame.] The most comprehensive assessment of food types was included in the EFSA opinion (EFSA, 2013), with seven other studies focusing on concentrations present in beverages only (alcoholic: Lachenmeier et al., 2009; Li et al., 2021a; non-alcoholic: Leth et al., 2008; Lino et al., 2008; Basílio et al., 2020; van Vliet et al., 2020; or both: Kubica et al., 2015). This reflected the greater presence of aspartame in non-alcoholic beverages (particularly "light" or sugar-free varieties) across all studies and regions globally. Aspartame was also reported in European studies in food groups such as flavoured fermented milk products (e.g. flavoured yogurts and yogurt drinks), tabletop sweeteners, food supplements, sports foods (e.g. protein or amino acid products), chewing gums, some processed fruit and vegetables (e.g. canned, bottled, dried varieties and jellies, jams and marmalades), powdered

Table 1.3 Concentrations of aspartame in food

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Non-alcoholic beverages	Denmark, 2005	Total: 177			HPLC (NR)	Study also quantified acesulfame-K, cyclamate, and saccharin (intense sweeteners detected in 76 samples).	Leth et al. (2008)
Total detects		76 (67)					
Of which:		Of which:					
Light soft drinks with carbon dioxide		56 (52)	212 (31–560)				
Light soft drinks without carbon dioxide		20 (15)	85 (11–310)				
Non-alcoholic beverages	Portugal, 2006–2007	Total: 48 (39)			RP-HPLC, (LOQ, 3.5 mg/L)	Drinks sampled based on responses by teenagers to a questionnaire on intake of sugar-free soft drinks and nectar; LOD, NR.	Lino et al. (2008)
Light (diet, or sugar-free) soft drinks		25 (23)	89 (8.8–339)				
Soft drinks based on mineral waters		13 (8)	82 (19–154)				
Light (sugar-free) nectars		10 (8)	73 (39–129)				
Alcoholic beverages: fruit wines	Poland	19 (3)	[38] (35–42)		DART-MS/MS (LOD, 30 µg/L)	Aspartame present in combination with acesulfame-K. The mean value was calculated from the three positive samples.	Lachenmeier et al. (2009)
Carbonated cola beverages:	Germany and France, 2010–2011	129			^{1}H NMR (LOD, 3.5 mg/L)	All cola samples. Often co-occurrence of other sweeteners.	Maes et al. (2012)
Branded cola	Germany	19 (0)	ND				
	France	8 (0)	ND				
Branded cola light	Germany	13 (5)	[149] (143–165)	[146]			
	France	6 (6)	[250] (243–255)	[249]			
Branded cola zero	Germany	6 (6)	Overlap (overlapping spectra, no quantification possible)			Overlap, 6/6	

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Branded cola zero	France	8 (7)	[450] (416–601)	[427]		Overlap, 1/8	Maes et al. (2012) (cont.)
Branded cola light no caffeine	France	5 (5)	[237] (224–255)	[237]			
Branded cola vanilla	Germany	2 (0)	ND				
Cola discount	Germany	18 (0)	ND				
	France	13 (0)	ND				
Cola light discount	Germany	10 (10)	Overlap			Overlap, 10/10	
	France	0	0				
Cola zero discount	Germany	10 (10)	[138] (115–168)	[137]		Overlap, 5/10	
	France	6 (6)	[367] (330–388)	[374]			
Cola strong	Germany	3 (0)	ND				
Flavoured fermented milk products included heat-treated products	Europe (use levels from industry, analytical data from Austria, Netherlands, Slovakia, Italy), 2000–2012	Industry: NA Austria: 0 Netherlands: 12 (NR) Slovakia: 1 (1) Italy: 0	Industry: 50–1000 Austria: NA Netherlands: 54–132 Slovakia: 93.5 Italy: NA	NR	NR	Industry typical maximum values listed.	EFSA (2013)
Edible ices		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: 40–50 Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NR		"Typical-max" values.	
Processed fruit and vegetables		Industry: NA Austria: 137 (NR) Netherlands: 0 Slovakia: 1 (1) Italy: 0	Industry: 350–1000 Austria: 107–695 Netherlands: NA Slovakia: 74.5 Italy: NA	NR		"Typical-max" values.	
Cocoa and chocolate products as covered by Directive 2000/36/EC (European Parliament and Council, 2000)		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: 500–1000 Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NR		Industry reports for cocoa probably refer to Spain only.	

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Other confectionery without added sugar		Industry: NA Austria: 7 (NR) Netherlands: 4 (NR) Slovakia: 6 (NR) Italy: 0	Industry: 100–1000 Austria: 68.3 Netherlands: 12 Slovakia: 151–912 Italy: NA	NR			EFSA (2013) (cont.)
Chewing gum with added sugar		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: 600–1450 Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NR		"Typical-max" concentrations.	
Chewing gum without added sugar		Industry: NA Austria: 5 (NR) Netherlands: 0 Slovakia: 1 (1) Italy: 0	Industry: 3650–5420 Austria: 40–1747 Netherlands: NA Slovakia: 561 Italy: NA	NR		"Typical-max" concentrations.	
Breakfast cereals		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NR			
Processed fish and fishery products including molluscs and crustaceans		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NR			

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Tabletop sweeteners		Industry: NA Austria: 19 (NR) Netherlands: 1 (1) Slovakia: 0 Italy:10 (NR)	Industry: 8700 –36 000 (powder); 100 000–360 000 tablet form Austria: < 0.25 to 238 600 (tablet) Netherlands: 230 647 (tablet) Slovakia: NA Italy: 0–500 000 (tablet)	NR		"Typical-max" concentrations.	EFSA (2013) (cont.)
Mustard		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NA			
Soups and broths		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NA			
Sauces		Industry: NA Austria: 2 (NR) Netherlands: 0 Slovakia: 0 Italy: 0	Industry: 200 Austria: < 4 Netherlands: NA Slovakia: NA Italy: NA	NR		Limited representation of the European market. Levels refer to ketchup.	
Salads and savoury-based sandwich spreads		Industry: NA Austria: 6 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: < 20 Netherlands: NA Slovakia: NA Italy: NA	NA		Austria: only Feinkostsalat.	

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding 13/1/5) (European Commission, 1999)		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: 180 Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NA			EFSA (2013) (cont.)
Dietary foods for weight control diets intended to replace total daily food intake or an individual meal		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NA			
Fruit nectars as defined by Council Directive 2001/112/EC and vegetable nectars and similar products (Council of the European Union, 2001)		Industry: NA Austria: 19 (NR) Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: < 20 to 71 Netherlands: NA Slovakia: NA Italy: NA	NR			
Flavoured drinks with sweeteners		Industry: NA Austria: 272 (NR) Netherlands: 40 (NR) Slovakia: 60 (NR) Italy: 0	Industry: 100–600 Austria: < 1 to 496 Netherlands: < 3 to 470 Slovakia: 3.5–25.6 Italy: NA	NR			
Alcoholic beverages, including alcohol-free and low-alcohol counterparts		Industry: NA Austria: 47 (NR) Netherlands: 0 Slovakia: 4 (NR) Italy: 0	Industry: 100–600 Austria: < 1 to 104 Netherlands: NA Slovakia: 7.4–25.6 Italy: NA	NR			
Potato-, cereal-, flour-, or starch-based snacks		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NA			

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Processed nuts		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NA			EFSA (2013) (cont.)
Desserts excluding products covered in category 1, 3, or 4 (flavoured fermented milk products, edible ices, and processed fruits and vegetables)		Industry: NA Austria: 0 Netherlands: 0 Slovakia: 0 Italy: 0	Industry: NA Austria: NA Netherlands: NA Slovakia: NA Italy: NA	NA			
Food supplements as defined in Directive 2002/46/EC excluding food supplements for infants and young children		Industry: NA Austria: 3 (NR) Netherlands: 17 (NR) Slovakia: 0 Italy: 0	Industry: NA Austria: < 20–1845 Netherlands: < 20–2245 Slovakia: NA Italy: NA	NR			European Parliament and Council (2002)
Foods and beverages	Pingtung, China, 2012	42		NR	LC-MS/MS (LOQ, 0.1 mg/kg)	The article has a greater focus on method development rather than being a comprehensive survey. Aspartame was detected in only two cola drinks and one sample of marinated olives.	Chang and Yeh (2014)
Non-alcoholic beverages Of which: Lemon tea Sports drinks Soft drinks Juices		11 (2) Of which: 1 (0) 2 (0) 2 (2) 6 (0)	[76.8] (55.1–98.4)				
Alcoholic beverages Of which: Cocktail Plum flavoured wine Grape wines Flavoured beer		16 (0) Of which: 1 (0) 3 (0) 8 (0) 4 (0)		NR			

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Preserved fruit and vegetables		15 (1)	87.1	NR			Chang and Yeh (2014) (cont.)
Of which:		Of which:					
Pickled pepper		1 (0)					
Preserved fruits		11 (1)					
Candies		3 (0)					
Food and food supplements	Rome, Italy, 2014	Total: 290		NR	UPLC-MS/MS (LOQ, 10 µg/L; LOD, 3 µg/L)	Sampling informed by national food consumption survey. All food contained aspartame at less than maximum levels established by the European Commission. Within the flavoured beverage category, heterogeneity in occurrence was identified between categories – aspartame was more likely to be present in cola-type beverages than in iced tea or sports drinks.	Janvier et al. (2015)
Beverages:		78					
Of which:		Of which:					
Flavoured drinks		57 (20)	162 (120)				
Fruit nectars		18 (3)	39 (49)				
Syrups		3 (0)	0				
Jams		14 (0)	0				
Ketchups		1 (0)	0				
Confectionery		84					
Of which:		Of which:					
Hard candy		42 (16)	773 (320)				
Chewing gum		42 (42)	1922 (1245)				
Ice-creams		3 (3)	89 (16)				
Yogurts		42 (5)	187 (24)				
Tabletop sweeteners		14 (2)	388 (32)				
Food supplements		54 (11)	5797 (3814)				
Alcoholic and non-alcoholic drinks	Gdansk, Poland, NR	24 (2)	[38] (9.88–66)	NR	RP LC/MS (LOD, 1.63 ng/mL; LOQ, 4.90 ng/mL)	Paper more focused on method development.	Kubica et al. (2015)
Carbonated non-alcoholic		5 (0)	NA				
Non-carbonated non-alcoholic		11 (0)	NA				
Carbonated alcoholic		5 (2)	[38] (9.88–66)				
Instant drink powders		3 (0)	NA				

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Foods and beverages (no supplements)	Republic of Korea, domestic and imported products, NR	Total: 908		NR	HPLC-ELSD and LC-high-resolution MS (LOD, 3.4 mg/kg; LOQ, 10.3 mg/kg)	Mean of all samples including non-detects. Number of detects was calculated by the Working Group but presented as detection rate (%) in the publication. Means of positive samples were 1.3–23 times as high as means of all samples. *Takju* is a grain-based, non-distilled alcoholic beverage.	Lee et al. (2017)
Snacks		51 [7]	28.4 (ND–310)				
Candy		41 [8]	74.5 (ND–2251)				
Chewing gum		43 [9]	59.3 (ND–1057)				
Chocolate		13 [5]	11.2 (ND–48)				
Processed cocoa products		33 [5]	269 (ND–5649)				
Pickled food		46 [4]	6 (ND–185)				
Yogurt		33 [3]	17 (ND–250)				
Carbonated beverages		47 [3]	10 (ND–202)				
Yogurt flavoured beverages		14 [6]	66 (ND–352)				
Beverages (other)		46 [2]	96 (ND–2474)				
Takju		42 [32]	47 (ND–144)				
Food and food supplements	Dublin, Ireland, NR	Total, 375 (210)		NR	UPLC-MS/MS (NR)	Numbers of sampled foods (occurrence) do not match up; not clear from text how many detects for some foods. Aspartame alone was detected in 35 samples. In addition, it was present in combinations with acesulfame-K, saccharin, sucralose, and/or cyclamate in 175 samples.	Buffini et al. (2018)
ER or NAS dairy products		75 (44)	225.7 (88.7)				
ER or NAS carbonated flavoured drinks		98 (26)	277 (237.7)				
ER or NAS flavoured drinks		98 (60)	179.4 (86.4)				
Solid food supplements		21 (11)	9800 (1.9)				
Tabletop sweeteners		18 (5)	306.3 (396.6)				
Sauces		15 (0)	ND				
Other		50 (20)					
ER or NAS jams, jellies, marmalades		8 (0)	414.7 (127.7)				

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
ER or NAS cocoa and chocolate products		13 (NR)	255.4 (98.9)				Buffini et al. (2018) (cont.)
ER or NAS non-chocolate confectionery		4 (NR)	1294.5 (33.47)				
ER or NAS ice-cream cornet and wafers		5 (0)	ND				
ER soups and broths		1 (0)	ND				
Cider and perry		1 (1)	126				
Mixtures of alcoholic and non-alcoholic drinks		2 (0)	ND				
Potato-, cereal-, or flour-based snacks		6 (1)	45				
ER or NAS desserts		4 (1)	135				
Syrup-type or chewable food supplements		3 (NR)	18.9 (3.0)				
Non-alcoholic beverages	Portugal, NR	56 (21)		NR	LC-UV (LOD, 59.8 mg/L)	Small convenience sample. Mean and range values were calculated for positive samples.	Basílio et al. (2020)
Traditional soft drinks		27 [10]	139 (< LOQ to 419.3)				
Tea-based soft drinks		10 [4]	90.7 (< LOQ)				
Soft drinks based on mineral waters		4 [3]	< LOQ (< LOQ)				
Sport drinks		4 (0)	ND				
Energy drinks		2 (2)	479 (278–680)				
Nectars		9 [2]	203 (< LOQ to 315)				

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Non-alcoholic beverages	10 European area countries (Belgium, Denmark, Finland, France, Germany, Spain, Sweden, Netherlands, Türkiye, UK). NR	Total: 111 (95)	30–527 103–1790 µmol/L	NR	HPLC-MS/MS LOD, [0.6 µg/L] 0.002 µmol/L LOQ, [2.1 µg/L] 0.007 µmol/L	Total Phe also available in this study. Groupings not always self-evident, e.g. some cola drinks in the "other category". Numbers sampled per category were unclear, with some overlap between categories. Value used for conversions calculated by the Working Group: 1 mol = 294.30 g aspartame. Numbers per country ranged from Denmark (2), France (4), Türkiye (4), Sweden (4), Germany (7), Spain (7), UK (9), Belgium (15), Finland (21), to Netherlands (38).	van Vliet et al. (2020)
Orange drinks with sugar		8 (4)	[41] [0–108] 0–368 µmol/L	[29]			
Orange drinks without sugar		5 (5)	[230] [75–306] 255–1040 µmol/L	[275]			
Lemon drinks with sugar		12 (8)	[105] [0–232] 0–790 µmol/L	[105]			
Lemon drinks without sugar		7 (7)	[303] [104–462] 594–1570 µmol/L	[302]			
Cola drinks with sugar		2 (0)	ND				
Cola drinks without sugar		36 (35)	[0–527] 0–1790 µmol/L	NR			
Other drinks including "energy drinks"		40 (35)	[0–480] 0–1630 µmol/L	NR			
Alcoholic drinks	China, NR	17	LOD		RT QTRAP MS (LOD, 0.03 mg/L)	LOD presented as 30 µg/L in the paper.	Li et al. (2021a)
White liquor		6 (0)	ND				
Beer		2 (0)	ND				
Whiskey		1 (1)	< LOQ				
Red wine		4 (0)	ND				
Yellow wine		3 (0)	ND				

Table 1.3 (continued)

Sample type	Location and collection date	No. of samples (no. of detects)	Mean (SD or range, as available) mg/kg or mg/L	Median (IQR) mg/kg or mg/L	Analytical technique (LOD or LOQ)	Comments	Reference
Foods and beverages	Germany, 2000–2022	5703 [3720]			Various methods, most commonly HPLC (EN 12856) (LOD, NR)	Most important sample groups out of a data set of 53 116 food monitoring results, of which 14% were positive for aspartame. In column 3, the number of quantifiable samples is shown, not the number of detects.	Schorb et al. (2023)
Chewing gum		312 (241)	1543 (1042)	1369			
Sports foods		297 (125)	1453 (1461)	1030			
Fibre supplements		20 (11)	1248 (175)	1276			
Powdered drink bases		195 (162)	1068 (672)	1133			
Candies		603 (339)	473 (332)	440			
Diet soft drinks		2783 (2021)	91 (101)	60			
Soft drinks		1167 (574)	59 (74)	34			
Flavoured milk drinks		268 (207)	48 (17)	47			
Mixed beer drinks		58 (40)	24 (15)	26			

DART-MS/MS, direct analysis in real time with tandem mass spectrometry; ELSD, evaporative light scattering detector; ER, energy reduced; ^{1}H NMR, ^{1}H nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; IQR, interquartile range; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; NA, not applicable; NAS, no added sugar; ND, not detected; NNS, non-nutritive sweetener; NR, not reported; Phe, phenylalanine; QTRAP, Q Trap mass spectrometry; RP, reverse phase; RT, real time; SD, standard deviation; UPLC, ultra-performance liquid chromatography; UV, ultraviolet.

drink bases, and confectionery without added sugar (EFSA, 2013; Janvier et al., 2015; Buffini et al., 2018; Schorb et al., 2023). This was broadly similar for the most comprehensive study in Asia (Republic of Korea), but the presence in processed cocoa products and *takju* (a type of rice alcohol) was also noted (Lee et al., 2017). Although aspartame was approved for use in many food categories under European Union law, at the time of the analyses submitted to inform the EFSA opinion (EFSA, 2013) it appeared that aspartame was often not present. For example, no detection data were reported in 14 out of the 23 food categories studied, e.g. for mustards, edible ices, cocoa and chocolates, breakfast cereals, processed fish, soups and sauces, sandwich spreads, foods for special medical purposes and weight control diets, processed nuts, or starch-based snacks (EFSA, 2013). [Analytical data were reported from four countries – Austria, the Kingdom of the Netherlands (hereafter "the Netherlands"), Slovakia, and Italy – with variations in the number of samples analysed. The Working Group noted that it was unclear whether detection was not reported in the 14 food categories because the foods were not analysed or because aspartame was not detected.] Aspartame was detected in similar food groups in a large survey (n = 53 116) in Germany in 2000–2022 in which only 14% of samples were positive for aspartame (Schorb et al., 2023).

Aspartame concentrations were typically highest in tabletop sweeteners, chewing gums, and beverages. The highest concentrations were reported in tabletop sweeteners (solid form): industry use levels were reported as 100 000–360 000 mg/kg and analytical values from Italy (tablet form) as 0–500 000 mg/kg (EFSA, 2013); later studies revealed mean concentrations of 388 000 mg/kg in Italy (Janvier et al., 2015) and 306 300 mg/kg (tablet/powder form) in Ireland (Buffini et al., 2018). [The Working Group noted that in the European Union, there was no maximum permitted level concentration outlined for tabletop sweeteners, unlike for other food categories; addition is quantum satis (no numerical maximum is defined; see Section 1.5), and this may explain the variation in concentrations present.]

There was scant information on chewing gum, with recent data suggesting that, when present, aspartame concentrations ranged from 0–1057 mg/kg in the Republic of Korea (Lee et al., 2017) to a mean value of 1922 mg/kg in Italy (Janvier et al., 2015). EFSA reported industry use levels of up to 5420 mg/kg, yet the cited analytical data suggested a range of 40–1747 mg/kg in five samples from a contributing Austrian study (EFSA, 2013). An average of 1543 mg/kg (median, 1369 mg/kg; maximum, 4617 mg/kg) was found in chewing gum (n = 312) on the German market during 2000–2022 (Schorb et al., 2023).

Information on concentrations present in food supplements and foods targeted at individuals participating in sport was scant. Available information indicated concentrations in food supplements of < 20–2245 mg/kg in the Netherlands (EFSA, 2013), a mean of 5797 mg/kg in Italy (Janvier et al., 2015), mean concentrations of 9.8 and 18.9 mg/kg in Ireland (reflecting solid and syrup/chewable food supplements, respectively (Buffini et al., 2018), and mean concentrations of 1248 and 1453 mg/kg for fibre supplements and sports foods (e.g. protein and amino acid powder to prepare drinks), respectively, in Germany (Schorb et al., 2023).

Of note, although studied less frequently in the publications identified, lower concentrations of aspartame were also measured in other food categories e.g. dairy products (including fermented milks and yogurts), ice creams, processed fruits and vegetables, chocolates and processed cocoa products, pickled foods, and confectioneries and candies (EFSA, 2013; Janvier et al., 2015; Lee et al., 2017; Buffini et al., 2018; Schorb et al., 2023).

(iii) Beverages

Beverages were the most frequently studied food category. Concentrations in beverages depended on categorization; the categories of "light" or "reduced sugar" or "no added sugar" were not always transparent and thus comparison was difficult. Studies published before 2015 indicated mean concentrations in any beverage type of up to 212 mg/L (Leth et al., 2008), 89 mg/L (Lino et al., 2008), 35–42 mg/L (range) (Lachenmeier et al., 2009), [279] mg/L (Maes et al., 2012), and 496 mg/L (upper limit of range) (EFSA, 2013). Studies published after 2015 indicated mean concentrations in beverages of 162 mg/L (Italy; Janvier et al., 2015), 277 mg/L (Ireland; Buffini et al., 2018), 139 mg/L (Portugal; Basílio et al., 2020), 92 mg/L (Republic of Korea; Lee et al., 2017), and 91 mg/L (Germany; Schorb et al., 2023). However, a recent study spanning 10 countries in the European area (Belgium, Denmark, Finland, France, Germany, the Netherlands, Spain, Sweden, Türkiye, and the UK) indicated the presence of aspartame in both sugar-containing and non-sugar-containing beverages (van Vliet et al., 2020). Van Vliet et al. (2020) reported consistently higher aspartame levels in orange, lemon, and cola drinks without sugar [0–527 mg/kg] than in sugar-containing versions [0–232 mg/L]. Other beverages containing aspartame included energy drinks, with levels of [80–160 mg/L] (n = 10) (van Vliet et al., 2020) and 278–680 mg/L (n = 2) (Basílio et al., 2020) in European studies. Of note, older studies (Leth et al., 2008; Lino et al., 2008; Maes et al., 2012) reported consistently high levels of detection (75–100% of beverages tested). More recent studies revealed greater variability. Van Vliet and colleagues recorded [86%] detection across beverage categories of 111 drinks with and without sugar (van Vliet et al., 2020). In contrast, detection levels in beverages were 38% in a Portuguese study targeting non-alcoholic beverages (Basílio et al., 2020), 8% in a Polish study on 24 soft and alcoholic drinks (Kubica et al., 2015), [66%] in an Irish study on non-alcoholic beverages (n = 196) (Buffini et al., 2018), [10%] and 76% in a comprehensive analysis of non-alcoholic and alcoholic (*takju*) beverages in the Republic of Korea, respectively (Lee et al., 2017), and 5% in a small study (n = 17) focusing on alcoholic beverages in China (Li et al., 2021a). Data for 2008–2016 from the Danish Veterinary and Food Administration Laboratory, which was responsible for monitoring food additives including intense sweeteners in non-alcohol beverages, suggested that, from 2010, aspartame was not present singly in any beverage, but was used in combination with other sweeteners (Nielsen and Zederkopff Ballin, 2009; Villadsen and Jakobsen, 2012; Jensen and Christiansen, 2014, 2016). [The Working Group noted that although the total samples analysed in these reports were not directly comparable year on year and that across all years there remained considerable variability in the concentrations of aspartame added to beverages, these reports may be suggestive of reduced use levels in recent years.] One study reported aspartame levels in beverages in one urban area of Nigeria (Shinggu and Bekab, 2018). [The Working Group noted that only five samples were analysed, which limits informativeness and comparability to the other studies.]

[For all studies on beverages, the Working Group also noted that the difference in detection rates and concentrations present may be explained by non-representative sampling, often relying on small numbers of samples of beverages. Further, the Working Group noted a lack of information relating to the use of aspartame as part of a mix of sugar and artificial sweeteners in beverages.]

(iv) General considerations

[The Working Group noted considerable limitations across all data (i.e. for foods and beverages). Differences in the categorization of foods and sampling protocols made strict comparisons

difficult. Four studies used consistent categorization of foods according to European Union legislation (EFSA, 2013; Janvier et al., 2015; Buffini et al., 2018; Schorb et al., 2023), but most other studies had their own categorization process. A variety of analytical methods were also employed with associated differences in detection sensitivity and with some papers focusing on method optimization rather than a comprehensive assessment of retail products. Where provided, sample sizes were in many instances low, except for beverages. The Working Group also noted the variability in concentrations present for a single food category, particularly for tabletop sweeteners, and between countries where specific formulations (e.g. for beverages) may differ.]

1.4.2 Occupational exposure

There was one report from NIOSH on occupational exposure to aspartame for workers in a plant that produced dry dessert and drink mixes in Ohio, USA (NIOSH, 1992). Workplace air measurements on a total of 148 personal breathing zone and general area air samples, across three shifts on four consecutive days were performed. Aspartame concentrations in personal breathing zone samples and area air samples ranged from not detected (ND) to 545 μg/m^3 and from ND to 83 μg/m^3, respectively. Four operations were identified as being of major interest because of higher exposure potential, namely, weigh-out, blending and packing operator, and packing helper. For these operations, full-shift breathing zone personal samples were collected, and the highest values obtained for each operation were 151 μg/m^3, 102 μg/m^3, 100 μg/m^3, and 20 μg/m^3, respectively. The short-term personal sampling performed in the same operations supported the findings; the highest peak concentrations obtained during blending and weigh-out were 213 and 432 μg/m^3, respectively. Thus, tasks for which aspartame powder was manually handled involved higher exposures.

In 2018–2020, the only aspartame manufacturer in the European Union employed 63–112 persons, some of whom may have been occupationally exposed to aspartame if the risk management measures in place did not provide the effectiveness needed to control exposure by inhalation (European Commission, 2022).

[The Working Group noted the lack of comprehensive exposure data in an occupational context. The Working Group also noted that, despite the lack of exposure data, occupational exposure to aspartame may occur during synthesis of the compound and production of aspartame-containing products.]

1.4.3 Exposure of the general population

Data on estimated aspartame exposure in the general population are presented in Table 1.4 and Fig. 1.3. Levels of exposure of the general population have been assessed in several studies, mostly in the last two decades. Foods, including tabletop sweeteners, and beverages are the main sources of exposure to aspartame (see Sections 1.2 and 1.4.1(e)). The target population of these reports included different sex and age groups (toddlers, children, adolescents, adults, and the elderly), as well as selected populations such as low-income groups and pregnant or lactating women. Although many of the early reports were based mainly on selected volunteer studies (convenience samples), later reports included nationally representative studies or large cohort studies. Most of the studies assessed dietary exposure using dietary assessment data such as multiple-day 24-hour recalls or records, and food frequency questionnaires (FFQs), whereas others were market basket methods and surveys such as food label surveys (market share data) and household surveys. In most reports, estimates were made from the analysis of total diet studies or using a tiered (or scenario) approach

Table 1.4 Measurement of aspartame exposure in the general population

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	Portuguese National Survey, 2015–2016 Overall	5005	Tier 2.1: 3.09 Tier 2.2: 0.31 Tier 3: 0.14		Tier 2.1: 8.92 Tier 2.2: 1.39 Tier 3: 0.59	Adults: 1-day food diaries Children: two 24-hour recalls	Tier 2.1: actual national food consumption data combined with the maximum permitted usage levels for the additive for all foods reported. Tier 2.2: actual national food consumption data combined with the maximum permitted usage levels for the additive for the foods identified as having non-nutritive intense sweetener according to the brand information. Tier 3: actual national food consumption data combined with the actual usage levels of the additive, given by analytical values of aspartame occurrence in foods	Carvalho et al. (2022)
	Children	521:	Tier 1: 8.78 Tier 2: 0.54 Tier 3: 0.30		Tier 1: 17.72 Tier 2: 2.34 Tier 3: 1.31			
	Adolescents	632	Tier 1: 5.68 Tier 2: 0.59 Tier 3: 0.22		Tier 1: 12.02 Tier 2: 2.32 Tier 3: 0.90			
	Adults	3102	Tier 1: 2.74 Tier 2: 0.30 Tier 3: 0.13		Tier 1: 6.95 Tier 2: 1.33 Tier 3: 0.55			
	Elderly	750	Tier 1: 1.36 Tier 2: 0.14 Tier 3: 0.08 (units: mg/kg bw per day)		Tier 1: 3.75 Tier 2: 0.65 Tier 3: 0.37 (95th percentile, units: mg/kg bw per day)			
Dietary exposure	Pubertal and paediatric endocrine outpatient clinics in multiple centres in Taiwan, China, dates not available	102 children and adults	1.58 mg/day			FFQ	Validation study for FFQ	Chu et al. (2022)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	Beirut and Mount Lebanon, February to August 2020	384 adult Lebanese individuals	98.9 mg/day (1.38 mg/kg bw per day)			FFQ	77.13 mg/day of aspartame from beverages, 21.11 mg/day from food, and 0.66 mg/day from pills and powders.	Daher et al. (2022)
Dietary exposure	French population-based cohort NutriNet-Santé, 2009–2021	102 865 adults	9.35 mg/day			Three non-consecutive web-based 24-hour-dietary records		Debras et al. (2022b)
Dietary exposure	Pregnant Brazilian women, cross-sectional study that used data from the Multicenter Study of Iodine Deficiency (EMDI-Brazil), February 2019 to May 2020	243 pregnant women	Scenario 1: 0.57 Scenario 2: 0.67 Scenario 3: 2.9 (units: mg/kg bw per day)	Scenario 1: 0.00 Scenario 2: 0.00 Scenario 3: 1.82 (units: mg/kg bw per day)	Scenario 1: 2.22 Scenario 2: 2.94 Scenario 3: 7.42 (95th percentile, units: mg/kg bw per day)	24-hour dietary recall	Scenario 1: foods and beverages with confirmed use of aspartame in the list of ingredients. Tabletop sweeteners were also included. Scenario 2: use of aspartame in the food and beverages was defined on the basis of high probability of containing aspartame. Foods and beverages that indicated probability of use such as "diet", "light" etc., were added to those in Scenario 1.	Duarte et al. (2022)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure (cont.)							Scenario 3: all foods and beverages identified in scenarios 1 and 2, added to those in which there was uncertainty of the use of the sweetener, but whose food group indicated the possibility to contain it	Duarte et al. (2022) (cont.)
Dietary exposure	South-eastern Santiago, Chile, 2016–2017	961 low-and middle-income pre-schoolers	2016: 24.6 mg/day 2017: 39.6 mg/day			24-hour dietary recalls		Rebolledo et al. (2022)
Dietary exposure	Brazil, nationwide survey in 2008–2009	34 003 participants	0.08 mg/kg bw per day			1-day dietary records	Intake in general consumers from processed food and beverages and tabletop usage in beverages	Barraj et al. (2021)
Dietary exposure	France, NutriNet-Santé cohort, 2009–2020	106 489 adults	8.63 mg/day 0.13 mg/kg bw per day	0.0 mg/day	95th percentile: 49.92 mg/day, 0.72 mg/kg bw per day	Three non-consecutive web-based 24-hour dietary records		Chazelas et al. (2021)
Dietary exposure	NHANES, USA, 2013–2016					2 complete days of dietary recalls	Scenario 1: brand loyal-deterministic. Reported maximum global use level applied to all beverages.	Tran et al. (2021)
	Toddlers aged 12–35 months	126	Scenario 1: 7.4 Scenario 2: 3.4 Scenario 3: 1.7		23.7 10.8 4.3			

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure (cont.)	Children aged 3–9 yr	579	Scenario 1: 4.4 Scenario 2: 2.2 Scenario 3: 1.4		11.6 6.1 4.3		Scenario 2: brand loyal-deterministic. Reported maximum global use level applied to LNCS CSD; average use level applied to all other beverages. Scenario 3: market share deterministic. Reported maximum global use level applied to LNCS CSD; average use level applied to all other beverages; corresponding fraction of specific LNCS-containing market share data applied to each beverage type.	Tran et al. (2021) (cont.)
	Adolescents aged 10–17 yr	665	Scenario 1: 2.8 Scenario 2: 1.4 Scenario 3: 1.1		7.0 3.9 3.4			
	Adults aged 18–64 yr	2331	Scenario 1: 3.7 Scenario 2: 2.6 Scenario 3: 2.3		10.6 8.5 8.1			
	Elderly aged 65–74 yr	436	Scenario 1: 3.7 Scenario 2: 2.5 Scenario 3: 2.3		13.2 9.0 8.4			
	Very elderly aged 75+ yr	279	Scenario 1: 2.7 Scenario 2: 1.9 Scenario 3: 1.8 (units: mg/kg bw per day)		6.5 5.5 5.4 (95th percentile, units: mg/kg bw per day)			
	UK, NDNS RP, 2008/2009–2016/2017					A 4-day estimated diet diary		
	Toddlers 18–35 months	423	Scenario 1: 12.5 Scenario 2: 5.9 Scenario 3: 4.8		34.4 15.6 13.3			
	Children 3–9 yr	1921	Scenario 1: 7.9 Scenario 2: 4.0 Scenario 3: 3.4		23.3 11.5 10.3			
	Adolescents 10–17 yr	2225	Scenario 1: 4.0 Scenario 2: 2.4 Scenario 3: 2.1		11.5 6.8 6.3			
	Adults 18–64 yr	5019	Scenario 1: 4.7 Scenario 2: 2.4 Scenario 3: 2.3		11.6 6.0 5.6			

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure (cont.)	Elderly aged 65–74 yr	818	Scenario 1: 5.5 Scenario 2: 2.6 Scenario 3: 2.4		13.0 6.2 5.8			Tran et al. (2021) (cont.)
	Very elderly aged 75+ yr	574	Scenario 1: 5.5 Scenario 2: 2.6 Scenario 3: 2.4 (units: mg/kg bw per day)		11.0 5.0 4.7 (95th percentile, units: mg/kg bw per day)			
Dietary exposure	Children aged 6–12 yr residing in Santiago, 2018	250		0.88 (0.25–2.10) mg/kg bw per day		FFQ		Martínez et al. (2020)
Dietary exposure	USA, 2002 and 2018 (Nielsen Homescan Consumer panels)	Number not available				Household purchase data	Drop in prevalence of households purchasing products containing aspartame from 60% to 49%. Aspartame had highest volume per capita purchased of all NNS both years (94.7 g/day and 80 g/day)	Dunford et al. (2020)
Dietary exposure	Pregnant women from the two main cities in Chile, 2016	601	0.91 mg/kg bw per day			Survey		Fuentealba Arévalo et al. (2019)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	A representative sample of Irish adults aged 18–90 yr, the National Adult Nutrition Survey (NANS 2011)	1500	Total population Tier 1: 1.05 Tier 2: 0.62 Tier 3: 0.28 Consumers only Tier 1: 1.04 Tier 2: 0.66 Tier 3: 0.80 (units: mg/kg bw per day)		Total population Tier 1: 5.18 Tier 2: 4.63 Tier 3: 3.57 Consumers only Tier 1: 8.64 Tier 2: 8.65 Tier 3: 7.11 (99th percentile, units, mg/kg bw per day)	4-day, semi-weighed food diaries	Tier 1: a crude assessment that assumed that all foods permitted to contain the additives of interest always did contain them, and at their maximum permitted level. Tier 2: refined assessments estimated intakes of the six sweeteners using food consumption data up to brand level with additive occurrence data from a survey of products currently available on the Irish market. Tier 3: sweetener concentration data (including data from all brands analysed) with sweetener occurrence data from a food label survey.	Buffini et al. (2018)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	Children and adolescents aged 2–18 yr, First Food and Nutritional/ Nutrition Survey of Buenos Aires City, Argentina, 2011	2664				1 day 24-hour recall interview		Garavaglia et al. (2018)
	Age 2–4 yr		6.8	5.5 (2.9–9.3)				
	Age 5–12 yr		4.2	3.2 (1.7–5.3)				
	Age 13–18 yr		2.9 (units: mg/kg bw per day)	2.2 (1.3–4.1) (units: mg/kg bw per day)				
Dietary exposure	Adult participants residing in south-western Virginia, USA, 2015	125	FFQ: 38.7 (0.0–694.5) Dietary recall: 36.5 (0.0–526.4) (units: mg/day)	FFQ: 7.1 Dietary recall: 1.2 (units: mg/day)		FFQ, dietary recall		Myers et al. (2018)
Dietary exposure	Spain, ANIBES study (anthropometric data, macronutrients and micronutrients intake, practice of physical activity, socioeconomic data and lifestyles), a cross-sectional study of a representative sample of the Spanish population (aged 9–75 yr), 2013	2009			Prevalence: 10.7%	3-day dietary records		Samaniego-Vaesken et al. (2018)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	Republic of Korea, Korea National Health and Nutrition Survey (KNHNES), 2010–2013	20 788				Food consumption data (total diet study)		Kim et al. (2017)
					95th percentile			
	Whole population Age < 2 yr Age 3–6 yr Age 7–12 yr Age 13–19 yr Age 20–64 yr Age > 65 yr		0.33 1.28 0.97 0.71 0.54 0.23 0.14 (units: mg/kg bw per day)		1.49 3.98 3.40 2.60 2.15 1.09 0.69 (units: mg/kg bw per day)			
Dietary exposure	INRAN-SCAI, 2005–2006	3323	Step 1: 0.97 Step 2: 0.78 (units: mg/kg bw per day)		95th percentile: 3.85 3.46 (units: mg/kg bw per day)	Food label survey (market share data)	Step 1: all food categories and sweeteners according to the legislation and maximum levels. Step 2: only food categories and sweeteners according to the label survey and maximum levels.	Le Donne et al. (2017)
Dietary exposure	University students Chile, male Chile, female Peru, male Peru, female Guatemala, male Guatemala, female Panama, male Panama, female	1229 155 317 64 140 36 217 84 216	 0.7 (0.07–1.8) 1.0 (0.9–2.3) 0.6 (0.0–3.7) 0.3 (0.01–3.3) 0.4 (0.1–1.7) 2.1 (0.1–4.7) 0.9 (0.3–2.3) 0.9 (0.3–2.8) (units: mg/kg bw per day)			Survey		Durán Agüero et al. (2017)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	USA, Nurses' Health Study (NHS), female registered nurses, aged 30–55 yr, 1994	77 218	All: 102 mg/day			FFQ		Schernhammer et al. (2012)
	USA, Health Professionals Follow-Up Study (HPFS), male health professionals, aged 40–75 yr, 1994	47 810	All: 114 mg/day					
Dietary exposure	Belgium, children with diabetes mellitus, 2014				95th percentile for all, consumers only	FFQ		Dewinter et al. (2016)
	Age 1–6 yr Age 7–12 yr Age 13–18 yr	242	0.91 1.07 0.68 (units: mg/kg bw per day)		2.89, 3.89 2.07, 3.46 1.41, 2.72 (units: mg/kg bw per day)			
Dietary exposure	Ireland, pre-schoolchildren (the National Pre-School Nutrition Survey, NPNS, 2010–2011	500				4-day weighed food diary	Scenario 1: the relevant MPL. Scenario 2: the relevant MPL, taking into account the occurrence of the sweetener in food. Scenario 3: sweetener concentration data; in cases where no analytical data were available for a specific food category, the MPL was applied.	Martyn et al. (2016)
	Total population		Scenario 1: 4.23 Scenario 2: 3.50 Scenario 3: 0.93 Scenario 4: 0.66 (units: mg/kg bw per day)		95th percentile: Scenario 1: 16.42 Scenario 2: 16.07 Scenario 3: 3.17 Scenario 4: 2.72 (units: mg/kg bw per day)			

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure (cont.)	Sweetener consumers only		Scenario 1: 4.63 Scenario 2: 5.06 Scenario 3: 1.02 Scenario 4: 0.76 (units: mg/kg bw per day)		95th percentile: Scenario 1: 18.21 Scenario 2: 18.20 Scenario 3: 3.30 Scenario 4: 2.82 (units: mg/kg bw per day)		Scenario 4: sweetener concentration data, taking into account the occurrence of the sweetener in food; in cases where no analytical data were available for a specific food category, the MPL was applied.	Martyn et al. (2016) (cont.)
Biological level (urine and blood)	Tianjin, China, May 2010 to June 2010 Urine and blood in the general population	54	Not detected			NA	Fasting (> 8 hours) samples	Zhang et al. (2016)
Dietary exposure	French survey on individual dietary consumption (BEBE-SFAE dietary survey), randomly sampled children under age 3 yr, 2005					3 consecutive days, food diary		Mancini et al. (2015)
	1–4 months 5–6 months 7–12 months 13–36 months	124 127 195 259	0.19 2.14 7.41 14.11 (units: mg/kg bw per day)	0 0 4.109 12.632 (units: mg/kg bw per day)	0 8.58 19.03 23.93 (90th percentile, units: mg/kg bw per day)			
Biological level (breast milk)	USA, lactating volunteer women, year not indicated	20	Not detected			NA		Sylvetsky et al. (2015)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	USA, men and women aged 47–95 yr, the Cancer Prevention Study II (CPS-II) nutrition cohort, 1999–2009	100 442 (43 350 men and 57 092 women)	46.6 mg/day	12.6 mg/day (median in quintile 3)	Median in quintile 5 (highest): 145 mg/day	FFQ		McCullough et al. (2014)
Dietary exposure	Norway, dietary surveys, Småbarnskost 2007 and Norkost 3					NR	Scenario 1: Content: average content of aspartame (adjusted for sale). Consumption: actual consumption (the real distribution of consumed beverages added sweeteners from the dietary survey). Scenario 2: Content: average content of aspartame (adjusted for sales). Consumption: it was assumed that all consumed soft drinks, "saft", or nectar contained sweeteners (no sugar).	VKM (2014)
	Age 2 yr	542 1216 542 1216	Scenario 1: 1.50 Scenario 2: 1.73 Scenario 3: 1.79 Scenario 4: 2.03		Scenario 1: 4.32 Scenario 2: 5.29 Scenario 3: 5.11 Scenario 4: 6.26			

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure (cont.)	Women aged 18–29 yr Men aged 18–29 yr Women aged 30–70 yr Men aged 30–70 yr	39 93 39 93 31 100 31 100 209 350 209 350 165 365 165 365	Scenario 1: 2.46 Scenario 2: 2.61 Scenario 3: 2.92 Scenario 4: 3.10 Scenario 1: 1.94 Scenario 2: 3.24 Scenario 3: 2.31 Scenario 4: 3.85 Scenario 1: 2.56 Scenario 2: 2.28 Scenario 3: 3.04 Scenario 4: 2.71 Scenario 1: 2.34 Scenario 2: 2.22 Scenario 3: 2.79 Scenario 4: 2.64 (units: mg/kg bw per day)		Scenario 1: 9.37 Scenario 2: 9.61 Scenario 3: 11.15 Scenario 4: 11.45 Scenario 1: 4.33 Scenario 2: 8.92 Scenario 3: 5.15 Scenario 4: 10.61 Scenario 1: 7.88 Scenario 2: 6.54 Scenario 3: 9.38 Scenario 4: 7.79 Scenario 1: 6.79 Scenario 2: 6.53 Scenario 3: 8.08 Scenario 4: 7.78 (95th percentile, units: mg/kg bw per day)		Scenario 3: Content: the highest value for the amount of added aspartame in soft drinks and "saft" was used for the calculation. Consumption: actual consumption (the real distribution of consumption of beverages added sweeteners from the dietary survey). Scenario 4: Content: the highest value for the amount of added aspartame in soft drinks, "saft", and nectar was used for the calculation. Consumption: it was assumed that all consumed soft drinks, "saft", or nectar contained sweeteners (no sugar).	VKM (2014) (cont.)
Dietary exposure	Italy, INRAN-SCAI, age 18–97 yr, 2005 Aged 1–17 yr, 2005 UK, National Diet and Nutrition Surveys (NDNS)	3323	Tier 2: 0.957 Tier 3: 0.182 Tier 2: 3.098 Tier 3: 0.622		Tier 2: 4.055 Tier 3: 1.176 Tier 2: 12.159 Tier 3: 3.884	3-day estimated food diary 4-day or 7-day weighed food diary	Tier 2: Calculated by combining consumption data with the MPLs Tier 3: Calculated by combining consumption data with concentration data	Vin et al. (2013)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure (cont.)	Aged > 65 yr, 1994–1995	1687	Tier 2: 1.526 Tier 3: 0.307		Tier 2: 4.376 Tier 3: 1.152			Vin et al. (2013) (cont.)
	Aged 19–64 yr, 2000–2001	1724	Tier 2: 2.931 Tier 3: 0.572		Tier 2: 9.629 Tier 3: 2.004			
	Aged 4–18 yr, 1997	1701	Tier 2: 7.445 Tier 3: 1.622		Tier 2: 18.782 Tier 3: 4.906			
	Age 1.5–4.5 yr, 1992–1993	1675	Tier 2: 12.123 Tier 3: 3.069		Tier 2: 28.042 Tier 3: 8.738			
	France, INRAN-SCAI	4079				7-day estimated food diary		
	Age 18–79 yr, 2005		Tier 2: 1.290 Tier 3: 0.530		Tier 2: 4.620 Tier 3: 2.500			
	Age 3–17 yr, 2005		Tier 2: 2.210 Tier 3: 1.000		Tier 2: 6.610 Tier 3: 3.850			
	Ireland, North/South Ireland Food Consumption Survey (NSIFCS), National Children's Food Survey (NCFS), National Teens Food Survey (NTFS)					7-day estimated or weighed food diary		
	Age 18–64 yr, 1997–1999	1379	Tier 2: 4.928 Tier 3: 1.018		Tier 2: 16.175 Tier 3: 3.653	7-day estimated food record		
	Age 13–17 yr, 2005–2006	441	Tier 2: 5.851 Tier 3: 0.989		Tier 2: 14.041 Tier 3: 2.600	7-day estimated food record		
	Age 5–12 yr, 2003–2004	594	Tier 2: 10.498 Tier 3: 1.962 (units: mg/kg bw per day)		Tier 2: 33.034 Tier 3: 5.137 (97.5th percentile, units: mg/kg bw per day)	7-day weighed food record		

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	Belgian Food Consumption Survey, Belgian inhabitants aged ≥ 15 yr, 2004	3245	1.95 mg/kg bw per day	1.74 mg/kg bw per day	4.11, 4.76 (90th, 99th percentile, units: mg/kg bw per day)	2 non-consecutive 24-hour recalls		Huvaere et al. (2012)
Dietary exposure	Japan, National Nutrition Survey, adults aged > 20 yr, 2001–2003	NA		0.14 mg/day		Market basket method	The daily intake based on the analysis of individual foods was more than twice that of the market basket method; therefore, the analysis of individual foods was used.	Kawasaki et al. (2011)
Dietary exposure	Denmark, random sample of people aged 1–80 yr, 1995	3098				A questionnaire with 20 beverages (7 consecutive-day record)	Scenario 1: calculated using analytical determined mean value for all the light samples in the category. Scenario 2: calculated using analytical determined mean value for all the light samples with the content of the specific sweetener.	Leth et al. (2008)
	Both sexes, aged 1–80 yr		Scenario 1: 0.03 Scenario 2: 0.04		0.52, 1.77 0.64, 2.11			
	Both sexes, aged 1–3 yr		Scenario 1: 0.40 Scenario 2: 0.50		1.61, 3.26 2.08, 4.28			
	Both sexes, aged 1–6 yr		Scenario 1: 0.29 Scenario 2: 0.40		1.43, 2.80 1.76, 3.81			
	Boys, aged 7–10 yr		Scenario 1: 0.29 Scenario 2: 0.40		1.22, 2.63 1.62, 3.37			
	Girls, aged 7–10 yr		Scenario 1: 0.26 Scenario 2: 0.34 (units: mg/kg bw per day)		1.02, 1.73 1.30, 2.21 (90th, 99th percentile, units: mg/kg bw per day)			

Table 1.4 (continued)

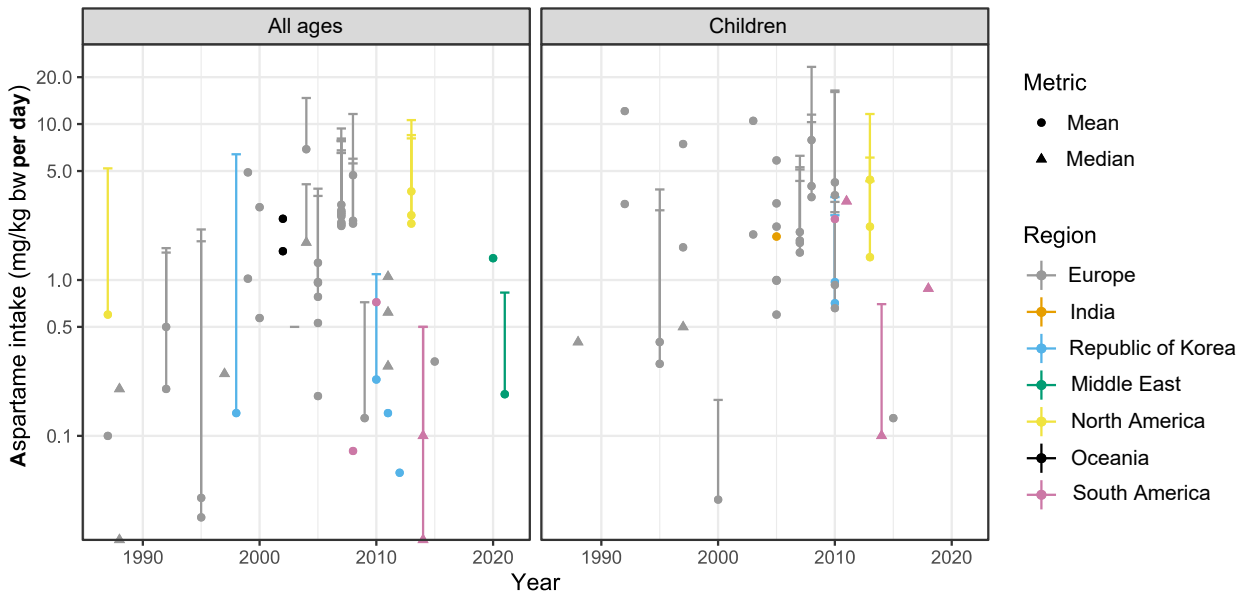
Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	USA (California, Florida, Pennsylvania, New Jersey, North Carolina, Louisiana, and metropolitan Atlanta and Detroit), NIH-AARP Diet and Health Study cohort, age 50–71 yr, 1995–1996	473 984	All: 111 Users: 205 (units: mg/day)			FFQ		Lim et al. (2006)
Dietary exposure	Republic of Korea, National Health and Nutrition Survey, 1998	11 525				24-hour recall		Chung et al. (2005)
	All participants Age 1–2 yr Age 3–6 yr Age 7–12 yr Age 13–19 yr Age 20–29 yr Age 30–49 yr Age 50–64 yr Age > 65 yr		7.7, 0.14 10.5, 0.86 13.1, 0.71 11.6, 0.34 14.5, 0.26 8.3, 0.14 5.2, 0.084 3.8, 0.060 3.4, 0.058 (units: mg/day, mg/kg bw per day)					
Dietary exposure	Rome, Italy, randomly selected teenagers, 2000	3982	All: 0.039 Consumers only: 0.054 (units: mg/kg bw per day)		0.170 (95th percentile, unit: mg/kg bw per day)	12-day (3 days of 4 periods) food records		Arcella et al. (2004)

Table 1.4 (continued)

Metabolite and sample type	Location, population group, and collection date	No. of samples	Mean (range)	Median (IQR)	Relevant percentiles	Method of dietary assessment	Comments	Reference
Dietary exposure	France, children with type I diabetes, aged 2–20 yr, 1997	227 (112 girls and 115 boys)	All: 69.4, 1.9 Consumers only: 82.0, 2.4 (units: mg/day, mg/kg bw per day)	All: 44, 1.1 Consumers only: 55.5, 1.4 (Units: mg/day, mg/kg bw per day)	All: 255.8, 7.8 Consumers only: 263.8, 7.8 (97.5th percentile, units: mg/day, mg/kg bw per day)	5-day diary record		Garnier-Sagne et al. (2001)
Dietary exposure	Rome, Italy, children, aged 13–19 yr, 1996	241	Consumers only: 0.03 mg/kg bw per day	Consumers only: 0.01 mg/kg bw per day	0.13 mg/kg bw per day (95th percentile)	14 consecutive-day food records		Leclercq et al. (1999)
Dietary exposure	Germany, 1988	2291 99	All: 0.05 Users: 1.21 (units: mg/kg bw per day)	All: 0.00 Users: 0.86 (units: mg/kg bw per day)	All: 0.00 Users: 2.75 (90th percentile, units: mg/kg bw per day)	24-hour food diary		Bär and Biermann (1992)

bw, body weight; CPS-II, Cancer Prevention Study II; CSD, carbonated soft drinks; EDMI, Estudo Multicêntrico de Deficiência de Iodo; FFQ, food frequency questionnaire; INRAN-SCAI, Italian National Food Consumption Survey; LNCS, low- and no-calorie sweeteners; MPL, maximum permitted level; NA, not applicable; NCFS, National Children's Food Survey; NDNS RP, National Diet and Nutrition Survey Rolling Programme; NHANES, National Health and Nutrition Examination Survey; NHS, Nurses' Health Study; NIH-AARP, National Institutes of Health-American Association of Retired Persons Diet and Health Study; NNS, non-nutritive sweetener; NR, not reported; NSIFCS, North/South Ireland Food Consumption Survey; NTSF, National Teens Food Survey; yr, year(s).

Fig. 1.3 Aspartame exposure reported in general population-based studies



Aspartame intake in the general population (all ages or children only) reported in the scientific literature (see Table 1.4). The scale is logarithmic; circles, triangles, and lines represent mean, median and 95th-percentile values for all participants surveyed (consumers and non-consumers). The dates represent the years for which the aspartame content in foods was calculated, if this information was available, otherwise it represents the year that the dietary intake was assessed.
Created by the Working Group.

in which the assumed levels of aspartame in food varied from the highest permitted level (the most conservative) to the actual levels of use (European Commission, 2001). For example, in a study among Italian adults (Italian National Food Consumption Survey, INRAN-SCAI) in 2005, the mean intake using conservative estimates was higher (0.957 mg/kg body weight (bw) per day) than the mean intake using actual levels (0.182 mg/kg bw per day) (Vin et al., 2013). [The Working Group noted that direct comparisons between studies should be made with caution because of these methodological differences.]

European countries, including Belgium, Denmark, France, Germany, Ireland, Italy, Norway, Portugal, and Spain started conducting assessments in the late 1980s. Early studies based on a small general population in Germany (Bär and Biermann, 1992) and in Italy (Leclercq et al., 1999) indicated rather low estimates (median, < 1 mg/kg bw per day) among consumers, although a study based on children with type I diabetes in France indicated a slightly higher estimate (1.4 mg/kg bw per day) (Garnier-Sagne et al., 2001). The studies based on larger sample sizes in Italy (Arcella et al., 2004) and Denmark (Leth et al., 2008) also reported the same level of low consumption among the general population. Other studies reported slightly higher estimates of > 1 mg/kg bw per day in some age groups in Belgium (Huvaere et al., 2012), the UK and Ireland (Vin et al., 2013), and Norway (VKM, 2014), even when actual consumption levels were used to estimate realistic intake level. A study in children aged < 3 years in France reported that mean estimated exposure was up to 14 mg/kg bw per day among children aged 13–36 months (Mancini et al., 2015). However, European studies conducted after 2010 reported lower mean estimates (less than or about 1 mg/kg bw per day) in

most tiers (Dewinter et al., 2016; Martyn et al., 2016; Buffini et al., 2018; Carvalho et al., 2022). A study in the USA and UK reported a consumption of up to 4.8 mg/kg bw per day for toddlers (aged 12–35 months in the USA or 18–35 months in the UK) (Tran et al., 2021). EFSA evaluated aspartame as a food additive in 2013 and assessed exposure of the general population in 17 European countries using data from 26 different dietary surveys (EFSA, 2013). The EFSA Scientific Panel on Food Additives and Nutrient Sources added to Food estimated exposure based on the maximum permitted levels and actual levels of aspartame for the general population. Mean levels of exposure for adults (aged 18–64 years) under the conservative approach using the maximum permitted levels were 0.8–8.6 mg/kg bw per day, depending on the dietary survey. High-level exposures, defined as the 95th percentile of consumers, were 2.5–27.5 mg/kg bw per day, depending on the dietary survey. [The Working Group noted that the methodology employed by EFSA for risk assessment was based on the assumption that food groups contained the maximum permitted concentration of aspartame. Therefore, this approach would lead to an overestimation of exposure. Other exposure estimates based on actual levels of use in the general population were highly variable but seemed generally to be much lower (between almost 0 and up to 5 mg/kg bw per day; see Fig. 1.3).]

Data from North America (USA and Canada) have been reported since the late 1990s among large-scale cohort populations (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014; Myers et al., 2018). Because of the use of FFQs and dietary recalls for estimation, values may not be comparable. Using a total diet study methodology, one study considering data from the National Health and Nutrition Examination Survey (NHANES) in 2013–2016 as the basis for the selection of products indicated that estimated mean levels in a market share deterministic scenario ranged from 1.1 mg/kg bw per day for adolescents to 2.3 mg/kg bw per day for the elderly and adults (Tran et al., 2021). Studies based on a representative large sample in Asian countries were limited to mainly to the Republic of Korea and indicated a mean intake of < 1 mg/kg bw per day for the whole population (Chung et al., 2005; Kim et al., 2017). More recently, data were reported from countries in South America (Brazil, Chile, Argentina) (Durán Agüero et al., 2017; Garavaglia et al., 2018; Fuentealba Arévalo et al., 2019; Martínez et al., 2020; Barraj et al., 2021; Rebolledo et al., 2022) and the Middle East (Lebanon) (Daher et al., 2022). These more recent data appeared to be generally lower (almost 0 to 2.1 mg/kg bw per day, although methods used varied), except for one study from Argentina that reported values of up to 6.8 mg/kg bw per day for children (aged 2–4 years).

Although it was difficult to compare temporal or geographical trends in exposure levels because of methodological differences, some studies have reported a decrease in aspartame use between 2002 and 2018 in the USA (Dunford et al., 2022). [The Working Group noted that mean or median exposure levels for adults in general were consistently at low levels (almost 0 to about 5 mg/kg bw per day, depending on the estimation scenario). Children, adolescents, and other populations such as people with diabetes tended to have elevated levels. Estimated levels will be higher when considering consumers only, but this information was only available in studies that measured intake at the individual level. The proportion of consumers of aspartame-containing products varied between countries and time points, which could explain some of the differences observed between countries, e.g. the very low average consumption levels reported in some studies. The Working Group also noted that there was considerable variability in the data underlying these estimates, and that very few studies used analytical data linked to actual brand consumption scenarios, probably because

of difficulties in collecting data and also in keeping the product database up to date.]

1.5 Regulations and guidelines

Adopted in 1995, the General Standards for Food Additives in the Codex Alimentarius of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) established the list of food additives, including aspartame, that are suitable for use in food (FAO/WHO, 1995). In addition to the foods in which aspartame could be used, this document also describes the maximum use levels (ranging from 300 mg/kg to 10 000 mg/kg) for each food category (FAO/WHO, 1995).

Within the European Union, several member states have authorized aspartame for use in foods and as a tabletop sweetener since the 1980s. Sweeteners were first regulated in the European Union in the 1990s with the entry into force of Directive 94/35/EC of the European Parliament and Council Directive 94/35/EC on sweeteners in foodstuffs, also known as the "Sweeteners Directive" (European Parliament and Council, 1994). In 2008, the European Parliament and the Council of the European Union adopted a framework regulation, Regulation (EC) No. 1333/2008, to harmonize all current uses of food additives, including sweeteners (European Parliament and Council, 2008). Annex II of this legislation, established by Commission Regulation (EU) No. 1129/2011, provides a Union list of sweeteners approved for use in foods, beverages, and tabletop sweeteners, and their conditions of use (European Commission, 2011). Under the Commission Regulation (EU) No. 1129/2011 on food additives for use in foodstuffs, maximum permitted levels were established, ranging from 25 to 6000 mg/kg in foods, with the exception of the tabletop sweeteners, for which it is authorized quantum satis (no numerical maximum is defined) (European Commission, 2011). The use of aspartame in organically produced foods is not approved (European Commission, 2008). Regarding the use of aspartame in pharmaceuticals, warnings for patients with phenylketonuria with a zero threshold for use were established, and specific information should be mentioned in the package leaflet (EMA/CHMP/302620/2017; European Medicines Agency, 2019). Similarly, in the European Union and USA, foods containing aspartame in the list of ingredients must indicate in the labelling that they include a source of phenylalanine (US FDA, 1974; European Parliament and Council, 1994, 2011).

In the USA, the United States Food and Drug Administration (US FDA) approved aspartame for restricted use in dry foods in 1974 and for uses under certain conditions, as a tabletop sweetener, in chewing gum, cold breakfast cereals, and dry bases for certain foods (i.e. beverages, instant coffee and tea, gelatins, puddings, and fillings, and dairy products and toppings) in 1981 (Office of the Federal Register, 1981). The US FDA approved the use of aspartame in carbonated beverages and carbonated beverage syrup bases in 1983, and its use as a general-purpose sweetener in 1996 (Office of the Federal Register, 1996).

Aspartame has been assessed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) on several occasions (JECFA, 1975, 1976, 1980, 1981), and an acceptable daily intake (ADI) of 40 mg/kg bw per day has been established (JECFA, 1981). EFSA also adopted this ADI of 40 mg/kg bw per day (EFSA, 2013). The US FDA has set the ADI for aspartame at 50 mg/kg bw (Office of the Federal Register, 1984). An ADI of 7.5 mg/kg bw per day was also established for the diketopiperazine of aspartame (JECFA, 1981). The purity of aspartame for use as a food additive is regulated in the European Union (see Section 1.1.4) (European Commission, 2012). Purity regulations are also available for aspartame in medicinal products (European Pharmacopoeia, 2020; United States Pharmacopeia, 2023).

[The Working Group noted that no threshold has been established for occupational exposure to aspartame.]

1.6 Quality of exposure assessment in key epidemiological studies of cancer and mechanistic studies in humans

1.6.1 Quality of exposure assessment in key cancer epidemiology studies

See Table S1.2 (Annex 1, Supplementary material for Section 1, Exposure Characterization, online only, available from: https://publications.iarc.who.int/627).

This section reviews the exposure assessment methods of studies of cancer in humans evaluated in Section 2 of the present monograph and was organized according to the exposure of interest stated in the original publications (aspartame, artificial sweeteners, or ASBs).

No validated biomarker of exposure was available for aspartame (see Section 1.3.4). Aspartame exposure in cancer epidemiology studies was assessed using self-report methods. Few studies specifically considered aspartame as the exposure; most derived aspartame exposure from the consumption of ASBs and the use of tabletop sweetener packets containing aspartame, assigning aspartame concentration values to these products. Other studies considered artificial sweeteners overall or ASBs. Studies were either cohort studies or case–control studies, and most used FFQs and/or 24-hour dietary recalls or records. The studies and their dietary assessment methods are described in detail below.

There are common limitations to the use of ASBs and packets of tabletop artificial sweeteners as sole sources and proxies for aspartame exposure. First, although ASBs, followed by tabletop artificial sweetener packets, can be considered as the main sources of aspartame (Section 1.4.1), other potential dietary sources also contribute to total aspartame exposure (e.g. "sugar-free", "diet", or "light" versions of foods such as yogurt, gelatin/pudding, ice cream, frozen desserts, hot chocolate, or breakfast cereals). These other sources have become more diversified and abundant over time, and their impact on total aspartame exposure has tended to increase. Additionally, most epidemiological studies did not account for aspartame from oral sources other than foods (including tabletop sweeteners) and beverages, i.e. they did not account for aspartame from dietary supplements, medicinal products, or personal care products. Second, even though aspartame was generally the main type of artificial sweetener used in ASBs at the time when the studies were conducted, the actual aspartame content (presence/absence and dose) of ASBs was variable across product types and brands, and over time and between countries (Section 1.2 and Section 1.4.1). After the introduction of aspartame in the early 1980s, individuals may have been exposed to multiple types and doses of artificial sweeteners depending on the evolution of both authorizations and use of artificial sweeteners. This is important to consider when looking at the timing of the baseline assessment and for the interpretation of the follow-up period as an ongoing exposure to aspartame. Finally, the range of ASBs included in the FFQs was variable across studies and may comprise only cola or only carbonated beverages and, less commonly, non-carbonated beverages. Hence there was potential for misclassification of aspartame exposure, with underestimation of exposure and misclassification of consumers as non-consumers because of the partial consideration of dietary sources, but also overestimation and misclassification of non-consumers as consumers because of the assumption that all ASBs contained aspartame, which was not verified in all time periods and regions.

Other limitations concerned the methods of dietary assessment and were shared by studies

using the same tool. FFQs, used by the majority of studies, capture the usual frequency of consumption over a period of time (usually the past 12 months) of a list of food items. In FFQs, the number and type of included items, the range of frequencies available as response options, as well as the possibility of choosing between several portion sizes (versus standard portion sizes applied to all) are important parameters influencing the quality of assessment of aspartame, artificial sweetener, or ASB intakes. A detailed assessment of aspartame exposure from FFQs would be improved by differentiation between sugar-sweetened and artificially sweetened versions for every food item for which these may exist, and by collection of the exact type but also the name and brand of the product usually consumed, since aspartame content varies between product types and between brands for the same type of product. FFQs are generally considered to be efficient tools for ranking individuals according to their dietary intakes, although they may be prone to recall error when participants are asked to provide a description of their diet during the past 1 year, since recall is known to be influenced by more recent intake (Fowke et al., 2004). FFQs are also less precise for the characterization of food and beverages ingested owing to the aggregation of products into food or line items. In contrast, 24-hour dietary records or recalls collect information on all food and beverages consumed during a 24-hour period. Hence the consumption of virtually all types of aspartame-containing product can be captured, depending on the level of detail of the nomenclature used and on the completeness of the underlying food composition databases. Several days of 24-hour dietary records or recalls at different times of the year are nonetheless necessary to consider day-to-day and seasonal variations in dietary intakes and provide a valid assessment of usual dietary intakes, especially when considering foods or beverages that are not consumed daily (including aspartame-containing products).

For both FFQs and 24-hour recalls or records, a major challenge involves the food composition databases available, since the detail on aspartame content (yes/no) and dose of each food and beverage to the level of product brand is ideally required because of the variability in aspartame concentrations in the food supply.

Patterns of consumption of aspartame-containing products may change over time, and this may not be captured in studies that used a single dietary assessment at baseline (especially when considering long follow-up periods). As a result of food innovations and reformulations, new aspartame-containing products may be released onto the market and the aspartame content of products may also vary over time. Beyond assessing changes in dietary intakes over time, food composition tables should also ideally be regularly updated to reflect the exact aspartame content of a given food product or the average aspartame content for a generic food item, considering available products on the market at the time of dietary assessment.

All the above-mentioned limitations are likely to induce measurement errors and some misclassification of participants with regard to consumption of aspartame, artificial sweeteners, or ASBs. Yet in prospective studies such misclassification would be mostly non-differential, i.e. would have a similar effect on the exposure assessment in participants who developed a cancer and in those who did not.

Lastly, the use of a prospective design in cohort studies, in which diet is assessed before cancer diagnosis, limits the risk of differential misclassification. In case–control studies, diet (current or in past years) is assessed after the diagnosis of cancer, and these studies are more at risk of recall bias, biased assessment, and differential misclassification. This may arise if participants with cancer overestimate or underestimate their consumption of artificial sweeteners or

ASBs compared with controls, if early cancer symptoms may induce changes in dietary habits, or if investigators are not blinded to the case or control status.

All studies are reviewed below, classified according to exposure and study design.

(a) Aspartame

(i) Prospective cohort studies

Five cohort studies investigated associations between exposure to aspartame and cancer risk. Four of the five were conducted in the USA, and of these three (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014) derived aspartame exposure from the consumption of ASBs and the use of aspartame tabletop sweetener packets, assessed through FFQs, whereas the remaining study (Fulgoni and Drewnowski, 2022) assessed exposure through aspartame-containing products reported in 24-hour dietary recalls. More recently, one study in France (Debras et al., 2022b) derived aspartame exposure from all aspartame-containing food and beverages assessed through repeated 24-hour dietary records.

[The Working Group noted that, of all the studies reviewed in Section 1.6.1, these five studies were the only ones that specifically focused on aspartame and provided the most accurate assessment of aspartame exposure. Debras et al. (2022b) performed the most comprehensive and up-to-date assessment of aspartame exposure from all possible sources, whereas the other studies (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014; Fulgoni and Drewnowski, 2022) only considered the two main sources of aspartame (ASBs and tabletop packets). More details, including the strengths and limitations of each of these studies, are provided below.]

The study by Debras et al. (2022b) was conducted in the NutriNet-Santé prospective cohort study and focused on breast and prostate cancers, obesity-related cancers combined, and all cancers combined. Aspartame intake (in mg/day) was assessed with repeated validated web-based 24-hour dietary records. Three 24-hour dietary records were sent to the participants every 6 months, covering 3 days (2 weekdays, 1 weekend day) randomly assigned over a 2-week period. The main model focused on aspartame estimated from the mean exposure over the first 2 years of follow-up, including a minimum of two dietary records (mean ± standard deviation (SD), 5.6 ± 3.0). In sensitivity analyses, a model accounting for all available dietary records throughout the follow-up was also presented. Measurement of aspartame was calculated from food composition data for all products as follows: presence/absence of additives in food products was retrieved from three large-scale databases: OQALI (a national database the management of which has been entrusted to the National Research Institute for Agriculture, Food and Environment and the French Agency for Food, Environmental and Occupational Health & Safety; INRAE, ANSES, 2023), Open Food Facts (Open Food Facts, 2023), and the Mintel Global New Products Database (Mintel Group Ltd, 2023); quantitative doses of additives were retrieved from ad hoc laboratory assays and completed with information for generic food categories provided by EFSA and JECFA. Changes over time and potential reformulations were accounted for by matching the date of consumption of each food or beverage to the closest date for composition data. The NutriNet-Santé study started in 2009 and is ongoing, with a continuous inclusion strategy. The end of the most recent follow-up for this study was in 2021, hence it had a maximum of 12 years of follow-up. [The Working Group noted that this study provided the most accurate assessment of aspartame exposure, considering all possible dietary sources and including a high level of detail through the use of repeated 24-hour dietary records that collected the brand of industrial product and a detailed food

composition database on variations in aspartame content over time. The baseline assessment between 2009 and 2021 reflected up-to-date consumption patterns of aspartame in France and allowed a sufficient delay between aspartame exposure and the development of cancer. Although this strategy did not allow the consideration of potential changes in dietary behaviours over time, potentially resulting in misclassification of participants, sensitivity analyses were conducted incorporating all 24-hour dietary records available during follow-up. Likewise, there was no information on lifetime exposure to aspartame before study entry, occurring between 2009 and 2021, which could lead to misclassification of participants who had changed or stopped their consumption of aspartame-containing products, although this was considered difficult to quantify.]

The study by Fulgoni and Drewnowski (2022) was conducted using data from the NHANES cycles and focused on cancer mortality. Aspartame exposure was assessed from one or two 24-hour dietary recalls performed by a trained interviewer during NHANES 1988–1994. For other NHANES cycles (1999–2018), only low-calorie sweeteners were considered without distinguishing aspartame, and therefore the analysis of aspartame relied on NHANES 1988–1994 only. Follow-up for cancer mortality was performed through 2019 using public-use mortality files from the National Center for Health Statistics. [The Working Group noted that the study methodology was not reported in sufficient detail. For example, it was unclear whether 2 days of records were used. In addition, although the authors reported identifying a list of products that potentially contained low-calorie sweeteners, for the aspartame assessment the NHANES 1988–1994 study documentation appeared to differentiate aspartame only for diet sodas, hot beverages, or added sweeteners, which was appropriate considering the authorization of aspartame in food products in the USA at that time. However, the assessment relied on a low number of dietary recalls (one or two) and the assessment was performed at baseline only, with up to 31 years of follow-up, during which the use of aspartame in food products changed. There was therefore high potential for non-differential misclassification.]

The study by McCullough et al. (2014) was conducted in the Cancer Prevention Study II (CPS-II) nutrition cohort and focused on lymphoid neoplasms (all types). The usual diet over the past year was assessed using a 152-item self-administered modified Willett FFQ, first at baseline in 1999 and then in 2003, and participants were followed until 2009. There were specific validated questions about the consumption of several types of artificially sweetened carbonated beverage (cola with caffeine, other carbonated beverages with or without caffeine) with frequency categories ranging from "never" to "≥ 4 per day" and a standard serving size (1 glass, bottle, or can, 355 mL). Additional questions related to the use of aspartame tabletop packets, with frequencies ranging from "never" to "≥ 6 per day". Long-term soda consumption patterns were available from data collected in 1982 (CPS-II mortality cohort) but aspartame exposure or intake was not calculated, since aspartame was not included in sodas in the USA before 1983. The mean daily exposure over the past year (in mg/day) was estimated in 1999 and 2003, assigning values for aspartame content of 180 mg/355 mL for low-calorie cola with caffeine, 90 mg/355 mL for other low-calorie sodas with caffeine, 70 mg/355 mL for other low-calorie sodas without caffeine, and 20 mg/packet of one of two branded commercial sweeteners containing aspartame. Estimates from 1999 were used to predict the risk for the 1999–2003 period and the mean of estimates for 1999 and 2003 was used to predict the risk for the 2003–2009 period. [The Working Group noted that the strengths of this study included the updated assessment of usual diet halfway through the maximum

10-year follow-up, allowing the consideration of potential variations in the consumption of ASBs or aspartame tabletop packets over time, thus limiting potential misclassification. Several types of soda were considered with corresponding assigned aspartame values, thus providing a more refined assessment of aspartame intake from different types of beverage. No information was available regarding lifetime aspartame exposure before study entry in 1999, which could misclassify participants who stopped their consumption of ASBs or aspartame tabletop packets. However, some information on patterns of sugar-sweetened and artificially sweetened soda consumption in the early 1980s was available, although artificially sweetened soda did not contain aspartame at that time; hence, this study provided a good assessment of aspartame exposure from artificially sweetened carbonated beverages and tabletop packets. However, other potential dietary sources of aspartame were not considered (although these were more limited), which may cause some non-differential misclassification of participants.]

The study by Schernhammer et al. (2012) was conducted in the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS) and focused on lymphoma and leukaemia. A validated self-administered semi-quantitative FFQ of about 130 items was used every 4 years to assess the usual diet of participants, including the frequency of consumption (nine frequencies from never to ≥ 6 times per day) of three types of diet soda (diet cola with caffeine, diet cola without caffeine, and other diet soda) with a standard serving size of 355 mL, starting from 1984 in the NHS and 1986 in the HPFS. The use of two branded commercial sweeteners containing aspartame (added at the table) was queried since 1994 and then every 4 years. Participants were followed until 2006. A cumulative average strategy was used to derive daily intakes of aspartame, whereby intakes were updated after every FFQ with the mean of all reported intakes up to that time. Values of aspartame content were assigned to diet sodas as a weighted average of the representative sodas in that category (i.e. 70–180 mg/serving) and to aspartame packets (20 mg). [The Working Group noted that the repeated assessment of aspartame exposure over a long period (up to 22 years of follow-up) and the use of a cumulative average analysis constitute major strengths of this study. In addition, these cohorts started in the 1980s, that is, concomitantly with the introduction of aspartame in the USA, thus potentially capturing lifetime exposure to aspartame from sodas (and exposure to tabletop aspartame since 1994). Finally, three different types of soda were considered, with corresponding assigned values for aspartame content. Hence, this study provided a good assessment of aspartame exposure from artificially sweetened sodas and tabletop packets. However, other potential dietary sources of aspartame, i.e. non-carbonated soft drinks or other types of foods containing aspartame (although they were limited at the time of this study) were not considered, which may cause some non-differential misclassification of exposure among participants.]

The study by Lim et al. (2006) was conducted in the National Institutes of Health-American Association of Retired Persons Diet and Health Study (NIH-AARP) and focused on haematopoietic cancers and glioma. Usual diet in the past 12 months was assessed at study baseline between 1995 and 1996 using a validated 124-item FFQ, and participants were followed until 2000. Three potentially aspartame-containing beverages were included – soda, fruit drinks, and iced tea – and participants were asked about the frequency of consumption of soft drinks overall (10 frequencies ranging from never to ≥ 6 times per day), the frequency of consumption of the sugar-free or diet versus regular versions of these beverages (usually, or more than half the time) and the portion size (three portion size ranges). The addition of tabletop sweetener packets of

aspartame to cups of coffee and hot tea was also queried. A quantitative average daily exposure to aspartame in milligrams per day was derived from the consumption of diet soft drinks using values of aspartame content assigned per 100 g of beverage (50 mg for diet soda, 14.95 mg for diet fruit drink, 25.55 mg for diet iced tea), and per tabletop sweetener packet (35 mg). [The Working Group noted that the sequential assessment of the frequency of firstly consumption of soft drinks and then of diet/sugar-free versions with imprecise frequencies was likely to induce inaccuracies and thus non-differential misclassification of participants. In addition, this study considered both carbonated and non-carbonated soft drinks but was less precise than the other US studies regarding types of carbonated soft drink (cola or not), which would have an impact on the aspartame exposure assigned to participants (non-differential misclassification). There was a maximum of 5 years between assessment of aspartame exposure and cancer diagnosis; over this limited period of time, the diet could be considered relatively stable, but the impact of aspartame exposure at baseline on the actual onset of cancer was questionable, also because no information was available regarding exposure to aspartame before the study baseline, which occurred 10 years after the introduction of aspartame in the USA. Hence the assessment of aspartame exposure in this study may be weaker than in the other studies described above.]

(ii) Case–control studies

Aspartame was also investigated in relation to cancer in three case–control studies.

Palomar-Cros et al. (2023) conducted a case–control study examining artificial sweetener and common cancers (colorectal, breast, prostate, and stomach, and chronic lymphocytic leukaemia) in Spain. A self-administered, semiquantitative FFQ with 140 food items, capturing usual dietary intake during the previous year, was used. Four items contributed to the assessment of artificial sweetener exposure (low- or no-calorie soft drink, "gaseosa" a typical Spanish soft drink, tabletop sweeteners containing saccharin, and other tabletop sweeteners). The classification of products according to the presence of aspartame was derived from food supply data (OpenFoodFacts, 2023). [The Working Group noted that the validity of the assumption that all low- or no-calorie soft drinks and tabletop sweeteners other than saccharin were aspartame was uncertain, given that this may not have been the case at the time of the exposure assessment (2008–2013). Other potential dietary sources of aspartame were not considered. Also, the retrospective assessment of diet from the year preceding cancer diagnosis could reflect changes in dietary habits as a result of pre-diagnosis symptoms. Non-differential and differential misclassification regarding aspartame exposure was therefore likely in this study.]

Cabaniols et al. (2011) conducted a case–control study on brain cancer in which consumption data were collected in 2005 in France. Aspartame consumption frequency was assessed using a self-administered questionnaire, followed by an in-person interview if information was missing. Participants were asked to recall intakes for the 5 years preceding diagnosis and were categorized as non-consumers (< 1 intake/week) and regular consumers (≥ 1 intake per week). [The Working Group noted that the strength of this assessment was its apparent focus on aspartame, but it was unclear whether this was limited to tabletop use or more broadly included consumption of products containing aspartame. Limitations of the exposure assessment included the retrospective recall of frequency of consumption and the lack of a quantitative assessment (intake was categorized into binary exposure (consumers/non-consumers) only and there was partial consideration of dietary sources). Hence, there was a high potential for misclassification of participants according to their exposure to aspartame in this study.]

Gurney et al. (1997) reported a population-based case–control study of childhood brain tumours in children in the USA. Case patients were born or were in utero in 1981 or later and diagnosed with a primary brain tumour between 1984 and 1991. Biological mothers were queried through in-person interview about their children's consumption of aspartame before the date of diagnosis as well as their own consumption during pregnancy and breastfeeding. Questions related to the age at first consumption of any food, chewing gum, or diet drink containing aspartame, or aspartame tabletop packets, time period of consumption, and frequency of consumption for children, as well as trimesters of consumption, time period of consumption, and frequency of consumption for mothers during pregnancy or while breastfeeding. [The Working Group noted that the period of exposure considered in this study covered lifetime exposure (starting in utero) of the children, which constitutes a major strength. However, only semiquantitative assessment of aspartame exposure could be derived from the questionnaire and, although the sources of aspartame were broadly covered, the lack of specificity regarding the food sources examined may have led to measurement errors. In addition, there was a strong potential for memory bias owing to the retrospective assessment of aspartame consumption several years in the past, and thus a high potential for misclassification of participants according to their exposure to aspartame in this study.]

(b) Artificial sweeteners

Associations between the consumption of artificial sweeteners overall (without assessment of aspartame exposure in particular) and several cancer sites were assessed in case–control studies conducted in various countries.

Singh et al. (2020) conducted a case–control study on thyroid cancer with cases diagnosed between 2004 and 2014 in the USA. Consumption of artificial sweeteners was assessed using a telephone-based, self-report questionnaire on the use of artificial sweeteners, and the total amount of artificial sweetener consumed and duration of consumption were calculated on the basis of consumption of tabletop sweeteners and ASBs. [The Working Group noted that consumption of artificial sweetener from other sources (e.g. snacks/ice creams) was not considered, and no analysis was provided on the individual types of artificial sweetener, including aspartame. However, the exposure period covered the time when ASBs contained mainly aspartame. For tabletop sweeteners, this was not the case, which may have led to overestimation of aspartame exposure (see Section 1.4.1). Misclassification and/or recall bias was likely to result from the retrospective assessment of diet, the measurement of total artificial sweeteners, and the lack of consideration of other sources of aspartame.]

Mahfouz et al. (2014) conducted a population-based case–control study in Egypt, focusing on colorectal cancers diagnosed between 2010 and 2011. Diet in the 2 years preceding cancer diagnosis was reported to be assessed using an FFQ from Melbourne University consisting of eight food groups from which the frequency of intake (daily, weekly, or monthly) and the number of servings were recorded. The study considered qualitatively the use of artificial sweeteners (yes/no) and the consumption of soft drinks (yes/no) without distinction between artificially sweetened and sugar-sweetened soft drinks. [The Working Group noted that overall this study was not very informative regarding aspartame exposure since there was a high potential for misclassification because of the lack of distinction between sugar-sweetened beverages and ASBs, and the lack of precision in the assessment of artificial sweetener use (including the type of artificial sweetener). In addition, there were inconsistencies in the description of the methods (including a misalignment between the reported use of an FFQ from Melbourne University and

the data reported in the study) and in the references cited.]

Kobeissi et al. (2013) conducted a hospital-based case–control study in Lebanon, focusing on urinary bladder cancer diagnosed between 2002 and 2008. A face-to-face interview (not blinded on the participant's status) collected data on the frequency of artificial sweetener consumption (never, rarely, frequently, always) before diagnosis or hospitalization. [The Working Group assumed that, in the absence of details regarding the sources of artificial sweetener, artificial sweetener consumption referred to tabletop artificial sweetener considered as the only source. No quantitative assessment of artificial sweetener exposure was possible and it was not known whether the artificial sweetener consumed actually contained aspartame. This study was therefore not very informative with regard to aspartame exposure because of the partial consideration of sources and especially the absence of consideration of ASBs, resulting in high misclassification potential.]

Andreatta et al. (2008) conducted a hospital-based case–control study in Argentina focusing on urinary tract tumours diagnosed between 1999 and 2006. Participants were interviewed by a nutritionist regarding their habitual use of artificial sweetener in infusions (tea, coffee, mate) in the 5 years preceding diagnosis or hospitalization: ever consumption (yes/no), brand name of artificial sweetener consumed, and duration of consumption (years). Ever use and duration of use were considered in analyses classifying participants into three categories: non-consumers of artificial sweeteners, short-term consumers (1–9 years), and long-term consumers (≥ 10 years). [The Working Group noted that this study considered only artificial sweeteners added to infusions, which, according to the authors, constituted the main source of artificial sweetener in this population. Aspartame was not specifically considered, and the authors noted that aspartame was only consumed by a small percentage of the population, saccharin and cyclamate being the most frequently used artificial sweeteners. Hence, data related to artificial sweetener consumption in this study were not very informative with regard to aspartame exposure.]

Two hospital-based case–control studies in Italy were conducted using similar methodologies. Gallus et al. (2007) focused on cancers of the oral cavity and pharynx, oesophagus, colon, rectum, larynx, breast, ovary, prostate, and kidney diagnosed between 1991 and 2004. Bosetti et al. (2009) focused on cancers of the stomach, pancreas, and endometrium diagnosed between 1997 and 2007 (stomach), between 1991 and 2007 (pancreas), or between 1992 and 2006 (endometrium). In both studies, the usual diet over the 2 years preceding diagnosis or hospital admission was assessed using a reproducible and validated 78-item FFQ. The FFQ included specific questions on weekly consumption of sachets or tablets of saccharin and/or other sweeteners, described as mainly aspartame by the authors. An average daily consumption of sachets or tablets of saccharin and other sweeteners was derived. [The Working Group noted that these studies included only tabletop sweeteners as potential sources of aspartame, and aspartame was not specifically considered either in the questionnaire or in analyses ("other sweeteners" only). Even though the authors stated that in this population "other sweeteners" were mainly aspartame, data to support this statement appeared to come from two studies in Italian adolescents, so the extrapolation to a middle-aged adult population may not be straightforward. The Working Group was not able to find evidence in adults in support of the claim that "other sweeteners" was mainly aspartame in Italy for that period. The authors also stated that the frequency of consumption of ASBs or products containing artificial sweeteners in the middle-aged and elderly in the studied Italian populations was rather low, but

no data were provided. There was a high potential for non-differential misclassification in this study.]

Ewertz and Gill (1990) conducted a case–control study of breast cancer in Denmark. Dietary intake was assessed retrospectively with a self-administered, semiquantitative FFQ collected from March 1983 to February 1984, that is, 1 year after diagnosis of the cases. Use of artificial sweeteners in coffee and tea was the only assessment of artificial sweeteners (binary assessment, yes/no) covering the 12 months before diagnosis. [The Working Group noted that there was no specific estimate of aspartame exposure; only sweetener use in tea and coffee was assessed as a proxy. Considering that aspartame only started to be used in the 1980s, it was very unlikely that the artificial sweeteners consumed before cancer diagnosis in this study would be aspartame. Hence this study was probably not informative regarding aspartame exposure. In addition, the case–control design meant that intake was assessed retrospectively after cancer diagnosis.]

Norell et al. (1986) conducted a population-based case–control study of pancreatic cancer in Sweden between 1982 and 1984. Exposure was assessed using a self-administered questionnaire and by telephone by a trained interviewer to clarify or complete specific items whenever necessary; there was a binary question on use of artificial sweeteners before diagnosis, and participants who had changed their diet because of recent illness were asked to report dietary habits before illness. Participants were classified as consumers or non-consumers, and there was no information on dose or duration. [The Working Group noted that the weaknesses of the exposure assessment included that there was no specific assessment of aspartame, the assessment relied on retrospective recall of frequency of consumption, and there was no quantitative assessment as participants were categorized as consumers versus non-consumers only. In addition, considering that aspartame had only started to be used in the 1980s, it was very unlikely that the artificial sweeteners consumed before cancer diagnosis in this study would have been aspartame. Hence this study was probably not informative regarding aspartame exposure.]

(c) Artificially sweetened beverages

The consumption of ASBs in relation to cancer was considered in several studies. In these studies, ASBs generally referred to soft drinks in which sugar had been replaced by artificial sweetener to maintain the sugary taste ("diet" or "sugar-free" version of soft drinks). It should be noted that some sugar-sweetened soft drinks ("regular" version) containing sugar also contain artificial sweetener. Such beverages were most likely not considered to be ASBs in the following studies. [The Working Group noted that ASB consumption can be considered as a proxy for exposure to artificial sweeteners (including aspartame), since these beverages generally constitute the main source of artificial sweeteners (Sections 1.4.1 and 1.4.3). However, this comes with limitations that applied to all the studies detailed below. Firstly, several other food products that also contain artificial sweeteners (including aspartame), such as tabletop sweeteners, artificially sweetened dairy products or desserts, were not considered. Secondly, the presence and dose of aspartame in different types of ASB and the share of ASBs actually containing aspartame have changed over time; this affects the relevance of considering ASB consumption as a direct proxy for aspartame exposure. Hence, the value of ASBs as a proxy measure for aspartame intake is dependent on the context (time period, country, and types of beverage considered). Sections 1.4.1 and 1.4.3 provide insights into patterns of use of aspartame.]

Considering the data available on aspartame use in ASBs in the USA between the introduction of aspartame in 1983 and the introduction of other

artificial sweeteners used in combination with aspartame in the mid-2000s (see Section 1.4.1), and the major contribution of ASBs to aspartame intake compared with other sources, ASB intake in some studies can be presumed to reflect most of the participants' exposure to aspartame because of the time period of dietary assessment and follow-up. This was the case for the following studies: Bao et al. (2008) (NIH-AARP cohort, dietary data collected in 1995–1996, follow-up until 2003); Schernhammer et al. (2005) (NHS and HPFS cohorts, dietary data collected at least every 4 years since 1980 and 1986, respectively, follow-up until 2000); You et al. (2022) (Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial, PLCO, cohort: dietary data collected at baseline between 1993–2001, follow-up until 2009); and Malik et al. (2019) (NHS and HPFS: dietary data collected every 4 years since 1980 and 1986, respectively; follow-up until 2010). The study by Jones et al. (2022) in the NIH-AARP and PLCO cohorts (dietary data collected in 1995–1996 and 1998, respectively) may also be presumed to reflect most of the aspartame exposure. However, part of the sample was followed for much longer after the relevant exposure window for aspartame from ASBs (follow-up through 2011 for NIH-AARP and through 2017 for PLCO; there was uncertainty regarding aspartame content in ASBs after the mid-2000s), and only one baseline dietary assessment was available. However, the authors presented the analysis separately by follow-up period (≤ 12 years and > 12 years of follow-up), making the analysis of the first 12 years of follow-up very relevant for using ASBs as a proxy for aspartame exposure.

(i) Prospective cohort studies

Several studies were conducted in the USA in the framework of the NHS, NHS-II, and HPFS cohorts (Schernhammer et al., 2005; Malik et al., 2019; Hur et al., 2021; Joh et al., 2021; Romanos-Nanclares et al., 2021; Wang et al., 2021; Yuan et al., 2022). In all three cohorts, diet was assessed through a validated self-administered, semi-quantitative FFQ repeated at least every 4 years since inclusion in 1980 for the NHS, 1986 for the HPFS, and 1991 for the NHS-II (61 items in 1980, 116 items in 1984 and 1986, and ≥ 130 items thereafter). ASB consumption was assessed using nine frequencies ranging from "never or less than once per month" to "6 or more times per day" and a standard serving of 355 mL (12 fl oz) corresponding to 1 glass, can, or bottle for caffeinated, caffeine-free, and non-carbonated low-calorie or diet beverages. In the NHS-II, a self-administered high school FFQ (HS-FFQ) was used in 1998 to collect data on the usual diet of participants when they were in high school (aged 13–18 years) between 1960 and 1982 (that is, before the use of aspartame in ASBs). The HS-FFQ comprised 124 food items commonly consumed at that time.

The study by Romanos-Nanclares et al. (2021) focused on breast cancer in the NHS and the NHS-II, with follow-up ending in 2016 (NHS) or 2017 (NHS-II), and 2006 when considering molecular subtypes of breast cancer. The main analyses used a cumulative average strategy, and other strategies were also considered in secondary analyses. These included: simple update (consumption reported on the most recent FFQ before each follow-up interval); latency (consumption reported at different latencies, i.e. 4–8, 8–12, 12–16, and 16–20 years, before a cancer diagnosis); and changes in consumption updated every 4 years to estimate the risk in the subsequent 4-year period (no change or relatively stable, increase or decrease from 1.0 serving/week to 0.5 serving/day, and increase or decrease by > 0.5 serving/day). The study by Hur et al. (2021) focused on early-onset colorectal cancer in the NHS-II, with follow-up ending in 2015. Adult ASB intakes were cumulatively averaged over questionnaire cycles. The study by Malik et al. (2019) focused on cancer mortality in the NHS and the HPFS, with follow-up ending in 2014. Analyses considered frequencies of ASB intake, with an update of intakes

at the beginning of each FFQ cycle as the main analysis. Secondary analyses used a cumulative average strategy whereby intakes from all FFQs up to the beginning of a follow-up interval were averaged, but also analyses with an 8-year lag, whereby exposures were evaluated in relation to outcomes 8 years later. Dietary intakes were not updated if the participant reported incidence of diabetes mellitus, stroke, coronary heart disease, or cancer. The study by Schernhammer et al. (2005) focused on pancreatic cancer in the NHS and the HPFS, with follow-up ending in 2000. The analyses used a cumulative average strategy, stopping updates after a diagnosis of diabetes mellitus was reported.

[The Working Group noted that in all four studies (Schernhammer et al., 2005; Malik et al., 2019; Hur et al., 2021; Romanos-Nanclares et al., 2021), the repeated assessment of ASB intake at least every 4 years since the 1980s and 1990s and the analytical strategy of updating diet data during follow-up constituted major strengths and allowed potential variations in trends in ASB intake over time to be taken into account. In addition, the time frame of these studies was very relevant to aspartame exposure via ASBs, since the studies started in the 1980s (overlapping the introduction of aspartame in ASBs in 1983), potentially capturing lifetime exposure to aspartame from ASB intake, and carried out diet assessment through the 1990s, when aspartame was almost the only sweetener used in ASBs. However, there was more uncertainty regarding aspartame content in ASBs from the mid-2000s, and the period between 1980 and 1983 is not relevant for aspartame exposure. Hence the quality of ASB intake assessment was high in these studies, which had a positive impact on the assessment of aspartame exposure from ASBs.]

The three remaining studies in the NHS, NHS-II, and/or HPFS (Joh et al., 2021; Wang et al., 2021; Yuan et al., 2022) were less informative with regard to aspartame exposure, considering their exposure of interest and/or analytical strategy. The study by Yuan et al. (2022) focused on colorectal cancer incidence and mortality in the NHS and the HPFS, with follow-up ending in 2014. The analyses considered the substitution of one serving of sugar-sweetened beverage per day with an equivalent amount of ASBs, without estimates for ASBs as such, using cumulatively averaged intakes as the main analyses and secondary analyses that considered average intake during the most recent 10 years (recent period) and > 10 years previously (distant period). [The Working Group noted that ASBs were not considered as the main exposure of interest in the analyses, which limited the relevance of the results for ASB intake and therefore even more so for aspartame exposure.] The study by Joh et al. (2021) focused on colorectal cancer precursors (polyp, adenoma) in the NHS-II in relation to ASB consumption in adolescence (between 1960 and 1982), with follow-up for health events between 1998 and 2015. Adult diet (cumulative average up to 2 years before the most recent endoscopy), including ASB intake, was considered as a covariate in the analyses. [The Working Group noted that soft drinks in the USA did not contain aspartame before 1983, hence no exposure to aspartame could be derived from the diet for adolescents. Besides, the recalled adolescents' diet (likely to suffer from substantial recall bias) only weakly correlated with the adults' diet, so that ASB intake reported in adolescence did not give insights into ASB intake in adulthood when ASBs potentially contained aspartame. This study was therefore not informative regarding aspartame exposure.] The study by Wang et al. (2021) focused on colorectal cancer in the NHS, NHS-II, and HPFS and considered a "sulfur microbial diet score", consisting of a weighted sum of standardized consumption of 10 food groups with weights reflecting positive or negative correlations with the proliferation of sulfur-producing bacteria in the gut. Low-calorie beverages were included in the score with a positive weight. A cumulative average of the sulfur microbial diet score was

derived for analyses. Participants were followed until 2014 (HPFS), 2016 (NHS), and 2017 (NHS-II). [The Working Group noted that ASBs were only included as a component of the score, so the extrapolation to aspartame exposure and therefore the informativeness of this study with regard to aspartame was very limited.]

Ringel et al. (2023) studied urinary tract cancers in the Women's Health Initiative Observational Study. Consumption of ASBs (defined as "diet drinks such as Diet Coke or diet fruit drinks") was assessed 3 years after study baseline, i.e. in 1996–2001. The frequency of consumption during the past 3 months was questioned using a reference serving size (12 fl oz can [355 mL]) and nine frequencies of servings: from never or < 1 serving/month to ≥ 6 servings/day. Analyses used three categories corresponding to rare (never or < 1 serving/week), frequent (1–6 servings/week) and daily (≥ 1 serving/day) consumption. The follow-up ended in 2020. [The Working Group noted that the time period of the assessment of ASB consumption was very relevant for aspartame exposure, since aspartame was almost the sole sweetener used in ASBs at the time of diet assessment. However, there was only one assessment at baseline, with up to 24 years of follow-up, so potential changes in ASB consumption over time could not be captured, and other sources of aspartame exposure were not considered (although these were more limited), which may have led to non-differential misclassification.]

McCullough et al. (2022) studied mortality from all cancers combined, obesity-related cancers combined, and 20 individual cancer types in the CPS-II study. ASB consumption was assessed at study baseline in 1982 via a question about the usual number of cups, glasses, or drinks consumed per day, and consumption duration for "diet soda or diet iced teas" (one pooled item). Previous intake was queried in case there had been a change in consumption during the past 10 years, and former drinkers (no current intake but non-zero amount for previous intake) were excluded. The consumption of ASBs was considered as categories – never drinkers (no past or current consumption), < 1 drink/day, 1 drink/day, and ≥ 2 drinks/day – and as continuous intake per 1 drink/day. Participants were followed until 2016 (median follow-up, 27.7 years). [The Working Group noted that the assessment of ASB intake was performed only at baseline in 1982, i.e. a time when aspartame was not used in ASBs. The relevance of this study to aspartame exposure depended on the stability of ASB consumption over time, because aspartame began to be included in ASBs in 1983 in the USA. Although no information was available regarding potential variations in ASB intake over time during up to 34 years of follow-up, some data from McCullough et al. (2014) in the CPS-II nutrition cohort suggested a stable ranking of participants with regard to ASB consumption between 1982 and 1999 for the subsample (n = 100 442) providing data at both time points. However, there was uncertainty regarding aspartame content in ASBs in the mid-2000s. Non-differential misclassification was therefore likely.]

Jones et al. (2022) performed a pooled analysis of data for liver cancer from the NIH-AARP and PLCO studies. For both studies, data on ASB intake were only available at baseline, an FFQ in 1995–1996 for the NIH-AARP study and a diet history questionnaire in 1998 for the PLCO study. Participants were followed until 2011 in the NIH-AARP and 2017 in the PLCO study. The consumption of soda and fruit punches as ASBs was considered as a mean daily frequency (from no consumption to ≥ 6 times/day). [The Working Group noted that ASB consumption was assessed during a very relevant period for aspartame exposure (1995–1996 and 1998 in the USA); however, there was more uncertainty regarding aspartame content in ASBs in the mid-2000s. The dietary assessment occurred only at baseline, and there was a follow-up of up to 16 years in the NIH-AARP

study and 19 years in PLCO. This increased the probability of misclassification of participants with regard to ASB intake and therefore aspartame exposure. However, the authors presented the analysis separately by follow-up period (≤ 12 years and > 12 years of follow-up). Hence the analysis on the first 12 years of follow-up was less prone to such misclassification and ASB consumption could be considered as a relevant proxy of aspartame exposure in this analysis. In addition, sodas and fruit punches (and not just carbonated beverages, as was the case in other studies) were also considered to be ASBs, which constituted a strength of this study.]

Zhang et al. (2021) focused on cancer mortality in the NHANES cohort study. The consumption of ASBs was assessed through 24-hour dietary recalls given by trained investigators at inclusion between 1999 and 2014. Participants included before 2003 completed only one 24-hour dietary recall administered in person, whereas participants included after 2003 completed two 24-hour dietary recalls (one administered in person and one administered by telephone 3–10 days after the first). Participants were followed through registries until 2015. The 24-hour dietary recalls were linked to a main food list of more than 2600 food items. A standard 12 fl oz [335 mL] serving was defined. ASBs were defined as "sugar-free soft drinks" and "carbonated water". [The Working Group noted that 24-hour dietary recalls allowed the consideration of different types of ASB. However, mean intakes were derived from a maximum of two 24-hour dietary recalls, which was likely to introduce measurement errors as a result of potential individual day-to-day variations in ASB intake (especially when only one recall was available). In addition, there was uncertainty regarding the aspartame content of ASBs in the USA after the mid-2000s (i.e. for a substantial part of the study assessment period, 1999–2014). Hence there was the potential for misclassification of participants with regard to ASB intake and therefore aspartame exposure.]

Inoue-Choi et al. (2013) focused on endometrial cancer in the Iowa Women's Health Study (IWHS). A 127-item Harvard semiquantitative FFQ assessing diet over the past 12 months was used at study baseline in 1986. Sugar-free beverages were included as low-calorie caffeinated and caffeine-free cola and other low-calorie carbonated beverages with a standard serving size. Mean intakes as servings per week were considered in the analyses. Participants were followed until 2010. In 2004, a follow-up survey including an FFQ was performed and a weak correlation was found for sugar-sweetened beverage intake (correlation coefficient, 0.23), with no information on ASBs. [The Working Group noted that only the baseline dietary assessment was used and that there was a follow-up of up to 24 years. Because only a weak correlation was reported for sugar-sweetened beverage intake between the baseline in 1986 and a reassessment of diet in 2004 (with no information on ASBs), there was potential for misclassification of participants with regard to ASB intake and therefore aspartame exposure. However, the dietary assessment was conducted at a very relevant period for aspartame exposure from ASBs.]

Bao et al. (2008) focused on pancreatic cancer in the NIH-AARP cohort. In this study, usual diet in the past 12 months was assessed once at study baseline between 1995 and 1996 using a 124-item FFQ calibrated against two non-consecutive 24-hour recalls. Participants were followed until 2003. Three beverage types potentially containing aspartame were included: soda, fruit drinks, and iced tea (in summer), and participants were asked about frequency of consumption of each type (10 frequencies ranging from never to ≥ 6 times/day). Participants were then asked whether they consumed the sugar-free/diet or regular version of these beverages "usually, or more than half the time" and which portion size (three portion size ranges). An average daily

diet soft drink consumption was derived for the analyses. [The Working Group noted that ASB consumption was assessed during a very relevant period for aspartame exposure (1995–2003 in the USA). This study considered both carbonated and non-carbonated diet soft drinks but was less precise regarding the type of carbonated soft drinks (cola or not), which was likely to affect the classification of participants by type of beverage consumed (and thus the potential corresponding aspartame exposure). The sequential assessment of the frequency of consumption of ASBs was likely to induce measurement errors and thus lead to non-differential misclassification of participants. There was a maximum of 8 years between the assessment of ASB intake and cancer diagnosis; over this limited period, the diet could be considered relatively stable, even though no information was available regarding ASB intake before the study baseline, which occurred 10 years after the introduction of aspartame.]

You et al. (2022) conducted a prospective analysis of the PLCO cohort to examine soft drink consumption and lung and total cancers. In 1993–2001, dietary intake was assessed with a 156-item FFQ. Soft drink (regular and diet) intake was calculated from information on frequency and serving size. Quantitative data on soft drink consumption were then categorized into non-consumers, consumers of regular only, consumers of diet only, and consumers of both or mixed consumption. [The Working Group noted that no quantification of ASB intake was used in the analyses. In addition, only baseline intakes were considered, and there was no consideration of intake before baseline; therefore, there was potential for non-differential misclassification. The timing of exposure assessment was consistent with aspartame being the major sweetener in ASBs in the USA.]

Several studies were conducted in the multi-country framework of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (Navarrete-Muñoz et al., 2016; Stepien et al., 2016; Mullee et al., 2019; Heath et al., 2021; Zamora-Ros et al., 2022), which gathers participants from 10 countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, UK). Usual diet was assessed at inclusion using country-specific instruments developed and validated within the various source populations and including hundreds of country- and region-specific foods. These extensive self-administrated quantitative dietary questionnaires collected data on usual portions and frequency of consumption of up to 260 food and drink items during different seasons of the year (Riboli et al., 2002). Data on artificially sweetened soft drinks were not available in Spain, nor in parts of Italy (Florence, Turin, Ragusa, Varese) and Sweden (Umeå). Artificially sweetened soft drink consumption was assessed between 1992 and 2000 as the consumption of several glasses per month, week, or day (typical glass sizes in each centre, about 250 mL) of "low-calorie or diet fizzy soft drinks". A mean daily intake of ASBs was derived for the analyses.

The study by Zamora-Ros et al. (2022) focused on thyroid cancer, with a mean follow-up of 14 years. The study by Heath et al. (2021) focused on renal cell carcinoma, with a mean follow-up of 15 years for incidence of renal cell carcinoma and 16 years for mortality. The study by Mullee et al. (2019) focused on cancer mortality, with a mean follow-up of 16.4 (range, 11.1–19.2) years. The study by Navarrete-Muñoz et al. (2016) focused on pancreatic cancer, with a median follow-up of 11.6 years. The study by Stepien et al. (2016) focused on cancers of the liver and biliary tract, with a mean follow-up of 11.4 years.

[The Working Group noted that, for all five studies, only one diet assessment was available at baseline, which was during a period that was relevant for aspartame exposure from ASBs, and the mean follow-up ranged from 11 to 16 years; as a result, potential variations in consumption of ASBs over time could not be captured. In addition, EPIC combined data from multiple

European countries, each having its own pattern of ASB consumption, and potentially its own products (with variable content in aspartame), which limited the direct extrapolation from ASB intake to aspartame exposure. The consumption of ASBs at baseline was relatively low (< 25% of total soft drinks) and no information was available on historic exposure before inclusion. Hence there was some potential for non-differential misclassification of participants with regard to ASB intake and aspartame exposure.]

Three other studies were conducted in Europe and considered beverages that potentially contained aspartame.

The study by Liu et al. (2022b) was conducted within the United Kingdom Biobank and focused on cancer mortality in relation to the consumption of artificially sweetened coffee. Coffee consumption was assessed using validated web-based 24-hour dietary recall questionnaires (Oxford WebQ), with a minimum of one questionnaire completed out of five maximum occasions during 1 year between April 2009 and June 2012 (to account for seasonal variations). The number of drinks of coffee in the previous 24 hours was recorded, together with the number of teaspoons of sugar or artificial sweeteners (any brand) added. An indication of portion size (e.g. mug or cup) was provided. Participants were classified as non-consumers, sole consumers (same kind of coffee over the dietary recalls: unsweetened, sugar-sweetened, artificially sweetened), or overlapped consumers, and analyses considered an average number of drinks (volume, about 250 mL) across multiple dietary recalls. Participants were followed until 2017–2018 (median, 7.0 years). [The Working Group noted that this study was not very informative with regard to aspartame exposure: (i) artificially sweetened coffee was the only ASB considered in this study, and other major sources of aspartame such as carbonated soft drinks (caffeinated or not) were not considered; (ii) there was no indication as to whether the artificial sweeteners used in coffee contained aspartame; and (iii) the mean number of dietary recalls used was relatively low (mean ± SD, 2.2 ± 1.2), which limited the consideration of day-to-day variations in coffee consumption.]

The study by Chazelas et al. (2019) was conducted in France within the NutriNet-Santé prospective cohort study (2009–2017) and examined cancers of the breast, colorectum, and prostate, and all cancers combined. Consumption of artificially sweetened soft drinks was assessed using repeated validated web-based 24-hour dietary records (minimum, 2; mean ± SD, 5.6 ± 3.0). Participants were asked at inclusion and then every 6 months to complete three non-consecutive 24-hour dietary records (2 weekdays, 1 weekend day) randomly assigned over a 2-week period. Baseline data were collected from 2009 through 2017, with follow-up to January 2018. The analyses considered only baseline ASB consumption, defined as the mean consumption over the first 2 years of follow-up. Exposure was assessed for 12 ASB items containing non-nutritive sweeteners, such as diet soft drinks, sugar-free syrups, and diet milk-based beverages, and consumption was categorized into sex-specific quartiles. [The Working Group noted that a major strength of this study was the use of repeated detailed 24-hour-dietary records reflecting up-to-date consumption patterns of ASBs in France. Considering the time period of dietary assessment, ASBs included a variety of products with aspartame only, artificial sweeteners other than aspartame only, or a mix of aspartame and other artificial sweeteners. Therefore, the consumption of ASBs in this study may not reflect only aspartame exposure but also exposure to other sweeteners, as confirmed in the later study by Debras et al. (2022b). In addition, only baseline dietary data were used in analyses, which did not allow the consideration of potential changes in dietary behaviours over time. Potential changes were nonetheless likely to be limited over the maximum of 9 years of follow-up. Likewise,

there was no information on lifetime exposure to aspartame before study entry, which occurred between 2009 and 2017. In terms of relevance for the present monograph, this publication was superseded by the publication of Debras et al. (2022b), which provided estimates specific to aspartame intake in the same cohort. The later publication demonstrated that: (i) aspartame represented only 58% of artificial sweetener intakes in this study; and that (ii) ASBs (with or with added sugar) represented only 57% of artificial sweetener intake.]

The study by Larsson et al. (2016) was conducted in Sweden and focused on cancers of the biliary tract and gallbladder in relation to consumption of sweetened beverages within the Swedish Mammography Cohort and the Cohort of Swedish Men. Usual diet was assessed in 1997 using a self-administered semiquantitative 96-item FFQ covering the past year, including one question about the usual consumption of a standard glass (200 mL) of sweetened beverages (not including fruit juices, energy and sports drinks, or sweetened coffee, tea, or milk). A mean daily intake was derived from the questionnaire. Participants were followed until 2012 (mean follow-up, 13.4 years). [The Working Group noted that no distinction was made between sugar-sweetened and artificially sweetened (low-calorie) beverages, rendering the extrapolation to aspartame exposure very limited. According to Swedish national consumption data, low-calorie soft drinks and "juice" drinks accounted for 9.9% (men) to 19.2% (women) of total soft drink and "juice" drink consumption in 1997.]

Finally, two prospective studies (Hodge et al., 2018; Bassett et al., 2020) were conducted in Australia within the Melbourne Collaborative Cohort Study (MCCS). The study by Hodge et al. (2018) examined associations between ASB consumption and obesity-related cancers, whereas the study by Bassett et al. (2020) focused on cancers not related to obesity. All methods of exposure assessment were the same across the two studies. Exposure to artificially sweetened soft drinks was assessed at baseline only (in 1990–1994) using a self-administered 121-item FFQ with separate questions on frequency of consumption of diet (artificially sweetened) soft drinks. The questionnaire asked respondents to recall their intake over the previous 12 months. Intake of ASBs was categorized into five groups based on frequency of reported consumption (never or < 1 time/month, 1–3 times/month, 1–6 times/week, 1 time/day, > 1 time/day). [The Working Group noted that the relative strengths of this assessment included the semiquantitative assessment for frequency of intake, which allowed multiple dose levels to be assessed. Limitations included that the assessment used for this analysis was conducted at baseline only (1990–1994), which did not allow the consideration of variations in ASB consumption over time, bearing in mind that the follow-up was up to 25 years in Bassett et al. (2020) and 23 years in Hodge et al. (2018). Hence, non-differential misclassification with regard to aspartame exposure was likely.]

(ii) Case–control studies

Four case–control studies (Nomura et al., 1991; Mayne et al., 2006; Chan et al., 2009; Davis et al., 2023) investigating ASBs were conducted in the USA.

Davis et al. (2023) conducted a hospital-based case–control study on pancreatic cancer and mortality in Buffalo, New York, USA, in which exposure was assessed between 1982 and 1998 using a 46-item FFQ on general dietary habits (including daily consumption of diet cola) in the few years before diagnosis. Participants were categorized in three groups: never, occasional (< 1 drink/day), and habitual (≥ 1 drink/day) consumers. [The Working Group noted that the retrospective assessment of ASB consumption during the few years before diagnosis may cause misclassification that could be both non-differential and differential, according to case or

control status. In addition, this study included only diet cola as an ASB and as a potential source of aspartame, which was a partial assessment that probably induced some misclassification. Additionally, for cancers diagnosed at the beginning of the study period (the first half of the 1980s), the assessment of ASB consumption was not relevant for aspartame exposure since aspartame was not included in ASBs in the USA before 1983). However, for the remaining study period, ASB consumption was relevant for aspartame exposure.]

Chan et al. (2009) conducted a population-based case–control study of pancreatic cancer in California, USA, in which exposure data were collected between 1995 and 1999. Total ASB consumption during the 12 months before diagnosis was assessed using a 131-item food questionnaire via in-person interviews with questions on low-calorie colas, low-calorie caffeine-free colas, and other low-calorie carbonated beverages). ASB frequency was categorized into three groups (0, < 1, ≥ 1 beverage/day). [The Working Group noted that although there was no specific estimate of aspartame exposure and ASBs were used as a proxy, the timing of exposure assessment was relevant for aspartame being the major artificial sweetener in ASBs. However, the retrospective assessment of diet after diagnosis may have led to differential or non-differential misclassification of participants with regard to ASB intake and therefore aspartame exposure.]

Mayne et al. (2006) conducted a population-based case–control study of oesophageal cancer in Connecticut, USA. Exposure was assessed in 1993–1995 using an in-person structured questionnaire covering the 3–5 years before diagnosis. The usual frequency of consumption of diet soft drinks or soda (per day, week, month, or year) was then categorized into quartiles, the top quartile being compared with non-consumers. [The Working Group noted that the limitations of the assessment included the retrospective assessment of diet after diagnosis, and that there was no consideration of lifetime consumption of ASBs, which made differential and non-differential misclassification of participants more likely with regard to ASB intake and therefore aspartame exposure. Nonetheless, ASB intake in this study was probably very relevant for aspartame exposure, considering the time frame of dietary assessment.]

Nomura et al. (1991) conducted a case–control study of bladder cancer in Hawaii, USA. Dietary intake was assessed via a diet history interview of 29 food items consumed during a usual week, or usual month for less frequently consumed items, on the basis of the participants' usual diet in the year before diagnosis, with assessment between 1977 and 1986. The consumption of ASBs (such as diet or low-calorie sodas) and frequency of use of saccharin, cyclamates, and other artificial sweeteners (but not aspartame) were calculated. Participants were categorized on the basis of frequency of consumption of ASBs as a non-user or user (1–2 can-years, 3+ can-years; can-years indicate number of servings/day × years). [The Working Group noted that the time frame of this study (i.e. cancers diagnosed between 1977 and 1987) was of questionable relevance for aspartame exposure, considering that the US authorization of aspartame for use in ASBs was in 1983. Other limitations were that diet was assessed retrospectively after diagnosis and that interviewers were not blinded to case or control status, both of which increased the potential for differential exposure misclassification.]

(d) Other related exposures

Exposures to agents previously evaluated by IARC that are potentially associated with the consumption of aspartame, artificial sweeteners, and ASBs include alcoholic beverages and tobacco smoking (both *carcinogenic to humans*, IARC Group 1, Volume 100E, IARC, 2012). These co-exposures were taken into account in almost all the studies described above. The consumption

of tabletop artificial sweeteners may also be associated with the consumption of very hot beverages (*probably carcinogenic to humans*, IARC Group 2A, Volume 116; IARC, 2018a), but this was rarely considered. Other potential co-exposures, as part of overall dietary patterns, include dietary cancer hazards such as red meat (IARC Group 2A, Volume 114; IARC, 2018b) and processed meat (IARC Group 1; IARC, 2018b), or environmental exposures. These factors were seldom considered in analyses. Other probable cancer risk factors that were not considered in analyses include, for instance, night shift work (IARC Group 2A, Volume 124; IARC, 2020). Other factors potentially associated with aspartame exposure include coffee intake, food additives, sugar and sugar-sweetened beverages, body fatness, and diabetes (which could induce some bias from reverse causation, whereby individuals at a higher risk of developing cancer because of higher body fatness or diabetes are more likely to consume ASBs and, more generally, artificial sweeteners).

1.6.2 Quality of exposure assessment in mechanistic studies in exposed humans

See Table S1.3 (Annex 1, Supplementary material for Section 1, Exposure Characterization, online only, available from: https://publications.iarc.who.int/627).

This section reviews the exposure assessment methods of mechanistic studies in exposed humans, evaluated in Section 4 of the present monograph. Studies included in this section were a mixture of those that examined mechanistic responses to: (a) added dose of aspartame (as a beverage or in capsule form and often investigating the impact of increasing doses); (b) added dose mixed with other artificial sweeteners (typically as a beverage); (c) intake of artificial sweeteners either from dietary intake of beverages only or from the total diet assigning intake estimates for aspartame; and (d) intake of aspartame from a commercial beverage but in situations where this acted as the control arm of interventions focusing on sugar-sweetened beverages rather than on aspartame per se.

There was a mix of challenge studies, randomized controlled trials, observational studies, matched control studies and one genome-wide association study (GWAS). There was a high degree of heterogeneity, with study participants including those who were generally healthy, groups with medical conditions (e.g. HIV infection), and those who self-selected for adverse responses to aspartame intake (e.g. headaches or urticaria).

Where present, dietary assessments typically used food diaries or records, FFQs, or 24-hour dietary recalls; associated challenges related to the accuracy and precision of each tool and concomitant food composition data for quantifying aspartame intake (see Section 1.6.1 for a detailed review). Habitual or background dietary intake was not always assessed, often because the study focus was on examining the impact of increasing doses of aspartame. Hence, habitual intakes were generally poorly characterized across all studies; in addition, there was no clear indication of co-exposure to other agents that may be carcinogenic. Differential misclassification of exposure among participants was unlikely in studies in which exposure to aspartame was allocated. However, in studies in which habitual or background diet was not assessed or controlled, there was a potential for diluted treatment effect. All studies are reviewed below and classified according to exposure and study design.

(a) Controlled doses of aspartame added to the diet

(i) Crossover challenge studies

Six studies adopted a crossover approach to investigating the impact of aspartame on health outcomes (Baraniuk et al., 1988; Garriga et al.,

1991; Nguyen et al., 1998; EFSA_UN07, 2011; Ahmad et al., 2020a, b). Two studies were carried out in the USA (Garriga et al., 1991; EFSA_UN07, 2011), one in France (Nguyen et al., 1998), and one was available only as an abstract with no information on location (Baraniuk et al., 1988). [The Working Group assumed that the study was performed in the USA because of the stated affiliation of the authors.] All studies involved adding doses of aspartame to usual daily intakes but over different time periods and different doses. The study populations included those who self-reported headaches related to aspartame ingestion (Baraniuk et al., 1988), those who self-reported urticaria related to aspartame ingestion (EFSA_UN07, 2011), a generally healthy adult population (Nguyen et al., 1998), and participants who had a confirmed positive adverse reaction of any sort to aspartame that was documented by the researchers (Garriga et al., 1991). Each study adopted an approach whereby added doses of aspartame were included as an acute challenge study over a single day (single meal approach), either as a beverage (Nguyen et al., 1998), capsule (Garriga et al., 1991; EFSA_UN07, 2011), or not stated (Baraniuk et al., 1988). Sample sizes ranged from 7 (Nguyen et al., 1998), 12 (Garriga et al., 1991), and 21 (EFSA_UN07, 2011), to 40 individuals (Baraniuk et al., 1988). EFSA_UN07 (2011) included two children in the study population. The doses under study ranged from 30 mg/kg bw (the exact doses administered were not available; Baraniuk et al., 1988), 250 mg per challenge (total amount) (Nguyen et al., 1998), and 950 mg/day for individuals with a body weight of > 40 kg (450 mg where body weight was < 40 kg) (EFSA_UN07, 2011) and 2000 mg per challenge (Garriga et al., 1991). Given the acute nature of these studies, there was no background information on habitual dietary aspartame exposure in any study. It was not possible to characterize usual dietary exposure, if any.

[The Working Group noted that, across the studies, the dose of aspartame was controlled and known, and therefore the certainty of exposure was high, and risk of misclassification (except for background exposure) was low.]

Two publications (Ahmad et al., 2020a, b) reported on primary and secondary outcomes for a crossover randomized controlled trial in which participants received a dose of aspartame of 14% (0.425 g) of the ADI, mixed with water, every day for 2 weeks. Among the inclusion criteria for the study was that participants were not regular users of non-nutritive sweeteners, i.e. artificial sweeteners (i.e. consuming less than one can of diet beverages and/or one spoonful of non-nutritive sweetener or the equivalent per week in food products, which was confirmed via screening with a web-based FFQ (Canadian Diet History Questionnaire II). The FFQ included questions on the type, quantity, and frequency of use of artificial sweeteners in tea, coffee, and other drinks, and on the consumption of diet beverages (including fruit drinks, diet soda, iced tea, and flavoured water). Participants were asked to avoid food or drink products that contained non-nutritive sweeteners for the study period. Compliance with this advice and the treatment was assessed via completion of a 3-day food diary (2 weekdays, 1 weekend day, during the 2-week study period), a daily beverage checklist over the 14-day intervention period, and return of beverage containers each week. Aspartame was the specific exposure tested in both studies.

In the EFSA clinical research report (EFSA_UN01, 2011), 10 participants with type 2 diabetes and 12 participants without type 2 diabetes received a single dose of aspartame-containing beverage (approximately 400 mg of aspartame was added to 300 mL of an unsweetened beverage, cherry-flavoured reconstituted drink mix). No measurement of background or usual diet was conducted in this study, and it was not reported whether participants were consumers or non-consumers of artificial sweeteners before the study. Aspartame was the specific exposure tested in this study.

Kashima et al. (2019) conducted a crossover randomized controlled trial in Japan in which participants (n = 9) received either 25 mL of water (control) or a 2.5% solution of *Gymnema sylvestre* (a plant that suppresses sweet taste sensation) as a mouth rinse, followed by 200 g (four doses of 50 g over 80 minutes) of either 0.09% aspartame or 15% glucose solution containing 100 mg of ^{13}C-labelled sodium acetate. There was no information provided on time between administration of each intervention arm. There was no measurement of background or usual diet conducted in this study, and it was not reported whether participants were consumers or non-consumers of artificial sweeteners before the study. Aspartame was the specific exposure tested in this study. There was no information on the purity of aspartame delivered; however, a branded commercial product containing aspartame was used (see Section 1.1.4 for purity requirements of commercial food products).

(ii) Randomized parallel-arm challenge studies

Six studies adopted a parallel-arm approach (Okuno et al., 1986; Leon et al., 1989; EFSA_UN08, 2011; Higgins et al., 2018; Higgins and Mattes, 2019; Suez et al., 2022). Four of these were carried out in the USA (Leon et al., 1989; EFSA_UN08, 2011; Higgins et al., 2018; Higgins and Mattes, 2019), one in Israel (Suez et al., 2022), and one in Japan (Okuno et al., 1986). [The Working Group noted for all studies that the dose of aspartame was controlled over a short period of time during which there was a high certainty regarding short-term exposure to aspartame.]

Higgins et al. (2018) completed a three-parallel-arm study whereby a total of 93 generally healthy participants were randomized to consume one of three doses of aspartame as a beverage and/or capsule – 0 mg/day (dextrose capsule), 350 mg/day (beverage) or 1050 mg/day (350 mg as a beverage plus 700 mg as a capsule) – over 12 weeks. There was no record of dietary intakes of aspartame from any other sources during this time, although participants were selected at screening to be low- or non-consumers of low-calorie sweeteners.

In the trial described in EFSA_UN08 (2011), 108 generally healthy adults were randomized in a double-blind fashion to consume either a placebo or a total of 75 mg/kg per day of aspartame over three doses each day for 24 weeks. This dose was considered by the authors to be approximately equivalent to the amount of aspartame in 10 L of aspartame-sweetened beverage per day for a 70 kg person at that time (around 1985). Aspartame was delivered as capsules (300 mg/capsule) and the placebo was microcrystalline cellulose. No data were provided on the purity of aspartame delivered. Use of aspartame-containing products was recorded at each visit, and participants were encouraged to continue their usual diet but to avoid aspartame-containing products (list provided). There was no detailed record of habitual intakes of aspartame (if any) or of exposure to any other artificial sweetener during the study period (EFSA_UN08, 2011).

Okuno et al. (1986) described two studies. The first examined the effect of a single dose of aspartame (500 mg mixed with water) in 7 individuals without diabetes (controls) and 22 individuals with untreated diabetes, whereas the second involved daily intake of aspartame (125 mg/day) as part of a jelly cake, for 2 weeks, by Japanese adults living with diabetes but with controlled glycaemia. There was no record of usual intakes of aspartame or other low-calorie sweeteners at baseline or throughout the study period. The authors reported that in the second study the administered dose was considered equivalent in sweetness to mean daily sugar consumption for Japanese adults (20–30 g of sugar).

Leon et al. (1989) conducted a randomized controlled trial in the USA in 108 male and female participants aged 18–62 years; aspartame delivered in capsule form at a dose of 75 mg/kg (taken at three times, spread across the day)

for 24 weeks. A sample of the aspartame was analysed and found to be 98% pure aspartame. Compliance was assessed by counting unused capsules at clinic visits every 3 weeks. In addition, blood and urine metabolites including the products of aspartame metabolism, i.e. aspartic acid, phenylalanine, and methanol, were used to assess exposure five or six times over the 24-week intervention. [Measures of compliance were a strength of this work.]

Higgins and Mattes (2019) conducted a randomized controlled trial (n = 154) with aspartame provided as a beverage in one of five intervention arms (the other arms were sucrose, saccharin, sucralose, or rebaudioside A), with daily consumption for 12 weeks (n = 30). Participants consumed between 1.25 L and 1.75 L of a coloured, fruit-flavoured beverage (made from a reconstituted drink mix) per day, according to body weight at baseline (body weight, 60–75 kg, 1.25 L/day; body weight, 76–90 kg, 1.50 L/day; body weight, > 91 kg, 1.75 L/day), with 0.58 g of aspartame. Food and energy intake were measured on 3 days (2 non-consecutive weekdays and 1 weekend day) during baseline and weeks 4, 8, and 12, using the Automated Self-Administered 24-hour Dietary Assessment Tool (ASA24), a standardized dietary assessment tool. There was also a brief questionnaire that measured habitual beverage intake over the past month, completed at baseline, and in weeks 4, 8, and 12, which was used to assess whether participants made other changes to beverage intake. *para*-Aminobenzoic acid was added to the supplied beverages, and urinary *para*-aminobenzoic acid was measured to determine compliance for the intervention beverages. Urinary concentrations of low-calorie sweeteners were not measured. Background diet assessments were not used for aspartame exposure. Blood was sampled to conduct an oral glucose tolerance test at baseline and week 12. A single fasting blood draw was collected at week 6 for measurement of whole-blood glycated haemoglobin (HbA1c) and serum glucose, insulin, triacylglycerol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol. [Measures of compliance were a strength of this work.]

Suez et al. (2022) conducted a randomized controlled trial with aspartame as one intervention arm (2 sachets, three times per day; total amount per day, 0.24 g) for 3 weeks, in Israel (120 participants overall, 20 participants per arm of intervention). Among the inclusion criteria for the study was being a non-consumer of non-nutritive sweeteners, which was assessed through completion of an FFQ based on products containing non-nutritive sweeteners on the Israeli market. Dietary intake during the trial was assessed with a smartphone food diary; however, this was not used to assess aspartame.

(iii) Genome-wide association study

Hwang et al. (2019) completed a genome-wide association study (GWAS) focusing on sugars and sweeteners, using data from the USA, UK, and Australia. The aim was to identify associations with genes involved in the peripheral receptor systems relating to human perception of sweetness, i.e. taste 1 receptor members 2 and 3 (*TAS1R2* and *TAS1R3*), and G protein subunit alpha transducing 3 (*GNAT3*). In the US sample, aspartame (or any other artificial sweetener) was not included in the taste test. In the cohort from the United Kingdom Biobank, only total sugar and sweets (confectionery) was assessed (not aspartame, ASBs, or artificial sweeteners). In the Australian cohort, aspartame was added as an additional dose as part of a taste test in teenagers. The taste battery included duplicated presentations of four sweet solutions (0.60 M glucose, 0.30 M fructose, 8.0×10^{-5} M neohesperidin dihydrochalcone, and 1.4×10^{-3} M aspartame), and five bitter solutions (propylthiouracil, sucrose octaacetate, quinine, caffeine, and denatonium benzoate), plus two water solutions as controls. [The Working Group noted that there was no indication of habitual or background intakes of

the teenagers and no information available for the other cohorts beyond the Australian teenagers.]

(b) Controlled doses of aspartame mixed with other artificial sweeteners added to the diet

Two randomized studies examined the impact of added doses of aspartame mixed with other artificial sweeteners on mechanistic outcomes (Sørensen et al., 2005; Kim et al., 2020). One study with a randomized controlled design was carried out in Denmark (Sørensen et al., 2005) and the second study had a randomized crossover design and was carried out in Australia (Kim et al., 2020).

Randomized controlled trial

The study by Sørensen et al. (2005) involved the secondary analysis of an existing randomized controlled trial in which the aspartame-consuming group acted as a control (Sørensen et al., 2005). For 10 weeks the control group of healthy adults consumed a mix of artificial sweeteners as foods and beverages at three different levels based on energy intakes dependent on body weight (694 kJ/day, 832 kJ/day, or 971 kJ/day). Aspartame was consumed as a mix along with cyclamate, acesulfame-K, and saccharin (54% aspartame). Foods containing artificial sweeteners were listed as soft drinks, fruit juices, yogurt, marmalade, ice creams, and stewed fruits, but exact compositions and intakes were not provided. There was no quantitative estimate of intakes of any artificial sweetener, including aspartame, or of usual dietary exposure. Participants were advised to continue their usual diet ad libitum. [The Working Group noted that there was co-ingestion of other sweeteners (cyclamate, acesulfame-K, saccharin).]

Kim et al. examined the impact of added doses of water or an artificially sweetened soft drink (containing a mix of acesulfame-K and aspartame) on glucose metabolism in healthy adults after consuming each for 2 weeks in a randomized crossover fashion (Kim et al., 2020). The dose of aspartame was 144 mg/L, which translated as a daily intake of 86.4 mg of aspartame per 600 mL of beverage. There was no record of usual dietary intakes of aspartame (or any other artificial sweetener) or of intakes during the study period, although participants were encouraged to keep diets constant and minimize eating out. Study participants were recruited on the basis of lack of use of non-nutritive sweeteners in the previous 2 weeks and, although no results were provided, changes in dietary intakes or consumption of soft drinks were recorded (Kim et al., 2020). [The Working Group noted that there was co-ingestion of acesulfame-K.]

(c) Estimated intake of aspartame from beverage consumption or total diet

Seven studies focused on intakes of aspartame or artificial sweeteners either from the total diet or from beverages only (Auerbach and Garfinkel, 1989; Suez et al., 2014; Frankenfeld et al., 2015; Hall et al., 2017; Hess et al., 2018; Tamez et al., 2018; Ramne et al., 2021). One study was carried out in Mexico (Tamez et al., 2018), one in Israel (Suez et al., 2014), one in Sweden (Ramne et al., 2021), and four in the USA (Auerbach and Garfinkel, 1989; Frankenfeld et al., 2015; Hall et al., 2017; Hess et al., 2018). All were cross-sectional and observational in nature.

[Across the studies, common strengths included the assessment of habitual diet using a mix of FFQs, food diaries, and dietary recalls, and often assessing total dietary intakes of aspartame from a variety of foods rather than relying on intakes of specific food groups (e.g. beverages). However, common limitations also existed, including the potential for recall bias, timing of exposure relative to the introduction of aspartame into the food supply, reliance on food composition databases that may not reflect product reformulations and composition changes for aspartame, and the potential for non-differential exposure misclassification.]

Tamez et al. (2018) completed a cross-sectional analysis of beverage intake and biomarkers of cardiovascular risk among 825 generally healthy Mexican women. Intakes of sugar and ASBs were compared over the previous year using a 138-item FFQ from which three questions were extracted regarding beverage intake (colas, other sodas, and diet sodas). Intake of artificial sweeteners was estimated on the basis of diet soda intake, with a serving size being 355 mL. The total questionnaire was previously validated with dietary histories and dietary recalls for total energy, carbohydrate, protein, and fat, but not specifically for sugar or artificial sweeteners. [There was no specific estimate of aspartame intake available, and so the study was of limited value since the focus was on total beverage intakes (sugar and diet), and it was not possible to determine aspartame exposure.]

Hess et al. (2018) completed a short-term assessment of intakes of non-nutritive sweeteners over a 2-week period and compared intakes with biomarkers of metabolic syndrome among 125 adults. The participants completed three 24-hour dietary recalls (2 weekdays and 1 weekend day). The authors adopted a previously published approach of identifying consumers as those who ingested from their total diet a quantity equivalent to that of non-nutritive sweeteners present in 1 fl oz [29 mL] of dietary beverages (equivalent to 17 mg of aspartame together with other sweeteners, including cyclamate, saccharin and acesulfame-K) (Hedrick et al., 2017). The authors used a nutrition software platform (Nutrition Data System for Research) that had a publication date of 2015, indicating that compositions of aspartame (and other sweeteners) may relate to that time period. The authors aggregated the groups as either consumers (≥ 17 mg/day aspartame) or non-consumers (presumably < 17 mg/day aspartame) to examine the relationship with the metabolic syndrome, but the analysis was limited to 33 aspartame consumers and intake patterns were not characterized further.

Hall et al. (2017) evaluated dietary sweetener consumption among individuals infected with HIV and matched healthy controls, assessing the relationship to immune and inflammatory markers and coronary plaque characteristics. The data set focused on a cohort of 36 HIV-infected participants and 15 non-infected participants (controls) who consumed aspartame. Dietary intakes of aspartame were quantified using a 4-day food diary (3 weekdays, 1 weekend day) and the Nutrition Data System for Research (2012 version). Within the HIV-infected cohort, 29% were aspartame consumers, with a mean daily intake of 164 mg/day, whereas in the control group 27% were consumers, with a mean daily intake of 89 mg/day. No detail was provided on food or beverages contributing to these intakes.

Frankenfeld et al. (2015) examined aspartame intake using a paper-based, estimated (not weighed) 4-day food record, collected over consecutive days and analysed using appropriate food composition databases (Nutrition Data System for Research for nutrient analysis, 2010 version) for the period when the data were collected. All sources of aspartame from the diet were assessed, contingent on the available food composition data. No data were presented on completeness of the food records (i.e. whether participants completed all 4 days). [The Working Group noted that aspartame intakes were calculated in milligrams per day; however, for the analysis, participants were classified as consumer or non-consumers, with no consideration of dose.]

Ramne et al. (2021) examined ASB consumption among 1371 participants via two separate dietary assessment methods that were combined to reflect habitual consumption using data collected at baseline between 2013 and 2017. Participants completed a web-based 4-day food record and a web-based short FFQ covering the past 6 months; consumption frequencies addressed ASB intakes ranging from never or seldom to several times per day on an eight-level

scale. Data from each method were grouped into three levels and cross-tabulated to create a new classification of participants as non-consumers, medium consumers, and high consumers of ASBs. [The Working Group noted that no specific assessment of aspartame was conducted, and intakes of ASBs were used as a proxy for aspartame intake. Other artificial sweeteners were used at the time of intake data collection, which limited the certainty of the exposure estimate. No information or reference was provided to be able to examine the validity of the FFQ, and it was unclear how many items or questions on the short FFQ covered ASBs. Completeness of the food record was not reported.]

Suez et al. (2014) assessed long-term non-caloric artificial sweetener consumption among 381 non-diabetic individuals (males, 44%; females, 56%) from a single question in an FFQ with data collected in 2013 in Israel. Consumption of any non-caloric artificial sweetener was assessed, rather than specifically aspartame. [The Working Group noted that the dietary assessment was poorly described, and no further information on the FFQ was provided (e.g. validity, reference period, or specific details of the questionnaire). Furthermore, there was no reference cited from which these details could be obtained.]

Auerbach and Garfinkel (1989) conducted a retrospective case analysis of 149 non-smokers and smokers who had died from various causes between 1976 and 1984. Frequency of use of artificial sweeteners in soft drinks or added to coffee or tea or other beverages or foods was assessed by retrospective recall by family members of the cases. Frequency information was collected in broad categories (regular use, occasional use, or rarely used). [The Working Group noted that there was no specific assessment of aspartame, only of total artificial sweeteners. Given the dates when the study was conducted, and that aspartame was approved as a tabletop sweetener (relevant for tea and coffee exposure) in 1981 and in ASBs in 1983, significant exposure to aspartame was unlikely in the majority of this cohort.]

Yu et al. (2018) conducted a pooled analysis from two time points of the NHS and compared intakes of ASBs with several biomarkers of cardiometabolic health. Frequency of use of low-energy beverages or ASBs was assessed as a cumulative average of intakes from at least two FFQs, before blood collection, and ranked into five categories ranging from never/almost never to ≥ 1 time/day. Time points for collection of dietary intake were 1986–1990 and 1994–1998, and for blood biomarkers were 1989–1990 and 2000–2001. [The Working Group noted that there was no specific assessment of aspartame, only total artificial sweeteners. However, given the dates when the study was conducted in the USA, the timing of exposure was consistent with aspartame being the major artificial sweetener in beverages.]

(d) Intake of aspartame from commercial beverages used as the control arm in studies focusing on sugar-sweetened beverages

Randomized controlled trials

Four randomized controlled trials studies used commercially available beverages containing aspartame as a control in studies focused on sugar-sweetened beverages rather than aspartame per se (Hieronimus et al., 2020; Sigala et al., 2020, 2021, 2022). All studies were involving the same US study group and used the same aspartame-containing drink mix (fruit-flavoured, commercial) although the dose of aspartame was not specified. The study populations of healthy adults were all matched for sex, body mass index (BMI), fasting triglyceride, LDL-cholesterol, HDL-cholesterol, and insulin concentrations. In each study, 24-hour recalls were taken, but there was no detail provided on usual intakes of aspartame or of any other artificial sweetener. Participants were advised during the studies

to limit their consumption of sugar-containing beverages to one serving of 100% fruit juice, but no such guidance was provided regarding artificial sweeteners, and it was not possible to determine whether there were any changes in intake of aspartame in any of the groups beyond the beverages provided. [The Working Group noted that exposure was clearly defined in these study protocols and therefore there was a high certainty of aspartame exposure, despite the amount of aspartame exposure and background exposure being unknown.]

Bishop et al. (2002) conducted a randomized controlled trial (n = 6) in the UK comparing a carbohydrate solution with an artificially sweetened solution (the placebo); however, there was no information on the type of beverage used for the placebo. Diet was assessed (but not reported) for 2 days before the trial, to standardize intakes before the second trial. There was no assessment of long-term diet reported.

2. Cancer in Humans

Introduction

This section reviews studies of exposure to aspartame and to artificial sweetener formulations containing aspartame as the primary sweetener in relation to cancer incidence or mortality in humans. A systematic search was conducted in the PubMed database (NCBI, 2023) to identify cohort, case–control, and nested case–control studies evaluating exposure to aspartame or artificially sweetened beverages (ASBs) with cancer as an outcome. The search terms used and the resulting literature tree are available online from: https://hawcproject.iarc.who.int/lit/assessment/680/. Case reports, studies using ecological designs, and studies that did not include cancer as an end-point were not considered further.

The Working Group considered cohort and case–control studies with various exposure definitions aimed at capturing aspartame consumption. All identified studies were nutritional epidemiology studies, and no studies were identified in occupationally or environmentally exposed populations. Only a handful of studies assessed aspartame specifically. Many studies measured intake of ASBs, which were considered by the Working Group to be a proxy for aspartame exposure on the basis of the time period and country in which the study was conducted (see Section 1.4 and Annex 2, Scientific and other publicly available data on aspartame use in artificially sweetened beverages, also available from: https://publications.iarc.who.int/627). In addition to ASBs, some studies also measured consumption of tabletop sweeteners containing aspartame. Control for confounding, in particular, body mass index (BMI) and consumption of sugar or sugar-sweetened beverages, was carefully evaluated, as was effect modification by diabetes. Several studies provided information on use of artificial sweeteners that were likely to be a mix of aspartame and other artificial sweeteners. The Working Group also considered meta-analyses of aspartame or ASBs and cancer, but carefully reviewed whether the summary results were pertinent to exposure to aspartame versus exposure to other artificial sweeteners. A recent systematic review and meta-analysis by the World Health Organization (WHO) reported on non-sugar sweeteners and cancer risk (WHO et al., 2022). It is important to note that this systematic review was limited to studies with interventions or exposures within the respective acceptable daily intakes (ADIs) established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), so may not include some studies for which it was concluded that the ADI may have been exceeded. Additionally, this meta-analysis was focused on all types of non-sugar sweetener and therefore was only considered relevant to the present evaluation if

the summary estimates included studies relevant to aspartame exposure only (either directly or by proxy with ASBs).

The studies are presented below in sections by cancer end-point and are ordered by study design and then publication year (oldest to most recent). Cohort studies are described first, followed by case–control studies and meta-analyses of potential relevance.

2.1 Cancer of the liver, colon and rectum, pancreas, and other organs of the digestive tract

2.1.1 Cancer of the liver

See Table 2.1.

Three prospective cohort studies including a total of four cohorts examined the association between aspartame exposure or ASB consumption and risk of liver cancer (Stepien et al., 2016; Jones et al., 2022; McCullough et al., 2022).

A study in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (Stepien et al., 2016) examined soft drink consumption and liver cancer risk among 477 206 men and women from 10 European countries. Over a mean follow-up of 11.4 years, 191 cases of hepatocellular cancer were diagnosed. Usual diet was assessed at study enrolment between 1992 and 1998 using country-specific diet assessment instruments. Artificially sweetened soft drink intake was assessed by asking participants to recall the number of glasses (approximately 250 mL) of "low-calorie or diet fizzy soft drinks" per month, week, or day (Mullee et al., 2019). This quantity was then converted to 330 mL servings per week for the analysis (Stepien et al., 2016). In the analysis restricted to the centres with available information on artificially sweetened soft drink consumption, each 330 mL serving of artificially sweetened soft drink per week was associated with an increased risk of hepatocellular cancer (hazard ratio, HR, 1.06; 95% CI, 1.03–1.09; 151 cases). [The Working Group noted that a correction to the number of hepatocellular carcinomas reported in the original paper was published on 17 April 2024 and the original paper was updated.] Individuals who were obese or reported a history of diabetes consumed artificially sweetened soft drinks more frequently than sugar-sweetened soft drinks. [Strengths included prospective analysis of a geographically heterogeneous population with detailed and validated dietary instruments specific to each country (which potentially had its own products with variable content in aspartame) during relevant periods of aspartame exposure. However, towards the end of the follow-up period for countries that started recruitment later (i.e. towards 2000), co-exposure to other artificial sweeteners was likely. The study controlled finely for BMI (height and weight were measured by trained investigators), diabetes status, smoking, lifetime alcohol exposure, physical activity, and education. The authors also evaluated potential confounding by total sugar, sugar-sweetened beverages, coffee consumption, and other dietary factors and noted that these did not appreciably change risk estimates. The study did not control for hepatitis B virus/hepatitis C virus (HBV/HCV) status, which is a strong risk factor for liver cancer, but whether it was associated with ASB consumption is unclear. Moreover, the prevalence of HBV/HCV infection in the EPIC cohort is very low (around 3%) (Trichopoulos et al., 2011). A limitation of this study was that baseline-only assessment of ASB intake may contribute to non-differential measurement error and bias risk estimates towards the null.]

Jones et al. (2022) pooled data from two cohorts in the USA, the National Institutes of Health-American Association of Retired Persons Diet and Health Study (NIH-AARP) and the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial, which included 553 874 participants of whom 1060 developed liver cancer (ICD-O-3, C22.0 and C22.1, liver and intrahepatic bile

Table 2.1 Epidemiological studies on consumption of aspartame and cancers of the liver, pancreas, and other digestive organs

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2005) USA Enrolment, 1976 (NHS), 1986 (HPFS/ follow-up, 1980–2000 (NHS), 1986–2000 (HPFS) Cohort	88 794 women and 49 364 men; female registered nurses aged 30–55 yr in the NHS and male health professionals aged 40–75 yr in the HPFS Exposure assessment method: prospective assessment of ASB consumption through repeated FFQs between 1980–1986 and 2000	Pancreas, incidence	Frequency of diet soft drink consumption (RR):			Age (years), sex, follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption, BMI	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption as a cumulative average from repeated diet assessments (using validated instruments) every 4 yr, the majority at a very relevant period for aspartame exposure from ASBs (the USA between 1980–1986 and 2000) potentially capturing lifetime exposure to aspartame. A key limitation was that other sources of aspartame were not considered (although these were more limited). *Other strengths*: updated smoking and history of diabetes covariates, stopped updating exposure information with new report of diabetes mellitus; control for BMI at baseline.
			< 1/mo	155	1		
			1–12/mo	116	1.08 (0.85–1.38)		
			> 3/wk	108	1.02 (0.79–1.32)		
			Trend-test *P* value: 0.98				
		Pancreas, incidence	Frequency of diet soft drink consumption, women (RR):			Age (years), follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption, BMI	
			< 1/mo	77	1		
			1–12/mo	62	1.10 (0.78–1.55)		
			> 3/wk	66	1.12 (0.79–1.59)		
			Trend-test *P* value: 0.64				
		Pancreas, incidence	Frequency of diet soft drink consumption, men (RR):				
			< 1/mo	78	1		
			1–12/mo	54	1.08 (0.76–1.53)		
			> 3/wk	42	0.89 (0.60–1.33)		
			Trend-test *P* value: 0.52				
		Pancreas, incidence	Frequency of diet cola consumption (RR):			Age (years), sex, follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption, BMI	
			< 1/mo	182	1		
			1–12/mo	105	0.90 (0.67–1.21)		
			> 3/wk	92	0.85 (0.59–1.21)		
			Trend-test *P* value: 0.39				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2005) (cont.)		Pancreas, incidence	Frequency of diet cola consumption, women (RR):			Age (years), follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption, BMI	*Other limitations*: potential for confounding from unmeasured factors; diet soft drink intake was not high; limited number of cases in some stratified analyses resulted in wide confidence intervals.
			< 1/mo	83	1		
			1–12/mo	63	0.84 (0.49–1.42)		
			> 3/wk	59	0.83 (0.43–1.59)		
			Trend-test *P* value: 0.96				
		Pancreas, incidence	Frequency of diet cola consumption, men (RR):				
			< 1/mo	99	1		
			1–12/mo	42	0.86 (0.58–1.26)		
			> 3/wk	33	0.85 (0.54–1.32)		
			Trend-test *P* value: 0.38				
		Pancreas, incidence	Frequency of other diet soft drink consumption (RR):			Age (years), sex, follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption, BMI	
			< 1/mo	211	1		
			1–12/mo	111	1.14 (0.85–1.53)		
			> 3/wk	57	1.40 (0.93–2.11)		
			Trend-test *P* value: 0.13				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2005) (cont.)		Pancreas, incidence	Frequency of other diet soft drink consumption, women (RR):			Age (years), follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption, BMI	
			< 1/mo	87	1		
			1–12/mo	74	1.44 (0.85–2.44)		
			> 3/wk	44	1.50 (0.76–2.97)		
			Trend-test *P* value: 0.54				
		Pancreas, incidence	Frequency of other diet soft drink consumption, men (RR):				
			< 1/mo	124	1		
			1–12/mo	37	0.89 (0.59–1.34)		
			> 3/wk	13	1.62 (0.88–2.97)		
			Trend-test *P* value: 0.15				
		Pancreas, incidence	Frequency of diet soft drink consumption, BMI < 25 kg/m^2 (RR):			Age (years), sex, follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption	
			< 1/mo	86	1		
			1–12/mo	59	1.24 (0.88–1.74)		
			> 3/wk	37	0.95 (0.63–1.41)		
			Trend-test *P* value: 0.69				
		Pancreas, incidence	Frequency of diet soft drink consumption, BMI ≥ 25 kg/m^2 (RR):				
			< 1/mo	66	1		
			1–12/mo	56	0.96 (0.67–1.38)		
			> 3/wk	67	1.03 (0.72–1.47)		
			Trend-test *P* value: 0.88				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2005) (cont.)		Pancreas, incidence	Frequency of diet soft drink consumption, low physical activity (men, < 11.5 MET/wk; women, < 7.7 MET/wk) (RR):			Age (years), sex, follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption	
			< 1/mo	72	1		
			1–12/mo	53	1.07 (0.74–1.54)		
			> 3/wk	51	0.99 (0.68–1.44)		
			Trend-test *P* value: 0.84				
		Pancreas, incidence	Frequency of diet soft drink consumption, high physical activity (men, ≥ 11.5 MET/wk; women, ≥ 7.7 MET/wk) (RR):				
			< 1/mo	83	1		
			1–12/mo	63	1.07 (0.77–1.50)		
			> 3/wk	57	1.03 (0.72–1.47)		
			Trend-test *P* value: 0.94				
		Pancreas, incidence	Frequency of diet soft drink consumption, women, BMI, < 25 kg/m^2 (RR):			Age (years), follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption	
			< 1/mo	55	1		
			1–12/mo	34	1.05 (0.68–1.62)		
			> 3/wk	30	1.02 (0.64–1.62)		
			Trend-test *P* value: 0.93				
		Pancreas, incidence	Frequency of diet soft drink consumption, women, BMI ≥ 25 kg/m^2 (RR):				
			< 1/mo	21	1		
			1–12/mo	28	1.31 (0.74–2.32)		
			> 3/wk	34	1.32 (0.75–2.33)		
			Trend-test *P* value: 0.62				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2005) (cont.)		Pancreas, incidence	Frequency of diet soft drink consumption, women, low physical activity (< 7.7 MET/wk) (RR):			Age (years), follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption	
			< 1/mo	24	1		
			1–12/mo	25	0.72 (0.41–1.28)		
			> 3/wk	31	1.05 (0.63–1.76)		
			Trend-test *P* value: 0.38				
		Pancreas, incidence	Frequency of diet soft drink consumption, women, high physical activity (≥ 7.7 MET/wk) (RR):				
			< 1/m	53	1		
			1–12/mo	37	1.52 (0.98–2.36)		
			> 3/wk	35	1.21 (0.75–1.94)		
			Trend-test *P* value: 0.72				
		Pancreas, incidence	Frequency of diet soft drink consumption, men, BMI < 25 kg/m^2 (RR):				
			< 1/mo	31	1		
			1–12/mo	25	1.65 (0.96–2.83)		
			> 3/wk	7	0.68 (0.29–1.57)		
			Trend-test *P* value: 0.34				
		Pancreas, incidence	Frequency of diet soft drink consumption, men, BMI ≥ 25 kg/m^2 (RR):				
			< 1/mo	45	1		
			1–12/mo	28	0.77 (0.48–1.25)		
			> 3/wk	33	0.81 (0.55–1.40)		
			Trend-test *P* value: 0.74				
		Pancreas, incidence	Frequency of diet soft drink consumption, men, low physical activity (< 11.5 MET/wk) (RR):				
			< 1/mo	48	1		
			1–12/mo	28	0.94 (0.58–1.52)		
			> 3/wk	20	0.71 (0.41–1.22)		
			Trend-test *P* value: 0.21				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2005) (cont.)		Pancreas, incidence	Frequency of diet soft drink consumption, men, high physical activity (≥ 11.5 MET/wk) (RR): < 1/mo 1–12/mo > 3/wk Trend-test *P* value: 0.67	 30 26 22	 1 1.26 (0.74–2.15) 1.19 (0.66–2.12)	Age (years), follow-up cycle, history of diabetes, smoking status, caloric intake, nonvigorous physical activity, other soft drink consumption	
Bao et al. (2008) USA Enrolment, 1995–1996/ follow-up through 2003 (mean, 7.2 yr) Cohort	487 922; NIH-AARP Diet and Health Study: AARP members (men and women) aged 50–71 yr residing in 8 study areas, excluding those with prevalent cancer, history of diabetes, proxy respondents, and persons reporting extreme energy intake	Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption (RR): Never drinkers (0 g/day) 1st quintile (0.1–26.3 g/day) 2nd quintile (26.4–76.9 g/day) 3rd quintile (77.0–258.9 g/day) 4th quintile (259.0–494.4 g/day) 5th quintile (494.5–4897.6 g/day) Trend-test *P* value: 0.46	 716 137 86 99 118 102	 1 1.14 (0.90–1.43) 0.91 (0.70–1.18) 0.96 (0.75–1.24) 1.17 (0.92–1.49) 1.14 (0.89–1.47)	Sex, age	*Exposure assessment critique*: Key strengths were that ASB consumption was assessed and studied during a very relevant period for aspartame exposure from ASBs (1995–2003 in the USA); three different types of ASB were considered; and the prospective assessment.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bao et al. (2008) (cont.)	Exposure assessment method: FFQ at baseline, average follow-up of 7.2 yr Consumption frequency of 3 types of soft drink (soda, fruit drinks, and iced tea) + frequency of consumption of diet vs regular versions + 3 portion sizes	Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption (RR):			Sex, age, race, education, BMI, alcohol, smoking, physical activity, energy-adjusted red meat consumption, energy-adjusted folate consumption, total energy, regular soft drinks	Key limitations were the sequential assessment of first the frequency of consumption of soft drinks and then that of diet/sugar-free versions, with imprecise frequencies inducing inaccuracies; other sources of aspartame were not considered (although more limited). *Other strengths*: large study; estimated 90% completeness of case identification via linkage with cancer registries, detailed adjustment for confounders, including BMI; control for diabetes at baseline by exclusion of participants who reported having diabetes at baseline. *Other limitations*: potential for bias from non-differential measurement error.
			Never drinkers	716	1		
			1st quintile (0.1–26.3 g/day)	137	1.23 (0.97–1.55)		
			2nd quintile (26.4–76.9 g/day)	86	0.97 (0.74–1.27)		
			3rd quintile (77.0–258.9 g/day)	99	1.03 (0.80–1.33)		
			4th quintile (259.0–494.4 g/day)	118	1.23 (0.96–1.56)		
			5th quintile (494.5–4897.6 g/day)	102	1.11 (0.86–1.44)		
			Trend-test *P* value: 0.68				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bao et al. (2008) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption, excluding the first 2 yr of follow-up (RR):			Sex, age, race, education, BMI, alcohol, smoking, physical activity, energy-adjusted red meat consumption, energy-adjusted folate consumption, total energy, regular soft drinks	
			Never drinkers	NR	1		
			1st quintile (0.1–26.3 g/day)	NR	1.22 (0.94–1.59)		
			2nd quintile (26.4–76.9 g/day)	NR	1.00 (0.74–1.35)		
			3rd quintile (77.0–258.9 g/day)	NR	1.00 (0.75–1.34)		
			4th quintile (259.0–494.4 g/day)	NR	1.35 (1.03–1.77)		
			5th quintile (494.5–4897.6 g/day)	NR	1.25 (0.94–1.66)		
			Trend-test *P* value: 0.19				
		Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption, non-obese (BMI, < 30 kg/m^2) (RR):			Sex, age, race, education, BMI, alcohol, smoking, physical activity, energy-adjusted red meat consumption, energy-adjusted folate consumption, total energy, regular soft drinks	
			1st quintile (median, 0 g/day)	NR	1		
			2nd quintile (median, 16.2 g/day)	NR	1.31 (1.03–1.68)		
			3rd quintile (median, 74.8 g/day)	NR	0.88 (0.67–1.17)		
			4th quintile (median, 260.6 g/day)	NR	1.20 (0.93–1.56)		
			5th quintile (median, 816.9 g/day)	NR	1.16 (0.88–1.53)		
			Trend-test *P* value: 0.59				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bao et al. (2008) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption, obese (BMI, ≥ 30 kg/m²) (RR):			Sex, age, race, education, BMI, alcohol, smoking, physical activity, energy-adjusted red meat consumption, energy-adjusted folate consumption, total energy intake, regular soft drinks	
			1st quintile (median, 0 g/day)	NR	1		
			2nd quintile (median, 16.2 g/day)	NR	0.91 (0.51–1.63)		
			3rd quintile (median, 74.8 g/day)	NR	0.94 (0.53–1.64)		
			4th quintile (median, 260.6 g/day)	NR	0.93 (0.54–1.61)		
			5th quintile (median, 816.9 g/day)	NR	0.89 (0.51–1.53)		
			Trend-test *P* value: 0.73				
		Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption, high physical activity (≥ 3 times/wk) (RR):			Sex, age, race, education, BMI, alcohol, smoking, physical activity, energy-adjusted red meat consumption, energy-adjusted folate consumption, total energy, regular soft drinks	
			1st quintile (median, 0 g/day)	NR	1		
			2nd quintile (median, 16.2 g/day)	NR	1.21 (0.89–1.66)		
			3rd quintile (median, 74.8 g/day)	NR	0.87 (0.61–1.23)		
			4th quintile (median, 260.6 g/day)	NR	1.01 (0.72–1.41)		
			5th quintile (median, 816.9 g/day)	NR	1.14 (0.81–1.62)		
			Trend-test *P* value: 0.52				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bao et al. (2008) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption, low physical activity (≤ 3 times/week) (RR):			Sex, age, race, education, BMI, alcohol, smoking, physical activity, energy-adjusted red meat consumption, energy-adjusted folate consumption, total energy, regular soft drinks	
			1st quintile (median, 0 g/day)	NR	1		
			2nd quintile (median, 16.9 g/day)	NR	1.27 (0.92–1.75)		
			3rd quintile (median, 74.8 g/day)	NR	1.04 (0.74–1.47)		
			4th quintile (median, 260.6 g/day)	NR	1.27 (0.92–1.76)		
			5th quintile (median, 816.9 g/day)	NR	1.12 (0.79–1.57)		
			Trend-test *P* value: 0.84				
		Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption, never-smoker or quit ≥ 10 yr ago (RR):			Sex, age, race, education, BMI, alcohol, smoking, physical activity, energy-adjusted red meat consumption, energy-adjusted folate consumption, total energy, regular soft drinks	
			1st quintile (median, 0 g/day)	NR	1		
			2nd quintile (median, 16.2 g/day)	NR	1.22 (0.92–1.64)		
			3rd quintile (median, 74.8 g/day)	NR	1.03 (0.76–1.40)		
			4th quintile (median, 260.6 g/day)	NR	1.11 (0.82–1.49)		
			5th quintile (median, 816.9 g/day)	NR	1.25 (0.92–1.71)		
			Trend-test *P* value: 0.32				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bao et al. (2008) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Diet soft drink consumption, current smoker or quit < 10 yr ago (RR):			Sex, age, race, education, BMI, alcohol, smoking, physical activity, energy-adjusted red meat consumption, energy-adjusted folate consumption, total energy, regular soft drinks	
			1st quintile (median, 0 g/day)	NR	1		
			2nd quintile (median, 16.2 g/day)	NR	1.21 (0.81–1.79)		
			3rd quintile (median, 74.8 g/day)	NR	0.80 (0.51–1.27)		
			4th quintile (median, 260.6 g/day)	NR	1.19 (0.80–1.79)		
			5th quintile (median, 816.9 g/day)	NR	0.92 (0.60–1.40)		
			Trend-test *P* value: 0.45				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Navarrete-Muñoz et al. (2016) Europe Enrolment, 1992–2000/ follow-up, through 2004 to 2009 (depending on country) Cohort	477 199 (142 202 men and 334 997 women); EPIC cohort study participants from 23 centres in 10 European countries (Denmark, France, Germany, Greece, Italy, Norway, Spain, Sweden, Netherlands, and United Kingdom), excluding participants with prevalent cancer other than nonmelanoma skin cancer at baseline or with extreme energy intake/expenditure; Additionally, data from Italy, Spain, and Umeå were excluded from ASB analyses	Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption (HR): Nondrinker 1st quintile (0.1–2.0 g/day) 2nd quintile (2.1–9.9 g/day) 3rd quintile (9.9–28.6 g/day) 4th quintile (28.7–92.2 g/day) 5th quintile (> 92.2 g/day) Continuous (per 100 g/day) Trend-test *P* value: 0.55	 340 54 50 53 42 47 586	 0.88 (0.58–1.35) 1 1.13 (0.69–1.86) 1.12 (0.71–1.77) 1.03 (0.64–1.67) 1.07 (0.67–1.73) 1.04 (0.98–1.10)	Centre, sex, age, educational level, physical activity, smoking status, alcohol consumption, sugar-sweetened soft drinks, juice consumption	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption (several types of beverage) in several western Europe countries at a period relevant for aspartame exposure (between 1991 and 2000). Key limitations were that no other sources of aspartame were considered; uncertainty regarding aspartame content of ASBs in every country; and there was only one assessment at baseline.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Navarrete-Muñoz et al. (2016) (cont.)	Exposure assessment method: questionnaire; exposure to ASBs overall assessed once at baseline through country-specific validated tools (mainly FFQ) covering the usual diet over the past year; no specific assessment of aspartame or AS content in ASB	Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption (HR):			Centre, sex, age, educational level, physical activity, smoking status, alcohol consumption, sugar-sweetened soft drinks, juice consumption, energy intake, diabetes, BMI	Other information: context of low ASB consumption in middle-aged adults; continuous analyses were per 100 g increment which spans from nondrinker to the highest quintile; analyses across countries were meta-analysed; no heterogeneity by country; adjustment set for sex-stratified models by 336 g increment were confirmed on a personal communication with authors. *Other strengths*: large cohort, geographical diversity, validated questionnaires, cancer subtype available; results adjusted for diabetes and BMI. *Other limitations*: co-exposure to other ASs likely towards the end of follow-up for countries that started recruitment later.
			Nondrinker	340	0.89 (0.58–1.36)		
			1st quintile (0.1–2.0 g/day)	54	1		
			2nd quintile (2.1–9.9 g/day)	50	1.12 (0.68–1.84)		
			3rd quintile (9.9–28.6 g/day)	53	1.09 (0.69–1.73)		
			4th quintile (28.7–92.2 g/day)	42	0.99 (0.61–1.60)		
			5th quintile (> 92.2 g/day)	47	0.99 (0.61–1.60)		
			Continuous (per 100 g/day)	586	1.02 (0.96–1.08)		
			Trend-test *P* value: 0.81				
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, women (HR):			Centre, age, education, smoking, alcohol consumption physical activity, BMI, diabetes, energy intake	
			Continuous (per 336 g/day)	373	0.69 (0.44–1.09)		
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, men (HR):				
			Continuous (per 336 g/day)	313	1.25 (1.03–1.52)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Navarrete-Muñoz et al. (2016) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, age < 62 yr at diagnosis (HR):			Centre, sex, age, educational level, physical activity, smoking status, alcohol consumption, sugar-sweetened soft drinks, juice consumption	
			Continuous (per 100 g/day)	215	1.00 (0.90–1.10)		
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, age ≥ 62 yr at diagnosis (HR):				
			Continuous (per 100 g/day)	471	1.05 (0.98–1.12)		
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, BMI < 25 kg/m^2 (HR):				
			Continuous (per 100 g/day)	297	0.95 (0.81–1.11)		
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, BMI ≥ 25 kg/m^2 (HR):				
			Continuous (per 100 g/day)	389	1.05 (0.99–1.12)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Navarrete-Muñoz et al. (2016) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, waist circumference normal/moderate (HR):				
			Continuous (per 100 g/day)	452	1.05 (0.98–1.13)	Centre, sex, age, educational level, physical activity, smoking status, alcohol consumption, sugar-sweetened soft drinks, juice consumption	
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, waist circumference large (HR):				
			Continuous (per 100 g/day)	180	1.02 (0.93–1.11)	Centre, sex, age, education, physical activity, alcohol consumption, sugar-sweetened beverage intake	
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, low physical activity (HR):				
			Continuous (per 100 g/day)	381	1.07 (1.00–1.15)	Centre, sex, age, educational level, physical activity, smoking status, alcohol consumption, sugar-sweetened soft drinks, juice consumption	
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, high physical activity (HR):				
			Continuous (per 100 g/day)	268	1.02 (0.93–1.12)		
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, never-smokers (HR):				
			Continuous (per 100 g/day)	257	1.00 (0.88–1.14)	Centre, sex, age, educational level, physical activity, alcohol consumption, sugar-sweetened soft drinks, juice consumption	
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, former smokers (HR):				
			Continuous (per 100 g/day)	201	1.05 (0.96–1.15)		
		Pancreas (exocrine, adenocarcinoma), incidence	Artificially sweetened soft drink consumption, current smokers (HR):				
			Continuous (per 100 g/day)	213	1.03 (0.93–1.13)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Stepien et al. (2016) Europe Enrolment, 1992–1998/ follow-up, through 2006 to 2010 (depending on country) Cohort	424 123; EPIC study participants from 23 centres in Denmark, France, Greece, Germany, Italy, Netherlands, Norway, Spain, Sweden, and the United Kingdom (for artificially sweetened soft drinks analysis, participants from selected centres in Italy (north and Ragusa) and Sweden (Umeå) were excluded); men and women generally aged 35–70 yr; excluding participants with prevalent cancer other than nonmelanoma skin cancer at baseline or with extreme energy intake/expenditure	Liver (HCC), incidence	Artificially sweetened soft drink consumption (HR): Continuous (per serving of 330 mL)/ wk	151	1.06 (1.03–1.09)	Sex, age, study centre, non-alcoholic energy intake, alcohol at enrolment, lifetime alcohol intake, smoking status and intensity, BMI, physical activity, education, diabetes status	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption (several types of beverages) in several western European countries at a period relevant for aspartame exposure (between 1991 and 2000). Key limitations were that no other sources of aspartame were considered; uncertainty regarding aspartame content in ASBs in each country; and there was only one assessment at baseline. Other information: context of low ASB consumption in middle-aged adults; did not present detailed data by ASB, e.g. unknown whether models controlled for biochemical measures of hepatitis and liver enzymes.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Stepien et al. (2016) (cont.)	Exposure assessment method: questionnaire; exposure to artificially sweetened soft drinks overall assessed once at baseline through country-specific validated tools (mainly FFQ) covering the usual diet over the past year; no specific assessment of aspartame or AS content of the artificially sweetened soft drinks						[Note: the Working Group noted that a correction to the number of hepatocellular carcinomas reported in the original paper was published on 17 April 2024 and the original paper was updated.] *Other strengths*: prospective design; diverse European population; control for multiple confounders, including BMI. *Other limitations*: small number of cases; co-exposure to other ASs likely towards the end of follow-up for countries that started recruitment later; did not control for HBV/HCV status.
Hodge et al. (2018) Australia Enrolment, 1990–1994/ follow-up through 30 June 2013 Cohort	35 593MCCS – a prospective cohort study of men and women aged 40–69 yr at recruitment and free of cancer, angina, heart attack, or diabetes at baseline; participants with extreme baseline energy intake were excluded	Stomach, (gastric cardia), incidence	Artificially sweetened soft drink consumption (HR): Never or < 1/mo 1–3/mo 1–6/wk ≥ 1/day Continuous (per beverage/ day) Trend-test *P* value: 0.46	123 9 23 10 165	1 0.86 (0.42–1.73) 1.46 (0.92–2.34) 1.03 (0.53–1.98) 1.24 (0.70–2.18)	Age, sex, socioeconomic index, country of birth, alcohol intake, smoking status, physical activity, Mediterranean diet score, sugar-sweetened soft drink consumption, waist circumference	*Exposure assessment critique*: Key strengths were that it was a prospective study; assessment after aspartame introduction in diet soft drinks in Australia (1987), and first half of follow-up largely overlapping with period of aspartame use in Australia.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hodge et al. (2018) (cont.)	Exposure assessment method: self-administered 121-item FFQ with separate questions on frequency of consumption in the past year of diet (artificially sweetened) soft drinks	Colon and rectum, incidence	Artificially sweetened soft drink consumption (HR): Never or < 1/mo 1–3/mo 1–6/wk ≥ 1/day Continuous (per beverage/ day) Trend-test *P* value: 0.46	 802 77 125 51 1055	 1 0.87 (0.68–1.11) 1.15 (0.95–1.40) 0.79 (0.60–1.06) 0.91 (0.71–1.17)	Age, sex, socioeconomic index, country of birth, alcohol intake, smoking status, physical activity, Mediterranean diet score, sugar-sweetened soft drink consumption, waist circumference	Key limitations were the FFQ assessment with no specific estimate of aspartame exposure, ASBs as a proxy, and exposure data at baseline only. Other information: other non-colorectal gastrointestinal cancers were included in the "overall obesity-related cancers" outcome but were not examined individually. *Other strengths*: adjustment for key confounders, including a measure of obesity (waist circumference); ability to examine stomach cancer subsite. *Other limitations*: small number of consumers; likely bias from non-differential exposure misclassification given single baseline assessment and long follow-up.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Chazelas et al. (2019) France Enrolment, 2009–2017/ follow-up, through 11 January 2018 Cohort	101 257; NutriNet-Santé prospective cohort (web-based); men and women aged ≥ 18 yr Exposure assessment method: participants were asked every 6 mo to complete a series of three validated web-based 24 h dietary records randomly assigned over a 2-wk period (2 weekdays, 1 weekend day); at least two 24 h dietary records during the first 2 yr of follow-up were considered in analyses (mean ± SD, 5.6 ± 3.0)	Colon and rectum, incidence	ASB intake (subdistribution HR):			Age, sex, energy intake without alcohol, sugar intake from other dietary sources, alcohol, sodium, lipid, fruit and vegetable intakes, BMI, height, physical activity, smoking status, number of 24 h records, family history of cancer, educational level, prevalent type 2 diabetes, hypertension, major cardiovascular event and dyslipidaemia at baseline	*Exposure assessment critique*: A key strength was that it was a prospective study using repeated dietary records. Key limitations were that there was no specific estimate of aspartame exposure, use of ASBs as a proxy (includes 12 ASB items, all beverages containing non-nutritive sweeteners, such as diet soft drinks, sugar-free syrups, and diet milk-based beverages); and exposure data at baseline only. *Other strengths*: large population-based prospective study; comprehensive adjustment for confounders. *Other limitations*: only 166 cases of colorectal cancer; low level of consumption of ASB, with median of 6.9 mL/day; non-representative population, predominantly women, may limit result generalizability.
			1st quartile (men, < 2.7 mL/day; or women, < 4.6 mL/day)	119	1		
			2nd quartile (men, 2.7 to < 4.7 mL/day; or women, 4.6 to < 7.7 mL/day)	29	0.65 (0.37–1.14)		
			3rd quartile (men, 4.7 to < 7.9 mL/day; or women, 7.7 to < 11.6 mL/day)	4	0.84 (0.21–3.42)		
			4th quartile (men, ≥ 7.9 mL/day; or women, ≥ 11.6 mL/day)	14	0.80 (0.44–1.46)		
			Continuous (per 10 mL/day)	166	1.02 (0.94–1.10)		
			Trend-test *P* value: 0.40				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Malik et al. (2019) USA Enrolment, 1976 (NHS), 1986 (HPFS)/ follow-up, 1980–2014 (NHS), 1986–2014 (HPFS) Cohort	37 716 men and 80 647 women; female registered nurses aged 30–55 yr in the NHS and male health professionals aged 40–75 yr in the HPFS, excluding those with history of diabetes, cardiovascular disease, or cancer at baseline, or with implausible dietary intake Exposure assessment method: prospective assessment of ASB consumption through repeated FFQs between 1980–1986 and 2010	Colon, mortality	ASB intake (HR): < 1 serving/mo 1–4 servings/mo 2–6 servings/wk 1 to < 2 servings/day ≥ 2 servings/day Continuous (per serving/ day) Trend-test *P* value: 0.69	 NR NR NR NR NR NR	 1 0.97 (0.81–1.16) 1.01 (0.86–1.18) 1.11 (0.89–1.37) 1.01 (0.77–1.31) 1.00 (0.91–1.07)	Age, race, smoking, alcohol intake, postmenopausal hormone use (NHS), physical activity, family history of diabetes; family history of myocardial infarction, family history of cancer, multivitamin use, aspirin use, baseline history of hypertension and hypercholesterolaemia, intake of whole grains, fruit, vegetables, or red and processed meat, total energy, BMI, SSB intake	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption from repeated diet assessments (using validated instrument) every 4 yr, the majority at a very relevant period for aspartame exposure from ASBs (the USA between the 1980s and 2010) potentially capturing lifetime exposure to aspartame. Key limitations were that other sources of aspartame were not considered (although these were more limited); and uncertainty regarding aspartame content in ASBs after the mid-2000s. *Other strengths*: large cohort with long follow-up. *Other limitations*: likely bias from non-differential misclassifications of exposure to aspartame; stratified numbers of deaths not provided for specific cancer sites.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mullee et al. (2019) Europe Enrolment, 1992–2000/ follow-up, through 2009–2013 (depending on study centre, mean 16.4 yr) Cohort	451 743; EPIC cohort study participants from 10 European countries (Denmark, France, Greece, Germany, Italy, Netherlands, Norway, Spain, Sweden, and the United Kingdom); men and women excluding those with prevalent cancer, heart disease, stroke, or diabetes, or with implausible dietary intake; for artificially sweetened soft drinks; participants from Italy, Spain, and Sweden were not included; 2095 colorectal cancer deaths	Colon and rectum, mortality	Glasses (250 mL) of artificially sweetened soft drink consumption (HR): < 1 glass/mo 1 to 4 glasses/mo > 1 to 6 glasses/ wk ≥ 1 glass/day Trend-test *P* value: 0.21	 NR NR NR NR	 1 1.08 (0.91–1.28) 1.02 (0.85–1.22) 1.22 (0.91–1.64)	Age, centre, sex, BMI, physical activity index, educational status, alcohol consumption, smoking status, smoking intensity, smoking duration, ever use of contraceptive pill, menopausal status, ever use of menopausal hormone therapy, intakes of total energy, red and processed meat, fruits and vegetables, coffee, fruit and vegetable juice, sugar-sweetened soft drinks	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption (several types of beverage) in several western European countries at a period relevant for aspartame exposure (between 1991 and 2000). Key limitations were that no other sources of aspartame were considered; uncertainty regarding the aspartame content in ASBs in every country; and there was only one assessment at baseline. Other information: context of low ASB consumption in middle-aged adults. *Other strengths*: large population-based cohort spanning multiple countries with different behaviours; large number of cases; results adjusted for appropriate potential confounders, including BMI.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mullee et al. (2019) (cont.)	Exposure assessment method: questionnaire; exposure to artificially sweetened soft drinks overall assessed once at baseline through country-specific validated tools (mainly FFQ) covering the usual diet over the past year; no specific assessment of aspartame or AS content of the artificially sweetened soft drinks						

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hur et al. (2021) USA Enrolment, 1989/follow-up, 1991 through June 2015 Cohort	95 464; NHS-II cohort; female registered nurses aged 25–42 yr at enrolment; excluded women with colorectal cancer or inflammatory bowel disease before baseline, or with implausible energy intake Exposure assessment method: semiquantitative FFQ every 4 yr since 1991 assessing the frequency of ASB consumption with standard serving size; cumulative average of intakes updated at every FFQ cycle	Colon and rectum, early onset (before age 50 yr), incidence	Low-calorie carbonated beverage intake (RR):			Age, total energy intake, race, height, BMI, menopausal status, menopausal hormone use, family history of colorectal cancer, pack-years of smoking, physical activity, regular aspirin use, regular non-steroidal anti-inflammatory use, current multivitamin use, intake of alcohol, intake of red and processed meat, dietary fibre, total folate, total calcium, Alternative Healthy Eating Index-2010 without alcohol or SSB, lower endoscopy for screening or other indications in past 10 yr	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption as a cumulative average from repeated diet assessments every 4 yr (from 1991 to 2015) at a very relevant period for aspartame exposure from ASBs in the USA, potentially capturing lifetime exposure to aspartame. Key limitations were that other sources of aspartame including non-carbonated ASBs were not considered (although these were more limited); there was uncertainty regarding aspartame content in ASBs after the mid-2000s; and the repeated intake data could have been better used by analysing as time-dependent data rather than just averaging. *Other limitations*: relatively small numbers of cases, only 109 early-onset colon or rectal cancers, i.e. diagnosed before age 50 yr.
			< 1 serving/wk	32	1		
			1 serving/wk to < 1 serving/day	33	1.20 (0.73–1.98)		
			1 to < 2 servings/day	19	0.86 (0.48–1.54)		
			≥ 2 servings/day	25	0.73 (0.42–1.27)		
			Continuous (per serving/day)	109	0.93 (0.83–1.04)		
			Trend-test *P* value: 0.11				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Jones et al. (2022) USA Enrolment, 1995–1996 (NIH-AARP), 1993–2001 (PLCO)/ follow-up, through 2011 (NIH-AARP), 1998–2011 (PLCO) Cohort	553 874; pooled analysis of two cohorts: (i) NIH-AARP Diet and Health Study – men and women aged 50–71 yr residing in six states and two metropolitan areas; (ii) PLCO – men and women aged 55–74 yr randomized to screening trial between 1993–2001 across 10 screening centres in the USA; all participants were without prior cancer diagnoses; proxy respondents were not included; 1060 incident liver cancers. Exposure assessment method: questionnaire; frequency of consumption of ASBs assessed in two cohorts at baseline in 1995–1996 and 1998, no indication of volume consumed	Liver (HCC), incidence	ASB consumption, non-diabetics, ≤ 12 yr follow-up (HR):			Age, sex, race/ ethnicity, BMI, smoking, alcohol use, study, total energy intake (kcal/day)	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption (soda and fruit punches) at a very relevant period for aspartame exposure from ASBs (the USA in 1995–1996 and 1998). Similar exposure assessment in both cohorts. Key limitations were that no other sources were considered (although these were more limited); there was only one assessment at baseline (possible variations over up to 19 yr of follow-up); and uncertainty regarding aspartame content in ASBs after the mid-2000s. *Other strengths*: prospective, pooled data from two large studies resulted in large sample size, stratification by diabetes status.
			Continuous (per time/day)	587	1.01 (0.92–1.11)		
		Liver (HCC), incidence	ASB consumption, non-diabetics, 12+ yr follow-up (HR):				
			Continuous (per time/day)	252	0.99 (0.86–1.15)		
		Liver (HCC), incidence	Artificially sweetened soda consumption, non-diabetics, ≤ 12 yr follow-up (HR):				
			Continuous (per time/day)	587	1.00 (0.90–1.11)		
		Liver (HCC), incidence	Artificially sweetened soda consumption, non-diabetics, 12+ yr follow-up (HR):				
			Continuous (per time/day)	252	0.98 (0.83–1.15)		
		Liver (HCC), incidence	Artificially sweetened fruit punch consumption, non-diabetics, ≤ 12 yr follow-up (HR):				
			Continuous (per time/day)	587	1.06 (0.80–1.41)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Jones et al. (2022) (cont.)		Liver (HCC), incidence	Artificially sweetened fruit punch consumption, non-diabetics, 12+ yr follow-up (HR):			Age, sex, race/ ethnicity, BMI, smoking, alcohol use, study, total energy intake (kcal/day)	*Other limitations*: single assessment of covariates; stratification by diabetes status may be misclassified due to potential changes in status during follow-up; the study did not control for coffee consumption or HBV/HCV infection status.
			Continuous (per time/day)	252	1.11 (0.74–1.65)		
		Liver (HCC)	ASB consumption, diabetics, ≤ 12 yr follow-up (HR):				
			Continuous (per time/day)	158	1.13 (1.02–1.25)		
		Liver (HCC), incidence	ASB consumption, diabetics, 12+ yr follow-up (HR):				
			Continuous (per time/day)	63	0.82 (0.64–1.05)		
		Liver (HCC), incidence	Artificially sweetened soda consumption, diabetics, ≤ 12 yr follow-up (HR):				
			Continuous (per time/day)	158	1.13 (1.01–1.27)		
		Liver (HCC)	Artificially sweetened soda consumption, diabetics, 12+ yr follow-up (HR):				
			Continuous (per time/day)	63	0.78 (0.59–1.03)		
		Liver (HCC), incidence	Artificially sweetened fruit punch consumption, diabetics, ≤ 12 yr follow-up (HR):				
			Continuous (per time/day)	158	1.17 (0.92–1.48)		
		Liver (HCC), incidence	Artificially sweetened fruit punch consumption, diabetics, 12+ yr follow-up (HR):				
			Continuous (per time/day)	63	1.01 (0.61–1.69)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) USA Enrolment, 1982/follow-up, through 2016 (median, 27.7 yr) Cohort	934 777 (416 313 men, 518 464 women); CPS-II prospective cohort; adults aged ≥ 28 yr; excluded participants with personal history at baseline of diabetes or cancer other than nonmelanoma skin cancer, men aged > 90 yr or women aged > 95 yr at enrolment, and those reporting only prior but not current consumption of either SSBs or ASBs Exposure assessment method: questionnaire; exposure to ASBs assessed in 1982 through a question about number of drinks per day of diet soda or ice teas (one pooled item) and potential changes over the past 10 yr; no specific assessment of aspartame content in ASBs	Larynx[a], pharynx, and oral cavity combined, mortality	ASB consumption, BMI adjusted (HR): Never < 1 drink/day 1 drink/day ≥ 2 drinks/day Continuous (per drink/day) Trend-test *P* value: 0.107	 NR NR NR 1852	 1 0.95 (0.79–1.13) 1.17 (0.97–1.41) 1.13 (0.94–1.37) 1.04 (0.99–1.09)	Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption. Key limitations were that there was only one dietary assessment at baseline in 1982 that was before the use of aspartame in ASBs, hence the relevance to aspartame exposure depends on the stability of ASB consumption over up to 34 yr of follow-up, but such information was not directly available; no other sources were considered (although these were more limited); and uncertainty regarding aspartame content in ASBs after the mid-2000s. Other information: exclusion of participants who reported only prior but not current consumption of either SSBs or ASBs at baseline.
		Larynx[a], pharynx, and oral cavity combined, mortality	ASB consumption, men, BMI adjusted (HR): Never < 1 drink/day 1 drink/day ≥ 2 drinks/day Continuous (per drink/day) Trend-test *P* value: 0.034	 NR NR NR 1215	 1 1.23 (0.98–1.53) 1.40 (1.10–1.78) 1.13 (0.87–1.48) 1.07 (1.00–1.14)	Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Larynx[a], pharynx, and oral cavity combined, mortality	ASB consumption, women, BMI adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	*Other strengths*: large, prospective cohort with long follow-up; ability to examine multiple cancer types, stratify by sex or BMI, and limit to never-smokers; comprehensive adjustment for confounders, including SSB consumption. *Other limitations*: potential for residual confounding because unable to distinguish gastric cardia and non-cardia cancers or oesophageal SCC and adenocarcinoma with different risk factors; unable to control liver cancer for HBV/HCV infection status.
			Never	NR	1		
			< 1 drink/day	NR	0.68 (0.51–0.89)		
			1 drink/day	NR	0.89 (0.66–1.20)		
			≥ 2 drinks/day	NR	1.06 (0.80–1.40)		
			Continuous (per drink/day)	637	0.99 (0.91–1.07)		
			Trend-test *P* value: 0.845				
		Oesophagus, mortality	ASB consumption, BMI adjusted (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.98 (0.85–1.11)		
			1 drink/day	NR	0.89 (0.75–1.05)		
			≥ 2 drinks/day	NR	0.92 (0.78–1.08)		
			Continuous (per drink/day)	2727	0.99 (0.94–1.03)		
			Trend-test *P* value: 0.155				
		Stomach, mortality	ASB consumption, BMI adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.92 (0.81–1.05)		
			1 drink/day	NR	0.92 (0.79–1.09)		
			≥ 2 drinks/day	NR	1.06 (0.91–1.24)		
			Continuous (per drink/day)	2798	1.00 (0.96–1.05)		
			Trend-test *P* value: 0.892				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Liver (HCC), mortality	ASB consumption, BMI adjusted (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.01 (0.90–1.15)		
			1 drink/day	NR	1.01 (0.87–1.17)		
			≥ 2 drinks/day	NR	0.95 (0.81–1.10)		
			Continuous (per drink/day)	2722	0.98 (0.94–1.02)		
			Trend-test *P* value: 0.578				
		Liver (HCC), mortality	ASB consumption, men, BMI adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.10 (0.92–1.32)		
			1 drink/day	NR	1.07 (0.87–1.32)		
			≥ 2 drinks/day	NR	1.00 (0.80–1.24)		
			Continuous (per drink/day)	1576	0.99 (0.93–1.05)		
			Trend-test *P* value: 0.701				
		Liver (HCC), mortality	ASB consumption, women, BMI adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.93 (0.79–1.11)		
			1 drink/day	NR	0.94 (0.76–1.16)		
			≥ 2 drinks/day	NR	0.90 (0.72–1.12)		
			Continuous (per drink/day)	1146	0.97 (0.92–1.04)		
			Trend-test *P* value: 0.270				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Liver (HCC), mortality	ASB consumption, never-smokers, BMI adjusted (HR):			Age, sex, race/ ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.98 (0.81–1.20)		
			1 drink/day	NR	1.01 (0.80–1.28)		
			≥ 2 drinks/day	NR	0.93 (0.72–1.20)		
			Continuous (per drink/day)	996	0.96 (0.89–1.04)		
			Trend-test *P* value: 0.637				
		Liver (HCC), mortality	ASB consumption, men, never-smokers (HR):			Age, race/ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	
			Never	NR	1		
			< 1 drink/day	NR	1.13 (0.80–1.60)		
			1 drink/day	NR	1.17 (0.80–1.73)		
			≥ 2 drinks/day	NR	1.44 (0.99–2.08)		
			Continuous (per drink/day)	413	1.08 (0.98–1.19)		
			Trend-test *P* value: 0.040				
		Liver (HCC), mortality	ASB consumption, men, never-smokers, BMI adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	1.03 (0.73–1.47)		
			1 drink/day	NR	1.04 (0.70–1.53)		
			≥ 2 drinks/day	NR	1.21 (0.83–1.77)		
			Continuous (per drink/day)	413	1.03 (0.93–1.15)		
			Trend-test *P* value: 0.335				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Liver (HCC), mortality	ASB consumption, women, never-smokers, BMI adjusted (HR):			Age, race/ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.96 (0.75–1.21)		
			1 drink/day	NR	0.99 (0.74–1.32)		
			≥ 2 drinks/day	NR	0.76 (0.54–1.08)		
			Continuous (per drink/day)	583	0.91 (0.81–1.01)		
			Trend-test *P* value: 0.169				
		Pancreas, mortality	ASB consumption (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	
			Never	NR	1		
			< 1 drink/day	NR	1.03 (0.97–1.11)		
			1 drink/day	NR	1.09 (1.01–1.18)		
			≥ 2 drinks/day	NR	1.16 (1.07–1.26)		
			Continuous (per drink/day)	9196	1.04 (1.02–1.06)		
			Trend-test *P* value: < 0.0001				
		Pancreas, mortality	ASB consumption, BMI adjusted (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.00 (0.94–1.07)		
			1 drink/day	NR	1.06 (0.98–1.14)		
			≥ 2 drinks/day	NR	1.11 (1.02–1.20)		
			Continuous (per drink/day)	9196	1.03 (1.01–1.05)		
			Trend-test *P* value: 0.008				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Pancreas, mortality	ASB consumption, men, BMI adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.96 (0.86–1.08)		
			1 drink/day	NR	1.10 (0.97–1.25)		
			≥ 2 drinks/day	NR	1.15 (1.01–1.30)		
			Continuous (per drink/day)	4339	1.04 (1.00–1.08)		
			Trend-test *P* value: 0.023				
		Pancreas, mortality	ASB consumption, women, BMI adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	1.01 (0.93–1.10)		
			1 drink/day	NR	1.02 (0.92–1.13)		
			≥ 2 drinks/day	NR	1.08 (0.97–1.19)		
			Continuous (per drink/day)	4857	1.02 (0.99–1.05)		
			Trend-test *P* value: 0.167				
		Pancreas, mortality	ASB consumption, never-smokers, BMI adjusted (HR):			Age, sex, race/ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.99 (0.90–1.10)		
			1 drink/day	NR	1.07 (0.95–1.20)		
			≥ 2 drinks/day	NR	1.19 (1.05–1.34)		
			Continuous (per drink/day)	3955	1.05 (1.02–1.09)		
			Trend-test *P* value: 0.005				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Pancreas, mortality	ASB consumption, men, never-smokers, BMI adjusted (HR):			Age, race/ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.93 (0.77–1.14)		
			1 drink/day	NR	1.00 (0.81–1.25)		
			≥ 2 drinks/day	NR	1.34 (1.10–1.65)		
			Continuous (per drink/day)	1456	1.08 (1.02–1.14)		
			Trend-test *P* value: 0.018				
		Pancreas, mortality	ASB consumption, women, never-smokers, BMI adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	1.01 (0.90–1.13)		
			1 drink/day	NR	1.09 (0.95–1.25)		
			≥ 2 drinks/day	NR	1.12 (0.96–1.30)		
			Continuous (per drink/day)	2499	1.03 (0.99–1.08)		
			Trend-test *P* value: 0.089				
		Pancreas, mortality	ASB consumption, normal weight (BMI, 18.5 to < 25 kg/m^2) (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.96 (0.87–1.06)		
			1 drink/day	NR	1.01 (0.89–1.14)		
			≥ 2 drinks/day	NR	1.07 (0.94–1.22)		
			Continuous (per drink/day)	4234	1.00 (0.97–1.04)		
			Trend-test *P* value: 0.432				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Pancreas, mortality	ASB consumption, overweight (BMI, 25 to < 30 kg/m²) (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.03 (0.93–1.14)		
			1 drink/day	NR	1.08 (0.96–1.22)		
			≥ 2 drinks/day	NR	1.18 (1.04–1.33)		
			Continuous (per drink/day)	3625	1.05 (1.01–1.08)		
			Trend-test *P* value: 0.006				
		Pancreas, mortality	ASB consumption, obese (BMI, ≥ 30 kg/m²) (HR):				
			Never	NR	1		
			< 1 drink/day	NR	1.10 (0.92–1.31)		
			1 drink/day	NR	1.02 (0.83–1.26)		
			≥ 2 drinks/day	NR	0.99 (0.80–1.21)		
			Continuous (per drink/day)	1022	1.01 (0.95–1.07)		
			Trend-test *P* value: 0.992				
		Bile duct or gallbladder, mortality	ASB consumption (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	
			Continuous (per drink/day)	1024	1.07 (1.01–1.14)		
			Trend-test *P* value: 0.011				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Bile duct or gallbladder, mortality	ASB consumption, BMI adjusted (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.12 (0.93–1.36)		
			1 drink/day	NR	1.18 (0.94–1.48)		
			≥ 2 drinks/day	NR	1.15 (0.90–1.46)		
			Continuous (per drink/day)	1024	1.05 (0.98–1.11)		
			Trend-test *P* value: 0.105				
		Bile duct or gallbladder, mortality	ASB consumption, never-smokers, BMI adjusted (HR):			Age, sex, race/ ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.05 (0.80–1.39)		
			1 drink/day	NR	1.14 (0.81–1.60)		
			≥ 2 drinks/day	NR	1.25 (0.88–1.77)		
			Continuous (per drink/day)	476	1.05 (0.96–1.16)		
			Trend-test *P* value: 0.170				
		Small intestine, mortality	ASB consumption (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	
			Continuous (per drink/day)	324	1.12 (1.01–1.23)		
			Trend-test *P* value: 0.162				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Small intestine, mortality	ASB consumption, BMI adjusted (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.98 (0.68–1.42)		
			1 drink/day	NR	1.09 (0.72–1.66)		
			≥ 2 drinks/day	NR	1.28 (0.85–1.93)		
			Continuous (per drink/day)	324	1.11 (1.00–1.22)		
			Trend-test *P* value: 0.244				
		Small intestine, mortality	ASB consumption, never-smokers, BMI adjusted (HR):			Age, sex, race/ ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.25 (0.75–2.07)		
			1 drink/day	NR	1.13 (0.60–2.13)		
			≥ 2 drinks/day	NR	1.20 (0.62–2.32)		
			Continuous (per drink/day)	138	1.10 (0.94–1.30)		
			Trend-test *P* value: 0.479				
		Colon and rectum, mortality	ASB consumption (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	
			Never	NR	1		
			< 1 drink/day	NR	0.99 (0.94–1.05)		
			1 drink/day	NR	1.03 (0.97–1.11)		
			≥ 2 drinks/day	NR	0.97 (0.90–1.04)		
			Continuous (per drink/day)	13 752	1.00 (0.98–1.02)		
			Trend-test *P* value: 0.704				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Colon and rectum, mortality	ASB consumption, BMI adjusted (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.96 (0.91–1.02)		
			1 drink/day	NR	1.00 (0.93–1.07)		
			≥ 2 drinks/day	NR	0.92 (0.86–0.99)		
			Continuous (per drink/day)	13 752	0.99 (0.97–1.01)		
			Trend-test *P* value: 0.034				
		Colon and rectum, mortality	ASB consumption, men, BMI adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.01 (0.92–1.11)		
			1 drink/day	NR	0.96 (0.86–1.07)		
			≥ 2 drinks/day	NR	0.95 (0.84–1.06)		
			Continuous (per drink/day)	6660	0.99 (0.96–1.02)		
			Trend-test *P* value: 0.276				
		Colon and rectum, mortality	ASB consumption, women, BMI adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.94 (0.87–1.01)		
			1 drink/day	NR	1.02 (0.93–1.11)		
			≥ 2 drinks/day	NR	0.90 (0.82–0.99)		
			Continuous (per drink/day)	7092	0.98 (0.96–1.01)		
			Trend-test *P* value: 0.052				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Colon and rectum, mortality	ASB consumption, never-smokers, BMI adjusted (HR):			Age, sex, race/ ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.97 (0.89–1.05)		
			1 drink/day	NR	1.04 (0.94–1.15)		
			≥ 2 drinks/day	NR	0.92 (0.82–1.03)		
			Continuous (per drink/day)	5799	0.98 (0.95–1.01)		
			Trend-test *P* value: 0.294				
		Colon and rectum, mortality	ASB consumption, men, never-smokers, BMI adjusted (HR):			Age, race/ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.03 (0.88–1.20)		
			1 drink/day	NR	0.95 (0.79–1.16)		
			≥ 2 drinks/day	NR	0.90 (0.74–1.11)		
			Continuous (per drink/day)	2107	0.96 (0.90–1.02)		
			Trend-test *P* value: 0.335				
		Colon and rectum, mortality	ASB consumption, women, never-smokers, BMI adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.95 (0.86–1.04)		
			1 drink/day	NR	1.07 (0.95–1.20)		
			≥ 2 drinks/day	NR	0.92 (0.80–1.06)		
			Continuous (per drink/day)	3692	0.99 (0.95–1.02)		
			Trend-test *P* value: 0.469				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Norell et al. (1986) Sweden 1982–1984 Case–control	Cases, 99; aged 40–79 yr diagnosed with cancer of the exocrine pancreas at three surgical departments in Stockholm and Uppsala; diagnosis based on resection or autopsy; of 120 confirmed cases, 21 were lost to the study, leaving 99 (55 men, 44 women) Controls: 301 (163 hospital-based, 138 population-based); population controls selected from parish registries, which list all inhabitants by date of birth; matched on sex, closest date of birth in the same parish; hospital controls were selected as a stratified (age, sex) random sample of patients aged 40–79 yr who had a diagnosis of inguinal hernia during the study period from the same three surgical departments; survey completed by proxy for 16 cases,	Pancreas (exocrine), incidence Pancreas (exocrine), incidence	Use of AS, population controls (RR): No Yes Use of AS, hospital controls (RR): No Yes	 78 18 78 18	 1 1.2 (90% CI, 0.7–2.0) 1 1.1 (90% CI, 0.6–2.0)	Age, sex, parish Age, sex, hospital	*Exposure assessment critique*: Key limitations were the retrospective recall with a single item on use of AS and not specifically aspartame; study participants could not consume aspartame before it was approved in the early 1980s, therefore, exposure of participants to AS does not reflect aspartame. *Other strengths*: population-based controls should reflect exposure experience of the study population (minimize selection bias). *Other limitations*: potential for bias from exposure misclassification due to long induction periods and variable exposure by time; potential for recall bias; limited number of exposed cases; reported results for ASs did not control for confounders other than sex, age, and hospital/parish.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Norell et al. (1986) (cont.)	2 hospital controls, and 1 population control Exposure assessment method: self-administered questionnaire including use of AS; exposure categorized as consumers vs non-consumers only						
Mayne et al. (2006) USA 1993–1995 Case–control	Cases: 255 gastric cardia adenocarcinoma; 352 noncardia gastric adenocarcinoma; 206 oesophageal SCC; 282 oesophageal adenocarcinoma; aged 30–79 yr with incident cancer in rapid reporting systems in three areas (Connecticut, New Jersey, and western Washington State); medical records and slides reviewed by study pathologists to confirm diagnoses	Stomach (gastric cardia, adenocarcinoma), incidence	Diet carbonated soft drink consumption (OR): No intake Top 20% of intake	 NR NR	 1 0.50 (0.31–0.81)	Age, sex, study centre, race, proxy interview status, BMI, mean caloric intake, consumption (each) of beer/wine/liquor, consumption of meat, cigarettes per day, education, income, frequency of reflux symptoms	*Exposure assessment critique*: Key limitations were the retrospective recall; the lack of a specific estimate of aspartame exposure with ASBs as a proxy (diet soft drinks or soda), but timing of exposure was consistent for aspartame being used as the major AS in beverages; lack of data on duration of exposure; the authors repeated analyses limiting to those without reflux symptoms.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mayne et al. (2006) (cont.)	Controls: 687; population-based controls were frequency-matched on the expected distribution of target cases by 5-year age group, sex, and geographical area (study site); controls aged 30–64 yr were identified by Waksberg's random-digit dialling method; controls 65–79 yr were identified by Health Care Financing Administration rosters Exposure assessment method: in-person, structured questionnaire of usual frequency of consumption of "diet soft drinks or soda" covering the period 3–5 yr before diagnosis	Stomach (gastric noncardia, adenocarcinoma), incidence	Diet carbonated soft drink consumption (OR):			Age, sex, study centre, race, proxy interview status, BMI, mean caloric intake, consumption (each) of beer/wine/liquor, consumption of meat, cigarettes per day, education, income, frequency of reflux symptoms	*Other strengths*: large sample size; separate cancer histologies and sites; population-based; control for key confounders, including BMI. *Other limitations*: 30% proxy interviews; potential for recall bias; reverse causation (potential to change behaviours based on reflux), and unmeasured confounders; selection bias due to low participation rate of controls (70.2%); use of random-digit dialling to identify controls may result in non-representative sample.
			No intake	NR	1		
			Top 20% of intake	NR	0.58 (0.38–0.90)		
		Oesophagus (SCC), incidence	Diet carbonated soft drink consumption (OR):				
			No intake	NR	1		
			Top 20% of intake	NR	0.43 (0.23–0.82)		
		Oesophagus (adenocarcinoma), incidence	Diet carbonated soft drink consumption (OR):				
			No intake	NR	1		
			Top 20% of intake	NR	0.52 (0.32–0.83)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gallus et al. (2007) Italy 1991–2004 Case–control	Cases: 598 (oral or pharyngeal), 304 (oesophagus), 1225 (colon), 728 (rectum); histologically confirmed cancers of oral cavity and pharynx (512 men, 86 women; median age, 58 yr), oesophagus (275 men, 29 women; median age, 60 yr), colorectum (1225 colon, 728 rectum; 1125 men, 828 women; median age, 62 yr); > 95% participation Controls: 1491 (oral/pharyngeal), 743 (oesophagus), 4154 (colorectum); controls admitted to same network of general and teaching hospitals as cases for acute, non-neoplastic disorders; total of 7028 patients (3301 men and 3727 women; 4838 included in more than one study); 24% trauma, 31% other nontraumatic orthopaedic	Oral or pharyngeal combined, incidence	Consumption of ASs other than saccharin (OR):			Age, sex, study centre, education, tobacco smoking, alcohol drinking, BMI, total energy intake, consumption of hot beverages	*Exposure assessment critique*: Key limitations were that there was no specific assessment of aspartame, aspartame was considered as "other sweeteners" but with unclear actual contribution; only one source was considered (tabletop sweeteners); and the retrospective assessment in a case–control study (potential for differential misclassification). Other information: according to the authors, limited consumption of sources of sweeteners (including ASBs) in the study population of middle-aged adults in Italy between 1991 and 2004). *Other strengths*: high response rates (< 5% refusals) for cases and controls reduced the potential for selection bias; large sample size with large case numbers for rarer cancers; control for key potential confounders, including BMI.
			Non-consumers	586	1		
			> 0 sachets or tablets/day	12	0.77 (0.39–1.53)		
		Oesophagus, incidence	Consumption of ASs other than saccharin (OR):				
			Non-consumers	294	1		
			> 0 sachets or tablets/day	10	0.77 (0.34–1.75)		
		Colon, incidence	Consumption of ASs other than saccharin (OR):			Age, sex, study centre, education, tobacco smoking, alcohol drinking, BMI, total energy intake, consumption of hot beverages	
			Non-consumers	1137	1		
			> 0 sachets or tablets/day	88	0.90 (0.70–1.16)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gallus et al. (2007) (cont.)	conditions, 17% acute surgical disorders, 28% miscellaneous other diseases; > 95% participation Exposure assessment method: questionnaire; assessment of use of tabletop sweeteners containing either saccharin or other sweeteners as sachets or tablets per week in the 2 yr before cancer diagnosis	Rectum, incidence	Consumption of ASs other than saccharin (OR):			Age, sex, study centre, education, tobacco smoking, alcohol drinking, BMI, total energy intake, consumption of hot beverages	*Other limitations*: potential for recall bias and reverse causation; exposure may have been related to disease conditions of controls.
			Non-consumers	689	1		
			> 0 sachets or tablets/day	39	0.71 (0.50–1.02)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bosetti et al. (2009) Italy 1997–2007 Case–control	Cases: 230 (stomach), 326 (pancreas); hospital-based, greater Milan area; cases had histologically confirmed cancer of the stomach (143 men, 87 women; median age, 63 yr) or pancreas (174 men, 152 women; median age, 63 yr) Controls: 547 (stomach), 652 (pancreas); controls selected from same network of general and teaching hospitals as cases for acute, non-neoplastic disorders, frequency-matched on age, sex, and study centre; 25% traumas, 32% nontraumatic orthopaedic conditions, 15% acute surgical disorders, 27% miscellaneous other diseases; > 95% control participation	Stomach, incidence	Consumption of low-calorie sweeteners other than saccharin (OR):			Age, sex, study centre, year of interview, education, BMI, tobacco smoking, history of diabetes, consumption of hot beverages, total energy intake	*Exposure assessment critique*: Key limitations were that there was no specific assessment of aspartame, aspartame was included in "other sweeteners" but with unclear actual contribution; only one source was considered (tabletop sweeteners); exposure assessment limited to users vs non-users, which increases the potential for exposure misclassification; and the retrospective assessment in a case–control study (potential for differential misclassification). Other information: according to the authors, low consumption of sources of sweeteners (including ASBs) in the study population of middle-aged adults in Italy between 1991 and 2007). *Other strengths*: > 95% participation rate among controls; adjustment for several key confounders, including BMI and diabetes status.
			Non-users	213	1		
			Users	17	0.86 (0.45–1.67)		
		Pancreas, incidence	Consumption of low-calorie sweeteners other than saccharin (OR):				
			Non-users	291	1		
			Users	35	1.16 (0.66–2.04)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bosetti et al. (2009) (cont.)	Exposure assessment method: questionnaire; assessment of use of tabletop sweeteners containing either saccharin or other sweeteners as several sachets or tablets per week in the 2 yr before cancer diagnosis; exposure to "other sweeteners" considered as ever users vs non-users only						*Other limitations*: potential for reverse causation, recall bias; exposure may have been related to disease conditions of controls; no distinction by stomach cancer subtype.
Chan et al. (2009) San Francisco Bay area, California, USA 1995–1999 Case–control	Cases: 532; men and women with incident adenocarcinoma of the exocrine pancreas were identified in 6 counties of the San Francisco Bay Area using rapid case ascertainment by the Northern California Cancer Center (in-area); cases verified by contacting participants' physicians and through SEER abstracts; eligible cases were aged 21–85 yr, residents of one of six counties,	Pancreas (exocrine, adenocarcinoma), incidence	Daily servings of sugar-free carbonated beverages (OR): 0 < 1/day ≥ 1/day Trend-test *P* value: 0.2	 310 124 92	 1 0.8 (0.7–1.1) 1.5 (1.1–2.1)	Age, sex, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	*Exposure assessment critique*: A key strength was the timing of exposure assessment that was consistent for aspartame as the major AS in beverages. Key limitations were the retrospective recall using an FFQ; and that there was no specific estimate of aspartame exposure, ASBs used as a proxy (low-calorie colas, low-calorie caffeine-free colas, and other low-calorie carbonated beverages).

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Chan et al. (2009) (cont.)	alive upon first contact, and could complete a first interview in English; 65 eligible out of area cases met the same criteria but were from counties adjacent to the six Bay Area counties Controls: 1701; control participants were frequency-matched to cases by sex and age within 5-year categories and were selected from the target population using random-digit dialling; controls aged > 65 yr were supplemented by random selection from Health Care Finance Administration lists (now the Centers for Medicare and Medicaid Services); 67% participation rate; no proxy interviews	Pancreas (exocrine, adenocarcinoma), incidence	Daily servings of sugar-free carbonated beverages, men (OR):			Age, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	*Other strengths*: population-based, large number of cases; detailed in-person interviews/no proxy interviews; models adjusted for BMI and history of diabetes. *Other limitations*: potential for recall bias, possible selection bias of controls (67% response rate), survivor bias; use of random-digit dialling to identify controls may result in non-representative sample.
			0	NR	1		
			< 1/day	NR	1.2 (0.8–1.7)		
			≥ 1/day	NR	1.8 (1.1–2.8)		
			Trend-test *P* value: 0.4				
		Pancreas (exocrine, adenocarcinoma), incidence	Daily servings of sugar-free carbonated beverages, women (OR):				
			0	NR	1		
			< 1/day	NR	0.6 (0.4–1.0)		
			≥ 1/day	NR	1.4 (0.9–2.3)		
			Trend-test *P* value: 0.3				
		Pancreas (exocrine, adenocarcinoma), incidence	Total servings of low-calorie cola (OR):			Age, sex, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			< 1/mo	381	1		
			1–3/mo	34	1.0 (0.7–1.5)		
			1–6/wk	54	0.9 (0.7–1.3)		
			≥ 1/day	57	1.7 (1.2–2.4)		
			Trend-test *P* value: 0.06				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Chan et al. (2009) (cont.)	Exposure assessment method: 131-item food questionnaire via in-person interviews, previous 12 mo to assess total sugar-free carbonated beverages/day (low-calorie colas, low-calorie caffeine-free colas, and other low-calorie carbonated beverages)	Pancreas (exocrine, adenocarcinoma), incidence	Total servings of low-calorie cola, men (OR):			Age, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			< 1/mo	NR	1		
			1–3/mo	NR	1.0 (0.6–1.8)		
			1–6/wk	NR	1.1 (0.7–1.7)		
			≥ 1/day	NR	1.8 (1.1–2.9)		
			Trend-test *P* value: 0.06				
		Pancreas (exocrine, adenocarcinoma), incidence	Total servings of low-calorie cola, women (OR):				
			< 1/mo	NR	1		
			1–3/mo	NR	1.0 (0.5–1.9)		
			1–6/wk	NR	0.8 (0.4–1.4)		
			≥ 1/day	NR	1.6 (0.9–2.8)		
			Trend-test *P* value: 0.4				
		Pancreas (exocrine, adenocarcinoma), incidence	Total servings of low-calorie caffeine-free cola (OR):			Age, sex, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			< 1/mo	437	1		
			1–3/mo	24	1.0 (0.6–1.6)		
			1–6/wk	43	0.8 (0.5–1.1)		
			≥ 1/day	22	1.1 (0.7–2.0)		
			Trend-test *P* value: 0.6				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Chan et al. (2009) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Total servings of low-calorie caffeine-free cola, men (OR):			Age, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			< 1/mo	NR	1		
			1–3/mo	NR	1.1 (0.5–2.2)		
			1–6/wk	NR	0.9 (0.5–1.4)		
			≥ 1/day	NR	1.1 (0.5–2.5)		
			Trend-test *P* value: 0.9				
		Pancreas (exocrine, adenocarcinoma), incidence	Total servings of low-calorie caffeine-free cola, women (OR):				
			< 1/mo	NR	1		
			1–3/mo	NR	0.9 (0.5–1.8)		
			1–6/wk	NR	0.7 (0.4–1.3)		
			≥ 1/day	NR	1.3 (0.6–2.7)		
			Trend-test *P* value: 0.7				
		Pancreas (exocrine, adenocarcinoma), incidence	Total servings of other low-calorie carbonated beverages (OR):			Age, sex, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			< 1/mo	418	1		
			1–3/mo	40	1.0 (0.7–1.5)		
			1–6/wk	47	0.9 (0.6–1.2)		
			≥ 1/day	21	1.4 (0.8–2.5)		
			Trend-test *P* value: 0.97				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Chan et al. (2009) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Total servings of other low-calorie carbonated beverages, men (OR):			Age, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			< 1/mo	NR	1		
			1–3/mo	NR	1.5 (0.9–2.5)		
			1–6/wk	NR	1.0 (0.6–1.6)		
			≥ 1/day	NR	1.8 (0.8–3.8)		
			Trend-test *P* value: 0.2				
		Pancreas (exocrine, adenocarcinoma), incidence	Total servings of other low-calorie carbonated beverages, women (OR):				
			< 1/mo	NR	1		
			1–3/mo	NR	0.7 (0.4–1.3)		
			1–6/wk	NR	0.6 (0.4–1.2)		
			≥ 1/day	NR	1.2 (0.4–3.2)		
			Trend-test *P* value: 0.2				
		Pancreas (exocrine, adenocarcinoma), incidence	Daily servings of sugar-free carbonated beverages, obese (BMI, ≥ 30 kg/m^2) (OR):			Age, sex, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			None	NR	1		
			≥ 1/day	NR	2.6 (0.9–7.7)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Chan et al. (2009) (cont.)		Pancreas (exocrine, adenocarcinoma), incidence	Daily servings of sugar-free carbonated beverages, overweight (BMI, 25.0–29.9 kg/m^2) (OR):			Age, sex, energy intake, BMI, race, education, smoking, history of diabetes, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			None	NR	1		
			≥ 1/day	NR	1.5 (0.9–2.6)		
		Pancreas (exocrine, adenocarcinoma), incidence	Daily servings of sugar-free carbonated beverages, normal weight (BMI, < 25.0 kg/m^2) (OR):				
			None	NR	1		
			≥ 1/day	NR	1.3 (0.8–2.1)		
		Pancreas (exocrine, adenocarcinoma), incidence	Daily servings of sugar-free carbonated beverages, non-diabetics (OR):				
			None	NR	1		
			≥ 1/day	NR	1.6 (1.1–2.3)		
		Pancreas (exocrine, adenocarcinoma), incidence	Daily servings of sugar-free carbonated beverages, diabetics (OR):			Age, sex, energy intake, BMI, race, education, smoking, physical activity, other food groups (red meat, white meat, vegetable and fruit, eggs, fish, dairy, whole grain, refined grain, and sweets), total sweetened beverages, sugar-type carbonated beverages	
			None	NR	1		
			≥ 1/day	NR	0.9 (0.3–2.6)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Davis et al. (2023) Buffalo, NY, USA 1982–1998 Case–control	Cases: 213; patients with pancreatic cancer who participated in PEDS; predominantly White (97%) and aged 30–89 yr at diagnosis Controls: 852; patients evaluated for pancreatic cancer with non-cancer diagnoses; frequency-matched to cases by sex, 5-year categories of age, and 5-year categories of year the survey was completed Exposure assessment method: questionnaire; exposure to ASBs assessed retrospectively (few years before diagnosis) between 1982 and 1998; frequencies of consumption of diet cola as ASB; no specific assessment of aspartame content in ASB	Pancreas, incidence	Diet cola consumption (OR): Never Occasional (< 1/day) Habitual (≥ 1/day)	 144 36 33	 1 0.96 (0.62–1.49) 0.92 (0.59–1.43)	Age, sex, smoking status, BMI categories, total vegetable servings/week, processed meat servings/week, family history of pancreatic cancer, regular cola consumption, regular non-cola soft drinks	*Exposure assessment critique*: Key strengths were that exposure data were collected during the admission process, presumably before diagnosis was known. Key limitations were the retrospective assessment of ASB consumption in a case–control study; the period for diet assessment (before cancer diagnosis in 1982–1998) included early years when no aspartame was found in ASBs (partly irrelevant data for aspartame exposure) and later years when ASBs almost exclusively contained aspartame (very relevant data for aspartame exposure); ascertained diet cola only (not other ASBs); no other sources were considered (although these were more limited).
		Pancreas, incidence	Diet cola consumption, never-smokers (OR): Never Occasional (< 1/day) Habitual (≥ 1/day)	 48 15 14	 1 1.31 (0.65–2.67) 1.23 (0.60–2.52)	Age, sex, BMI categories, total vegetable servings/ week, processed meat servings/week, family history of pancreatic cancer, regular cola consumption, regular non-cola soft drinks	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Davis et al. (2023) (cont.)		Pancreas, incidence	Diet cola consumption, former smokers (OR):			Age, sex, BMI categories, total vegetable servings/ week, processed meat servings/week, family history of pancreatic cancer, regular cola consumption, regular non-cola soft drinks	Other comments: in addition to the hospital-based case–control study, the authors also conducted a retrospective cohort mortality study among the 213 patients with pancreatic cancer (203 deaths); for this, they used clinical follow-up data including vital status and date of last contact, obtained from the cancer registry at Roswell Park (last two models). *Other strengths*: cases and controls from same catchment area; all evaluated at same hospital for suspected malignancy; models adjusted for BMI. *Other limitations*: small sample size, especially for stratified analyses, limiting power to detect associations; possibility of selection bias (50% participation rate in controls); potential bias if exposure was related to disease condition of controls.
			Never	72	1		
			Occasional (< 1/day)	16	0.69 (0.37–1.30)		
			Habitual (≥ 1/day)	11	0.54 (0.27–1.10)		
		Pancreas, incidence	Diet cola consumption, current smokers (OR):				
			Never	24	1		
			Occasional (< 1/day)	5	2.71 (0.71–10.35)		
			Habitual (≥ 1/day)	8	3.34 (1.12–9.98)		
		Pancreas, incidence	Diet cola consumption, normal weight (BMI, 18.5–24.9 kg/m^2) (OR):			Age, sex, total vegetable servings/ week, processed meat servings/week, family history of pancreatic cancer, regular cola consumption, regular non-cola soft drinks, smoking status	
			Never	60	1		
			Occasional (< 1/day)	8	0.61 (0.26–1.44)		
			Habitual (≥ 1/day)	11	1.29 (0.60–2.78)		
		Pancreas, incidence	Diet cola consumption, overweight/obese (BMI, ≥ 25.0 kg/m^2) (OR):				
			Never	79	1		
			Occasional (< 1/day)	28	1.19 (0.70–2.01)		
			Habitual (≥ 1/day)	21	0.89 (0.51–1.54)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Davis et al. (2023) (cont.)		Pancreas, incidence	Diet cola consumption, women (OR):			Age, total vegetable servings/week, processed meat servings/week, family history of pancreatic cancer, regular cola consumption, regular non-cola soft drinks, smoking status, BMI categories	
			Never	62	1		
			Occasional (< 1/day)	18	1.18 (0.62–2.24)		
			Habitual (≥ 1/day)	18	1.14 (0.61–2.14)		
		Pancreas, incidence	Diet cola consumption, men (OR):				
			Never	82	1		
			Occasional (< 1/day)	18	0.84 (0.46–1.53)		
			Habitual (≥ 1/day)	15	0.79 (0.42–1.49)		
		Pancreas, mortality	Diet cola consumption (HR):			Age, sex, smoking status, BMI categories, total vegetable servings/week, regular cola consumption, regular non-cola soft drinks, histology, stage, surgery status, radiation status	
			Never	137	1		
			Occasional (< 1/day)	35	1.14 (0.75–1.73)		
			Habitual (≥ 1/day)	31	1.05 (0.70–1.59)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) Spain 2008–2013 Case–control	Cases: 351 (stomach cancer), 1881 (colon and rectal cancer); aged 20–85 yr with newly diagnosed histologically confirmed cancer, resided in catchment area for at least 6 mo; no prior history of their cancer; enrolled as soon as possible after diagnosis; frequency-matched on age, sex, and region to population controls Controls: 3629; randomly selected from administrative records of selected primary health care centres within catchment area Exposure assessment method: self-administered, semiquantitative FFQ, 140 food items, assessing usual dietary intake during the previous year	Stomach, incidence	Consumption of aspartame-containing products (OR):			Age, sex, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, other ASs	*Exposure assessment critique*: A key strength was the categorization of intake of ASBs and tabletop sweeteners by type (aspartame vs others) using public data on ingredients in food supply, but it was unclear whether the assumption of aspartame content in products was correct. A key limitation was that beverages and tabletop sweeteners were assessed but there was no consideration of ASs in the rest of the food supply. *Other strengths*: relatively large sample size; histopathological confirmation of cancer cases; extensive assessment of confounding, including from BMI (captured in a combined score based on WCRF/AICR evidence on lifestyle factors; Romaguera et al., 2017); stratification by diabetes status to evaluate heterogeneity of associations.
			Non-consumers	290	1		
			Medium intake (< 3rd quartile among controls)	42	1.01 (0.69–1.46)		
			High intake (≥ 3rd quartile among controls)	19	1.09 (0.62–1.83)		
			Trend-test *P* value: 0.8				
		Stomach, incidence	Consumption of aspartame-containing products, participants without diabetes (OR):				
			Non-consumers	251	1		
			Medium intake (< 3rd quartile among controls)	28	0.84 (0.53–1.29)		
			High intake (≥ 3rd quartile among controls)	12	0.89 (0.43–1.66)		
			Trend-test *P* value: 0.5				
		Stomach, incidence	Consumption of aspartame-containing products, participants with diabetes (OR):			Age, sex, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, other ASs	
			Non-consumers	39	1		
			Medium intake (< 3rd quartile among controls)	14	2.02 (0.92–4.27)		
			High intake (≥ 3rd quartile among controls)	7	2.04 (0.70–5.40)		
			Trend-test *P* value: 0.05				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)		Stomach, incidence	Consumption of low- or no-calorie soft drinks (OR):			Age, sex, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, other sources of sweeteners, total caloric drinks	*Other limitations*: non-prospective study design (case–control); selection bias due to low participation among cases (participation rate was 68% for colorectal; 57% for gastric) and controls (mean participation rate was 53%); recall bias in exposure assessment; potential for bias from exposure measurement error and residual confounding due to other correlates of AS use among those with diabetes; relatively low exposure contrasts for aspartame-containing products; potential for chance findings due to small number in some strata.
			Non-consumers	295	1		
			Medium intake (< 3rd quartile among controls)	36	1.05 (0.70–1.54)		
			High intake (≥ 3rd quartile among controls)	20	1.31 (0.74–2.21)		
			Trend-test *P* value: 0.4				
		Stomach, incidence	Consumption of low- or no-calorie soft drinks, participants without diabetes (OR):				
			Non-consumers	254	1		
			Medium intake (< 3rd quartile among controls)	25	0.93 (0.57–1.46)		
			High intake (≥ 3rd quartile among controls)	12	1.17 (0.57–2.20)		
			Trend-test *P* value: 0.9				
		Stomach, incidence	Consumption of low- or no-calorie soft drinks, participants with diabetes (OR):				
			Non-consumers	41	1		
			Medium intake (< 3rd quartile among controls)	11	2.01 (0.86–4.46)		
			High intake (≥ 3rd quartile among controls)	8	1.86 (0.63–5.01)		
			Trend-test *P* value: 0.08				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)		Colon and rectum, incidence	Consumption of aspartame-containing products (OR):			Age, sex, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, family history of colorectal cancer, night shift work, other ASs	
			Non-consumers	1620	1		
			Medium intake (< 3rd quartile among controls)	172	0.76 (0.62–0.93)		
			High intake (≥ 3rd quartile among controls)	89	0.94 (0.71–1.25)		
			Trend-test *P* value: 0.1				
		Colon and rectum, incidence	Consumption of aspartame-containing products, participants without diabetes (OR):				
			Non-consumers	1358	1		
			Medium intake (< 3rd quartile among controls)	127	0.74 (0.59–0.93)		
			High intake (≥ 3rd quartile among controls)	57	0.87 (0.61–1.21)		
			Trend-test *P* value: 0.04				
		Colon and rectum, incidence	Consumption of aspartame-containing products, participants with diabetes (OR):				
			Non-consumers	262	1		
			Medium intake (< 3rd quartile among controls)	45	0.82 (0.52–1.27)		
			High intake (≥ 3rd quartile among controls)	32	1.09 (0.63–1.87)		
			Trend-test *P* value: 1.0				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)		Colon and rectum, incidence	Consumption of low- or no-calorie soft drinks (OR):			Age, sex, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, other sources of sweeteners, total caloric drinks	
			Non-consumers	1659	1		
			Medium intake (< 3rd quartile among controls)	150	0.82 (0.66–1.02)		
			High intake (≥ 3rd quartile among controls)	72	0.77 (0.56–1.05)		
			Trend-test *P* value: 0.02				
		Colon and rectum, incidence	Consumption of low- or no-calorie soft drinks, participants without diabetes (OR):				
			Non-consumers	1387	1		
			Medium intake (< 3rd quartile among controls)	107	0.75 (0.58–0.96)		
			High intake (≥ 3rd quartile among controls)	48	0.71 (0.48–1.01)		
			Trend-test *P* value: < 0.01				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)		Colon and rectum, incidence	Consumption of low- or no-calorie soft drinks, participants with diabetes (OR):			Age, sex, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, other sources of sweeteners, total caloric drinks	
			Non-consumers	272	1		
			Medium intake (< 3rd quartile among controls)	43	1.07 (0.68–1.69)		
			High intake (≥ 3rd quartile among controls)	24	0.95 (0.50–1.79)		
			Trend-test *P* value: 1.0				

AS, artificial sweetener; AICR, American Institute of Cancer Research; ASB, artificially sweetened beverage; BMI, body mass index; CI, confidence interval; CPS-II, Cancer Prevention Study II; EPIC, European Prospective Investigation into Cancer and Nutrition; FDA, Food and Drug Administration; FFQ, food frequency questionnaire; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; MCCS, Melbourne Collaborative Cohort Study; MET, metabolic equivalent tasks; mo, month(s); NHS, Nurses' Health Study; NHS-II, Nurses' Health Study II; NIH-AARP, National Institutes of Health-American Association of Retired Persons; NR, not reported; OR, odds ratio; PEDS, Patient Epidemiologic Data System; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer screening trial; RR, relative risk; SCC, squamous cell carcinoma; SD, standard deviation; SEER, Surveillance, Epidemiology, and End Results; SSB, sugar-sweetened beverage; vs, versus; WCRF, World Cancer Research Fund; wk, week(s); yr, year(s).

[a] The larynx is part of the respiratory system but is included here as reported in McCullough et al. (2022).

duct cancers combined). Results were stratified by diabetes status, determined a priori, because of potentially differing dietary behaviours. ASB consumption of > 2 servings/day was 24% among participants with diabetes, compared with 8% among those without diabetes; 19% of participants with diabetes reported not consuming ASBs, compared with 50% of those without diabetes. The remaining participants reported a consumption of either 1–2 servings/day or < 1 serving/day. The authors tested the proportional hazards assumption across the time scale used in the study (i.e. follow-up time). Because this assumption was found to be violated, the authors presented the associations separately by median follow-up: 0–12 years and ≥ 12 years. Among participants without diabetes, no association with ASB consumption was observed, regardless of follow-up period. Among participants with diabetes, positive associations with risk of liver cancer were reported for several exposure types for ≤ 12 years follow-up (HR per times/day for ASBs overall, 1.13; 95% CI, 1.02–1.25; HR for artificially sweetened soda, 1.13; 95% CI, 1.01–1.27; and HR for artificially sweetened fruit punch, 1.17; 95% CI, 0.92–1.48). All findings were null for ≥ 12 years follow-up. [The strengths of this study included a large sample size from the pooling of two large cohorts to examine the incidence of this relatively rare cancer. Another strength was that the baseline diet assessments in NIH-AARP and PLCO (1995–1996 and 1998, respectively) and the subsequent 12 years of follow-up coincided with a period in which the artificial sweetener used in ASBs was predominantly aspartame. Stratification by diabetes status helped to limit confounding by this important liver cancer risk factor and permitted examination of higher levels of diet soda intake. Diabetes appeared to be an effect modifier of the association, possibly due to greater ASB exposure in participants with diabetes; however, it was unclear whether biological mechanisms specific to individuals with diabetes could play a role in explaining these findings. The study controlled for multiple liver cancer risk factors but was unable to control for coffee consumption (which has been associated with lower liver cancer risk; IARC, 2018a) or HBV/HCV infection status. Exposure misclassification caused by a single assessment of diet at baseline was also a possibility but was likely to be non-differential.]

In the American Cancer Society's Cancer Prevention Study II (CPS-II) prospective cohort comprising 934 777 men and women in the USA, 2722 deaths from cancers of the liver and intrahepatic bile duct occurred over a median follow-up of 27.7 years (McCullough et al., 2022). A beverage grid on the baseline (1982) questionnaire (which included "diet soda or diet iced tea") queried the number of cups, glasses, or drinks usually consumed per day. If less than once per day, but at least three times per week, participants wrote "½". Overall, the frequency of ASB intake was not associated with liver cancer mortality risk (ICD-10, C22: cancer of the liver and intrahepatic bile duct). However, consumption of ≥ 2 drinks/day versus none was associated with increased risk among non-smoking men (HR, 1.44; 95% CI, 0.99–2.08; *P* for trend, 0.040). This was attenuated when BMI was included in the model (HR, 1.21; 95% CI, 0.83–1.77; *P* for trend, 0.335), suggesting some confounding by adiposity. [Mortality is a reasonable proxy for incidence for this cancer, which has a high fatality rate. The study controlled for BMI and sugar-sweetened beverages and excluded participants with a history of diabetes at baseline. However, it was unable to control for HBV/HCV infection status. With only a single baseline assessment of exposure in 1982, 1 year before the approval of aspartame use in beverages, and a median follow-up of 27.7 years, the study had potential for misclassification, which was likely to be non-differential and bias risk estimates towards the null. Although published data from more than 100 000 participants included in both CPS-II and the CPS-II nutrition cohort (a

subcohort of the parent CPS-II mortality cohort) (McCullough et al., 2014) suggested some decrease in ASB consumption between 1982 and 1999 in this population over 17 years, the relative ranking of intake category was consistent during this period.]

A meta-analysis (Jatho et al., 2021) evaluated artificially sweetened soft drink consumption and liver cancer (among other gastrointestinal tract cancers), but the Working Group judged this review to be uninformative for the evaluation of aspartame because it included estimates for regular soft drinks and combined results from prospective and case–control studies.]

2.1.2 Cancers of the colon and rectum

The Working Group identified six cohort studies (comprising seven unique cohorts) that assessed intake of aspartame, by proxy through ASBs, and its association with colon and/or rectal cancer incidence or mortality. These studies included cohorts from the USA (Malik et al., 2019; Hur et al., 2021; McCullough et al., 2022), Australia (Hodge et al., 2018), France (Chazelas et al., 2019), and seven European countries (Mullee et al., 2019). One case–control study assessed the association between artificial sweetener consumption and the incidence of colon and rectal cancer in Italy (Gallus et al., 2007). Another case–control study in Spain looked at associations between ASBs and tabletop sweeteners other than saccharin to represent aspartame intake (Palomar-Cros et al., 2023). A third case–control study from Egypt was also identified (Mahfouz et al., 2014). None of the studies with cancers of the colon or rectum as the outcome used specific aspartame intake as the exposure. [The Working Group noted that all the informative cohort and case–control studies used comprehensive adjustment for confounders, in all cases including adjustment for measures of obesity.]

Four additional studies were not considered to be informative since they did not directly investigate the association between aspartame (or a proxy) and colorectal cancer, or they measured dietary information before the commercial use of aspartame. In the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS) cohort, Yuan et al. (2022) estimated the effect of substituting sugar-sweetened beverages with ASBs on the risk of colorectal cancer, whereas Wang et al. (2021) and Nguyen et al. (2020) reported on the association between a dietary pattern (including low-calorie beverages) that promoted the proliferation of sulfur-producing bacteria in the gut and colorectal cancer. Joh et al. (2021) examined the association between intake of ASBs in adolescence and incidence of colorectal adenoma in adult women from the Nurses' Health Study II (NHS-II) cohort. The dietary data for adolescents were retrospectively collected for the period 1960–1982. [The Working Group noted that this period pre-dated the use of aspartame in soft drinks, making the data of questionable value for this investigation. The Working Group noted that colorectal adenoma is a valid outcome as defined in the Preamble to the *IARC Monographs* (see the front matter of the present volume; IARC, 2019), which allows the inclusion of epidemiological studies on benign neoplasms, preneoplastic lesions, malignant precursors, and other end-points when they relate to the agents reviewed.] The case–control study from Egypt (cases and controls were matched on age and sex) was considered to be uninformative because of the small number of cases (n = 150) and unclear exposure to aspartame (Mahfouz et al., 2014).

No association was seen between frequency of ASB consumption and colorectal cancer incidence in an Australian cohort (Hodge et al., 2018), or a French cohort (Chazelas et al., 2019). The Australian Melbourne Collaborative Cohort Study (MCCS), a cohort study that included 35 593 men and women followed for cancer incidence

until 30 June 2013, used a food frequency questionnaire (FFQ) to quantify the frequency of consumption of diet soft drinks at baseline in 1990–1994, after the approval of aspartame for use in soft drinks in Australia in 1986 (Hodge et al., 2018). [The Working Group noted that the limitations of this study were the nonspecific exposure and the single baseline assessment of intake.] The French NutriNet-Santé cohort included more than 100 000 men and women enrolled between 2009 to 2017 and followed until 11 January 2018; estimated consumption of 12 different types of ASB was based on multiple 24-hour dietary records collected every 6 months (Chazelas et al., 2019). Baseline diet was the average of all records over the first 2 years of follow-up, and all included participants had at least two records over this period (mean ± SD, 5.6 ± 3.0). [The Working Group considered the use of multiple records as a strength of this study. The inclusion of different beverages with different levels of aspartame and other artificial sweeteners was considered a weakness that could lead to misclassification of aspartame exposure.]

Malik et al. (2019) combined data from the HPFS (37 716 men) and the NHS (80 647 women) to evaluate ASB consumption and colon cancer mortality, finding no association. The main analysis updated ASB consumption every 4 years, whereas the secondary analysis for all-cancer mortality (also conducted in both the NHS and the HPFS) only used the cumulative average intake over time. Follow-up was from 1986 to the end of 2014 for the HPFS, and from 1980 to the end of 2014 for the NHS, thus the first dietary data in the NHS were collected before aspartame was approved for use in beverages. [The Working Group noted that although these cohorts used low-calorie beverages as a proxy for aspartame intake, these were the predominant source of aspartame in the USA, and aspartame was the predominant sweetener used in ASBs, at least for the period from the mid-1980s to the mid-2000s. The updating of exposure over time was also a strength.]

In the EPIC cohorts, which included 451 743 men and women from seven countries across Europe, data on consumption of artificially sweetened soft drinks defined as "low-calorie or diet fizzy soft drinks" were collected with cohort-specific dietary instruments between 1992 and 2000 (Mullee et al., 2019). In three additional countries included in EPIC (Italy, Spain, and Sweden), data on types of soft drinks were not collected in all recruiting centres, and data from these countries were excluded in this analysis. [The Working Group noted that data were collected during a relevant period for aspartame use in Europe, but the collection of intake data at a single time point, and the variation in products across countries over time may lead to exposure misclassification, which would be expected to be non-differential with respect to cancer outcome.] Cause-specific mortality follow-up was between baseline and 2009–2013 (depending on the study centre). A total of 2095 deaths from colorectal cancer were recorded. The frequency of artificially sweetened soft drink consumption was not associated with colorectal cancer mortality, although the hazard ratio in the group consuming ≥ 1 glasses/day was elevated (HR, 1.22; 95% CI, 0.91–1.64) relative to consumption of < 1 glass/month. [The Working Group noted that the study design, incorporating cohorts from multiple countries with different behaviours with standardized data collection methods, was a strength of this study.]

A study in 95 464 female registered nurses participating in the United States (US) NHS-II cohort investigated the association between consumption of low-calorie carbonated beverages and incidence of early-onset colorectal cancer (Hur et al., 2021). Participants were aged 25–42 years at baseline and self-reported dietary data, including consumption of low-calorie carbonated beverages, using a validated semiquantitative FFQ every 4 years from 1991 until the end of follow-up in June 2015. The

frequency with which low-calorie carbonated beverages were consumed during the previous 12 months was not associated with the incidence of early-onset (before age 50 years) colorectal cancer (Hur et al., 2021). This paper also looked at beverage consumption in adolescence on the basis of a retrospective questionnaire, but this intake occurred between 1960 and 1982, before the approval of aspartame for use in soft drinks.

McCullough et al. (2022) analysed data from the nationwide US CPS-II cohort, which included 934 777 men and women (aged ≥ 28 years) who completed a baseline questionnaire on diet, medical history, and demographic and lifestyle characteristics in 1982. Participants were followed for cause-specific mortality through 2016. Usual daily consumption of ASB, defined as diet soda or diet iced teas, was ascertained from the dietary questionnaire at baseline. Results showed an inverse association between frequency of consuming ASBs and colorectal cancer mortality, after adjusting for BMI (HR for ≥ 2 drinks/day versus never consuming ASBs, 0.92; 95% CI, 0.86–0.99; *P* for trend, 0.034), but no association without adjusting for BMI (HR for ≥ 2 drinks/day versus never consuming ASBs was 0.97; 95% CI, 0.90–1.04; *P* for trend, 0.704). [Data collection occurred the year before aspartame was approved for use in carbonated beverages in the USA. The Working Group considered that with only a single baseline assessment of exposure in 1982, 1 year before the approval of aspartame use in beverages, and a median follow-up of 27.7 years, the study had the potential for misclassification, which was likely to be non-differential and bias risk estimates towards the null. However, it was noted that the relative ranking of intake categories of ASB consumption over the 17-year period between 1982 and 1999 among 100 000 participants included in both the CPS-II and the CPS-II nutrition cohort (McCullough et al., 2014) was reasonably consistent over time, such that the reported intakes may partly reflect aspartame intake over the follow-up, during a period when ASBs were the predominant source of aspartame in the USA and aspartame was almost the only sweetener used in ASBs.]

Apart from the studies by Hur et al. (2021) (which used data from the NHS-II cohort) and Malik et al. (2019) (which used data from the combined NHS/HPFS cohort, updating or averaging intake from surveys repeated every 4 years), most other studies assessed intake on only one occasion. Chazelas et al. (2019) used multiple (at least two) 24-hour records to assess intake, but their assessment was based on a single value averaged over the first 2 years. [The Working Group noted that this could contribute to non-differential exposure misclassification if intake had changed over follow-up, and thus bias the results towards the null.]

Three case–control studies reported on the association between artificial sweetener consumption and colorectal cancer. [The Working Group noted important limitations common to all case–control studies: the risk of recall bias and the lack of temporal sequence, which may result in reverse causation. The direction of bias was unclear.] Data from a hospital-based unmatched case–control study across four areas of Italy indicated no association between use of artificial sweeteners and either colon (n = 1225) or rectal cancer (n = 728) (Gallus et al., 2007). In contrast to most of the cohort studies that used artificially sweetened soft drinks as the exposure, this study examined use of artificial sweeteners in sachets or as tablets as the exposure (Gallus et al., 2007). The data were collected between 1991 and 2004, as "saccharin" or "other sweeteners". On the basis of a study of Italian teenagers in 1996 (Leclercq et al., 1999) (which showed that most participants who consumed foods or beverages containing artificial sweeteners were consuming items containing aspartame), "other sweeteners" in the case–control study by Gallus et al. (2007) were assumed to be mainly aspartame. [The Working Group noted that it was not clear how valid this assumption was with regard

to sweeteners consumed in this form, although this would not be considered to create a spurious positive association.]

The Spanish multicase–control (MCC-Spain) study (Palomar-Cros et al., 2023) recruited 10 106 people aged 20–85 years with recently diagnosed cancer of the colorectum, breast, stomach, and prostate, and chronic lymphocytic leukaemia (CLL). Cases were frequency-matched to the population controls on age, sex, and region. Recruitment occurred from 2008 through 2013. Controls were randomly selected from primary care centres within the study centre catchment area. Dietary intake data were collected using a validated self-completed FFQ. After exclusion of participants who did not complete the FFQ (n = 1354), or who had missing covariate data (n = 29), or cases of CLL diagnosed > 1 year before the interview ([n = 271; personal communication with the authors]), 8452 cases and controls (including 1881 colorectal cancer cases and 3629 controls) remained across all studied outcomes. [The Working Group noted that exclusion rates by cancer type were not provided.] Aspartame intake was assessed as portions/day of low- or no-calorie soft drinks and tabletop sweeteners that were not saccharin. In a sensitivity analysis, low- or no-calorie soft drinks alone were evaluated. [The Working Group noted that it was unclear how valid was the assumption that it was aspartame that was used in all low- or no-calorie soft drinks and in tabletop sweeteners other than saccharin. Non-differential exposure misclassification was likely.] Intake of aspartame was modelled as non-consumers (reference group), moderate consumers (below the third quartile), and high consumers (third quartile or above). All models were adjusted for a score combining a set of modifiable lifestyle factors, including BMI, which have been established as risk factors for cancer by the World Cancer Research Fund/American Institute of Cancer Research (WCRF/AICR) (Romaguera et al., 2017). Analyses stratified by diabetes status were also performed. Compared with the non-consumers group, the overall odds ratio (OR) for colorectal cancer was 0.76 (95% CI, 0.62–0.93) for the moderate consumers and 0.94 (95% CI, 0.71–1.25) for the high consumers. [The Working Group noted that the results in the moderate consumers group were suggestive of a protective effect.] After stratification by diabetes, the odds ratio for high consumers was 0.87 (95% CI, 0.61–1.21) for people without diabetes and 1.09 (95% CI, 0.63–1.87) in people with diabetes. When only low- or no-calorie soft drinks were used as the exposure, the odds ratio for moderate consumers was 0.82 (95% CI, 0.66–1.02) and that for high consumers was 0.77 (95% CI, 0.56–1.05). [The Working Group noted the wide confidence intervals for most estimates. Limitations included low intake of products potentially containing aspartame, with a mean of 0.12 portions/day, which led to a relatively low exposure contrast, as well as uncertainty about how well these products reflected aspartame consumption. Strengths included the large number of cases and stratification by diabetes status.]

Three meta-analyses that examined the association between colorectal cancer and artificial sweetener consumption were considered uninformative (Jatho et al., 2021; Llaha et al., 2021; Tepler et al., 2021). This was because the exposures studied were not all appropriate proxies for aspartame intake [the Working Group noted, for example, that one study included in the meta-analysis by Jatho et al. (2021) did not separate artificially sweetened and sugar-sweetened beverages]; because the meta-analyses incorrectly combined outcomes from case–control and prospective studies with both cancer incidence and mortality as outcomes (Jatho et al., 2021); because they did not report results for colorectal cancer separately from other gastric luminal cancers (Tepler et al., 2021); or because an insufficient number of studies were identified to perform meta-analysis for colorectal cancer as an outcome (Llaha et al., 2021). A fourth meta-analysis (Yin et al., 2022), which included data

from three cohort studies, reported an overall inverse association between ASB consumption and cancer of the colon and rectum (relative risk, RR for highest versus lowest intake category, 0.78; 95% CI, 0.62–0.99, $P = 0.037$), with low heterogeneity between studies. [The Working Group noted that, although the title suggested that this meta-analysis was focused on artificially sweetened soft drinks as the exposure, studies in which the exposure was low-calorie sweeteners were also included. However, the colorectal cancer meta-analysis included only three cohorts (Hodge et al., 2018; Chazelas et al., 2019; Hur et al., 2021), for which the exposure was ASBs alone. The Working Group noted that data collection for these cohorts occurred over different time periods and did not always coincide with ASBs being the predominant source of aspartame, or with aspartame being the predominant type of artificial sweetener, most notably for the NutriNet-Santé report that did not specifically identify aspartame exposure (Chazelas et al., 2019).] The meta-analysis of ASB intake and colorectal cancer by Pan et al. (2023), which included two studies described above (Hodge et al., 2018; Chazelas et al., 2019) was not considered informative.

2.1.3 *Cancers of the pancreas and other digestive system organs (excluding liver and colorectum)*

See Table 2.1.

Five prospective cohort studies examined the association between consumption of ASBs or diet soft drinks and risk of cancers of the pancreas (Schernhammer et al., 2005; Bao et al., 2008; Navarrete-Muñoz et al., 2016; Hodge et al., 2018; McCullough et al., 2022) or other digestive organs (Hodge et al., 2018; McCullough et al., 2022). Seven case–control studies examined the association between aspartame exposure or ASB consumption and risk of cancers of the pancreas (Norell et al., 1986; Bosetti et al., 2009; Chan et al., 2009; Davis et al., 2023) or other digestive organs (Mayne et al., 2006; Gallus et al., 2007; Bosetti et al., 2009; Palomar-Cros et al., 2023). Four case–control studies (Norell et al., 1986; Gallus et al., 2007; Bosetti et al., 2009; Palomar-Cros et al., 2023) examined use of tabletop artificial sweetener; two of these studies specifically examined the category of "artificial sweeteners other than saccharin" (Gallus et al., 2007; Bosetti et al., 2009); Norell et al. (1986) examined artificial sweetener users/non-users, and Palomar-Cros et al. (2023) examined tabletop artificial sweeteners other than saccharin together with ASBs as "consumption of aspartame-containing products". The other three case–control studies (Mayne et al., 2006; Chan et al., 2009; Davis et al., 2023) assessed frequency of consumption of diet cola, sugar-free carbonated beverages, and diet carbonated soft drinks, respectively. The study by Larsson et al. (2016) was not considered informative as it was unable to separate consumption of sugar and ASBs.

[After the first small case–control study in 1986, in which artificial sweetener use was categorized as "yes/no" (Norell et al., 1986), advances in study design have included studies with larger sample sizes, prospective design, information on cancer subsite or histological subtype, estimation of ASB intake via repeated exposure assessments over time (one study, Schernhammer et al., 2005), and quantitative assessment of exposure using frequency and dose instead of "yes/no" user. None of the studies presented aspartame separately or estimated aspartame intake from all sources.]

In a study in two USA-based cohorts, the NHS cohort of 88 794 female nurses and the HPFS cohort of 49 364 male health professionals (Schernhammer et al., 2005), 379 cases of pancreatic cancer occurred during the 14–20-year follow-up period beginning in 1980 or 1986, respectively. The study assessed usual consumption of three types of diet soda (diet cola with caffeine, diet cola without caffeine, and other

diet soda), repeatedly every 4 years using a ~130-item FFQ, and the diet assessment was cumulatively updated. The exposure contrast was diet soft drink consumption of > 3 times/week versus < 1 time/month (median, 1.6 versus 0 drinks/day in women and 1.5 versus 0 drinks/day in men). No association with greater consumption of diet soft drinks was observed among men, women, men and women combined, or when stratified by BMI or physical activity. When examined separately by type of diet soda, neither diet cola nor other diet soft drinks was associated with pancreatic cancer risk. [A major strength of this study was the cumulatively updated diet assessment from the time of introduction of aspartame in beverages for up to two decades, potentially capturing lifetime exposure to aspartame from sodas. The study controlled for BMI at baseline and updated diabetes diagnoses during follow-up. Limitations were that diet soft drink intake was not high (highest category, ≥ 3 times/week), and the number of cases was limited in some stratified analyses, with wide confidence intervals, suggesting limited statistical power for some subgroups.]

Bao et al. (2008) followed 487 922 men and women in the NIH-AARP cohort in the USA for a mean of 7.2 years, during which time 1258 participants developed pancreatic cancer. Usual diet in the past 12 months was assessed at baseline between 1995 and 1996 when participants completed a 124-item FFQ. Participants were queried about frequency of consumption of beverages (ranging from "never" to "≥ 6 times per day") and whether they usually drank the sugar-free (diet) or the regular-calorie type of a particular beverage. Beverage consumption was expressed in gram weight. Compared with non-consumers of diet soft drinks, higher quintile consumption at baseline (fifth quintile, median, 816.9 g/day, versus non-consumers) was not associated with pancreatic cancer risk overall in minimally or fully adjusted models, or when stratifying by sex (data not provided), BMI, physical activity, or smoking status. Increased risk was observed only after excluding the first 2 years of follow-up in the fourth quintile (HR, 1.35; 95% CI, 1.03–1.77) and, with wider confidence intervals, in the fifth quintile (HR, 1.25; 95% CI, 0.94–1.66) versus non-consumers (*P* for trend, 0.19). [The strengths of this analysis included its large size and 90% completeness of case identification via linkage with cancer registries. The study controlled for BMI and excluded participants who reported having diabetes at baseline. The period of follow-up was relevant for diet soft drinks being a major contributor to aspartame in the diet, and for ASB intake reflecting aspartame, since aspartame was almost the only sweetener used in ASBs at that time. The main limitation was the potential for measurement error with the type of sequential assessment of frequency of consumption used in the study (i.e. whether the diet or regular version was usually consumed, instead of asking about beverage types individually), which was likely to result in non-differential exposure misclassification of participants.]

A study in the EPIC cohort (Navarrete-Muñoz et al., 2016) was conducted for seven European countries with information on artificially sweetened soft drink intake at baseline (i.e. it did not include Italy, Spain, and Sweden since some recruitment centres in these countries did not collect this information). Usual diet was assessed at study enrolment between 1992 and 2000 using country-specific diet assessment instruments. Artificially sweetened soft drink intake was assessed asking participants to recall the number of glasses (approximately 250 mL) of "low-calorie or diet fizzy soft drinks" per month, week, or day (Mullee et al., 2019). This quantity was then converted to 330 mL servings per week for the analysis (Navarrete-Muñoz et al., 2016). During a median follow-up of 11.6 years, 586 adenocarcinomas of the exocrine pancreas were verified (follow-up was reported for the whole EPIC cohort, not for the subset with information on ASB consumption). No association between

artificially sweetened soft drink consumption of > 92.2 g/day versus 0.1–2.0 g/day and pancreatic cancer risk was reported for models of men and women combined. However, a 25% increase in risk was observed in continuous models (per 336 g/day) among men but not women (*P* for interaction by sex, 0.004). [Personal communication with the authors confirmed that the correct sex-stratified model was that reported in supplemental Table 5 of their paper (Navarrete-Muñoz et al., 2016), which is the one reported in the text and tables of the present monograph.] Associations did not vary among strata defined by age of diagnosis, BMI, waist circumference, physical activity, and smoking status. [Strengths included the geographically heterogeneous populations with information on cancer subtype, and control for diabetes and BMI. Limitations included the low intake of diet soft drinks (the highest consumption category was > 92.2 g/day). EPIC combined data from multiple European countries, each with its own country-specific dietary questionnaire and potentially its own products with variable aspartame content, largely during a period relevant for aspartame exposure. However, towards the end of the follow-up period for countries that started recruitment later (i.e. towards 2000), co-exposure to other artificial sweeteners was likely. Baseline-only assessment of dietary intake may contribute to non-differential measurement error and bias risk estimates towards the null.]

In the MCCS in Australia, 165 cases of cancer of the gastric cardia occurred among 35 593 participants enrolled between 1990–1994 with up to 23 years of follow-up (Hodge et al., 2018). For artificially sweetened soft drink consumption of ≥ 1 drink/day versus never or < 1 drink/month, there was no association between artificially sweetened soft drink intake and risk of gastric cardia cancer. [Strengths included that the assessment was carried out after the introduction of aspartame into diet soft drinks in Australia (1987) and that the first half of follow-up largely overlapped with the period of aspartame use in Australia). The single baseline assessment and long follow-up probably resulted in non-differential measurement error that could bias results towards the null; the small number of cases (n = 10) among the most-exposed participants limited the model stability and ability to detect associations (e.g. the 95% confidence interval was wide; 95% CI, 0.53–1.98). BMI was not adjusted in the model because the study operated on the assumption that the association was mediated by BMI; however, adjustment for waist circumference, a measure of abdominal obesity, was performed.]

In the CPS-II prospective cohort comprising 934 777 men and women in the USA, there were 9196, 1024, 2798, 2727, and 324 deaths from pancreatic, gallbladder, stomach, oesophageal, and small intestine cancer, respectively, over a median follow-up of 27.7 years (McCullough et al., 2022). Deaths from oral, pharyngeal, and laryngeal cancers were combined, making a total of 1852. [The larynx is part of the respiratory system and, in the present monograph, laryngeal cancers are included in Section 2.4 (under "other solid cancers"). The grouping of data on oral, pharyngeal, and laryngeal cancers, as used in this study, was less informative than the individual cancer site-specific results.] ASB consumption of ≥ 2 drinks/day versus never was associated with a 16% increase in risk (95% CI, 1.07–1.26) of death from pancreatic cancer when there was no control for BMI, and an 11% increase in risk (95% CI, 1.02–1.20) when BMI was included in the multivariable-adjusted model. Overall, an increased risk of pancreatic cancer mortality was observed among both men and women, and no interaction with BMI was found (*P* for interaction, 0.678). For ≥ 2 drinks/day of ASB, risk was elevated among never-smokers for men, women, and both combined (HR for men, 1.34; 95% CI, 1.10–1.65; HR for women, 1.12; 95% CI, 0.96–1.30; and HR for men and women combined, 1.19; 95% CI, 1.05–1.34). [These estimates were

provided by personal communication with the author, after the estimates in the original publication (McCullough et al., 2022) were found to be incorrect.] No association was observed between ASB consumption and mortality from cancers of the oesophagus or stomach in categorical or continuous models. This study was unable to distinguish cancers of the gastric cardia from non-cardia cancers, or oesophageal squamous cell carcinoma from adenocarcinoma. [The Working Group noted that the absence of these cancer subtypes may mask associations, since risk factors are known to vary for gastric cardia and non-cardia cancers and for oesophageal squamous cell carcinoma and adenocarcinoma.] A 7% increase in risk was reported for gallbladder cancer mortality only in continuous models (HR per drink/day, 1.07; 95% CI, 1.01–1.14), which was attenuated after controlling for BMI (HR, 1.05; 95% CI, 0.98–1.11), with largely overlapping confidence intervals. Among never-smokers, the point estimate was the same, with slightly wider confidence intervals due to smaller numbers (HR, 1.05; 95% CI, 0.96–1.16; 476 deaths). The hazard ratio for the association between ASBs and small intestine cancer mortality in a continuous model (per drink/day) was 1.12 (95% CI, 1.01–1.23) and was similar when BMI was included in the model (HR, 1.11; 95% CI, 1.00–1.22). Among never-smokers, controlling for BMI, the association of ASB consumption with small intestine cancer mortality was similar, with wider confidence intervals (HR, 1.10; 95% CI, 0.94–1.30; 138 deaths). Among men, the association with mortality from oral, pharyngeal, and laryngeal cancers combined was 1.07 (95% CI, 1.00–1.14) per drink/day, controlling for BMI. The associations for women only and for men and women combined were null in the BMI-adjusted model. [Mortality is a reasonable proxy for incidence for these cancers that have a high fatality rate (pancreas, gallbladder, stomach, and oesophagus). The study controlled for BMI and excluded participants with a history of diabetes at baseline, minimizing confounding. However, with only baseline assessment of exposures and a median follow-up of 27.7 years, the study had the potential for misclassification and confounding, which was likely to be non-differential and bias risk estimates towards the null. A limitation was that aspartame was not approved in the USA for use in beverages until 1983, 1 year after the baseline exposure assessment for this cohort. The assessment of beverage intake 1 year before the approval of aspartame for addition to beverages increased the possibility of measurement error and confounding, which would most likely bias risk estimates towards the null. As noted above, published data for the 100 000 participants included in both the CPS-II and the CPS-II nutrition cohort (McCullough et al., 2014) suggested that there was some decrease in ASB consumption over the 17 years between 1982 and 1999 in this population, but that the relative ranking of intakes was consistent during this period.]

In a small case–control study conducted in Sweden in 1982–1984 that included 99 cases of cancer of the pancreas and 163 hospital-based and 138 population-based controls, null findings were reported for use of low-calorie sweeteners (sachets) and generic use of artificial sweeteners, respectively (Norell et al., 1986). [Limitations included the assessment of exposure as "use of artificial sweeteners" with non-quantitative responses of "yes" or "no" for past exposure. Participants were asked to recall intake before their illness, if they had changed their diet because of their illness. As aspartame was approved as a tabletop sweetener in Sweden in 1983, it was unlikely that prediagnostic intake reflected aspartame consumption.]

A population-based case–control study in the USA identified 255 cases of gastric cardia adenocarcinoma, 352 cases of non-cardia gastric adenocarcinoma, 206 cases of oesophageal squamous cell carcinoma, 282 cases of oesophageal adenocarcinoma, and 687 population-based controls (Mayne et al., 2006). Participants were

asked about the usual frequency with which they consumed diet soft drinks or soda (per day, week, month, or year) during the 3–5 years before diagnosis (cases) or interview (controls). High (top 20% of intake) versus no consumption of diet carbonated soft drinks was inversely associated with gastric cardia adenocarcinoma (OR, 0.50; 95% CI, 0.31–0.81), non-cardia gastric adenocarcinoma (OR, 0.58; 95% CI, 0.38–0.90), oesophageal adenocarcinoma (OR, 0.52; 95% CI, 0.32–0.83), and oesophageal squamous cell carcinoma (OR, 0.43; 95% CI, 0.23–0.82). Quantile cut-points were not provided for diet carbonated soft drinks. The authors controlled for frequency of reflux symptoms. [A strength of this analysis was the focus on gastric cancer subtype. The limitations included lack of data on duration of exposure. The patients diagnosed with cancer (cases) may have reduced their intake of carbonated beverages because of prediagnostic symptoms; therefore, their recall of their diet 3–5 years before diagnosis may not reflect intake in the distant past.]

In a hospital-based case–control study of 304 cases of oesophageal cancer and 743 controls in Italy (Gallus et al., 2007), no association was found between weekly consumption of sachets or tablets of "sweeteners other than saccharin" (assumed by the authors of the study to be mainly aspartame) and oesophageal cancer. The study also included 598 cases of oral and pharyngeal cancers and 1491 hospital-based controls; no evidence of an association between consumption of sachets or tablets of "sweeteners other than saccharin") and risk of these cancers was provided (Gallus et al., 2007). [The Working Group noted that only for 10 cases of oesophageal cancer and 12 cases of oral or pharyngeal cancer was any use of sachets or tablets per day reported, thus limiting study power. The exposure assessment of > 0 tablets or sachets per day versus non-daily use of tabletop sweeteners had the potential for exposure misclassification, which would tend to bias results towards the null.]

Another hospital-based case–control study based in northern Italy included 230 cases of gastric cancer matched to 547 hospital-based controls and 326 cases of pancreatic cancer matched to 652 controls (Bosetti et al., 2009). No association with increased risk of gastric or pancreatic cancer was observed for users versus non-users of sachets or tablets of "sweeteners other than saccharin", presumed by the author of the study to be mostly aspartame (17 and 35 cases of gastric and pancreatic cancer, respectively, reported use of tablets of "sweeteners other than saccharin"); the other sweetener assessed was saccharin. [Although the cases were histologically confirmed, the analysis presented all histological types combined, probably because of the small sample size. The exposure assessment of users versus non-users of other sweeteners increased the potential for exposure misclassification, which would tend to bias results towards the null.]

A population-based case–control study in the San Francisco Bay Area, USA, which included 532 cases with adenocarcinoma of the exocrine pancreas and 1701 controls, assessed sugar-free carbonated beverages overall and specific types (Chan et al., 2009). Diet was assessed using the Harvard FFQ instrument described above (Schernhammer et al., 2005). Among women and men combined, a higher risk of pancreatic cancer was found among consumers of ≥ 1 drink/day versus none (OR, 1.5; 95% CI, 1.1–2.1; *P* for trend, 0.2). For specific beverage types, risk was highest for low-calorie cola, with an 80% increase in risk (OR, 1.8; 95% CI, 1.1–2.9) among men, and a 70% increase in risk (OR, 1.7; 95% CI, 1.2–2.4) in men and women combined. Effect modification by diabetes history was reported: risk was elevated among participants without diabetes (OR, 1.6; 95% CI, 1.1–2.3) but not among those with diabetes (OR, 0.9; 95% CI, 0.3–2.6).

A hospital-based case–control study in Buffalo, New York, USA, which included 213 cases of pancreatic cancer and 852 cancer-free

controls, reported null associations between diet cola consumption and pancreatic cancer risk (Davis et al., 2023). Furthermore, no association between prediagnostic diet cola consumption and mortality from pancreatic cancer was observed in a retrospective cohort analysis of the 213 pancreatic cancer cases (203 pancreatic cancer deaths).

The MCC-Spain study enrolled 351 cases of stomach cancer and 3629 population-based controls between 2008 and 2013 and assessed consumption of aspartame-containing products as low- or no-calorie soft drinks and tabletop sweeteners other than saccharin (Palomar-Cros et al., 2023). Overall, there was no association between aspartame-containing products and the risk of stomach cancer. However, among participants with diabetes, high consumption of aspartame-containing products (sex-specific third quartile or above among controls) compared with non-consumers was associated with a higher risk of stomach cancer (OR, 2.04; 95% CI, 0.70–5.40; *P* for trend, 0.05), as was consumption of low- or no-calorie soft drinks (OR, 1.86; 95% CI, 0.63–5.01; *P* for trend, 0.08). [The limitations of the study by Palomar-Cros et al. (2023) included that the assumption that aspartame was used during this time period was questionable, since a mix of sweeteners may have been represented.]

[The strengths of these case–control studies included adjustment for BMI (Mayne et al., 2006; Gallus et al., 2007; Bosetti et al., 2009; Chan et al., 2009; Davis et al., 2023; Palomar-Cros et al., 2023) and adjustment or control for diabetes (Bosetti et al., 2009; Chan et al., 2009; Palomar-Cros et al., 2023) in some studies. A potential limitation of all included case–control studies was recall bias, which would be expected to bias estimates upward if cases were more likely than controls to recall artificial sweetener consumption, or downward if controls were more health-conscious and artificial sweetener use was viewed as healthy. Some of the case–control studies may have been subject to selection bias because of the low participation rate for controls – 67% in Chan et al. (2009); 50% in Davis et al. (2023); 70.2% in Mayne et al. (2006) (reported in Gammon et al., 1997); 53% in Palomar-Cros et al. (2023) (reported in Castaño-Vinyals et al., 2015) – if those who chose to participate varied from the source population in terms of sweetener use. Use of random-digit dialling to identify controls may result in a population that is not representative of the source population (Mayne et al., 2006; Chan et al., 2009). Cases were identified from population-based tumour registries (Mayne et al., 2006) or from patients admitted to specific hospitals or cancer centres (Norell et al., 1986; Gallus et al., 2007; Bosetti et al., 2009; Chan et al., 2009; Davis et al., 2023; Palomar-Cros et al., 2023) Artificial sweetener intake may have been enriched in the cases if diabetes status influenced hospital admission; however, diabetes status did not vary by case or control status in the study by Chan et al. (2009). In the hospital-based studies (Gallus et al., 2007; Bosetti et al., 2009; Davis et al., 2023), any potential associations between artificial sweetener use and the medical conditions of controls may lead to underestimation in associations with cancer if sweetener use (as a marker of diabetes or excess body weight, or other conditions) were higher in the controls (for example, non-traumatic orthopaedic conditions potentially related to excess body weight) than in the cases. The use of only tabletop sweeteners as potential sources of aspartame in the studies by Gallus et al. (2007), Bosetti et al. (2009), and Norell et al. (1986), may lead to misclassification of participants with regard to aspartame exposure. In the studies of Bosetti et al. (2009) and Gallus et al. (2007), diet soft drinks were not included, but the low frequency of consumption of diet soft drinks in middle-aged and elderly Italian participants at that time made their contribution less likely. Davis et al. (2023) examined only diet cola, which may have led to misclassification of exposure.]

Six meta-analyses of the association between ASBs and risk of cancers of the pancreas and other parts of the digestive tract were published (Jatho et al., 2021; Llaha et al., 2021; Tepler et al., 2021; Yin et al., 2022; and Pan et al., 2023). None reported a positive association with cancers of the digestive tract (excluding colorectal cancer).

One meta-analysis (Tepler et al., 2021) evaluated separately the associations between artificial sweeteners and risk of gastrointestinal luminal (i.e. organs with a lumen, an internal tubular channel, such as the intestine) and non-luminal (i.e. pancreatic) cancers. The study used the guidelines of the Cochrane Handbook (Higgins et al., 2023) and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), and evaluated study quality using the Newcastle–Ottawa Scale for quality and risk of bias assessment (all included studies were rated as "good"). The authors identified eight studies (four prospective, four case–control) of artificial sweetener consumption (exposed) or no consumption (non-exposed) in relation to gastrointestinal luminal or non-luminal cancers. The meta-analysis for non-luminal cancers included estimations only for pancreatic cancer, which was not associated with "artificial sweetener" consumption. This included three prospective studies and six case–control estimates (four estimates from the same case–control study). [The Working Group judged this meta-analysis to be uninformative because it included estimates for saccharin (Bosetti et al., 2009), which limited relevance for the agent under consideration, and it included four separate estimates of different and overlapping categories of ASB consumption from the same study (Chan et al., 2009) in the meta-analysis.]

Another meta-analysis (Jatho et al., 2021) that evaluated artificially sweetened soft drink consumption and gastrointestinal cancer included 38 studies (21 case–control and 17 cohort studies). [The Working Group judged this review to be uninformative for the evaluation of aspartame because all meta-analyses included estimates for regular soft drinks and combined results from prospective and case–control studies.]

A meta-analysis of sweetened beverages and cancer risk by Llaha et al. (2021) was conducted according to PRISMA guidelines. The only digestive cancer meta-analysed was pancreatic cancer (three cohort studies, two case–control studies). For high versus low intake of ASB, the relative risk was 1.07 (95% CI, 0.77–1.48). The I^2 (43.6) and P = 0.13 from the Cochran Q-test suggested between-study heterogeneity. In stratified analyses, estimates remained null by study type (RR for cohort studies, 1.05; 95% CI, 0.92–1.21; RR for case–control studies, 0.66; 95% CI, 0.37–1.17); by region [RR for USA, 1.08; 95% CI, 0.67–1.74; RR for Europe, 0.99; 95% CI, 0.61–1.60]; and by reference intake category (RR for high versus low, 0.93; 95% CI, 0.55–1.56; RR for high versus none, 1.27; 95% CI, 0.19–8.50). [The Working Group noted that not all studies included in the pancreatic cancer meta-analysis met the definition of the exposure in this monograph (one case–control study conducted in the 1970s assessed diet soda consumption before aspartame approval in the USA; Gold et al., 1985), limiting its informativeness.]

A dose–response meta-analysis of ASB consumption and cancer risk from prospective studies (Yin et al., 2022) used the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach to assess certainty of evidence. The only digestive cancer meta-analysed separately was pancreatic cancer (four cohorts, RR, 1.10; 95% CI, 0.92–1.31; P = 0.307; I^2 = 0.0%), with no linear or nonlinear relation suggested. The four cohorts, including EPIC (Navarrete-Muñoz et al., 2016), NIH-AARP (Bao et al., 2008), and NHS and HPFS (Schernhammer et al., 2005), were assigned as having moderate risk of bias, with the most common reason being lack of direct measurement of exposure (e.g. questionnaires). [Measurement

error from use of an FFQ would be expected to bias associations towards the null.]

A meta-analysis by Pan et al. (2023) included three cohort studies (HPFS, NHS, and NIH-AARP) on pancreatic cancer and did not report an association (RR, 1.03; 95% CI, 0.96–1.10). The authors did not detect a linear dose–response relation between ASB intake per 250 mL and pancreatic cancer risk. [The meta-analysis did not include all available studies. The authors used GRADE criteria to rate the certainty of evidence for pancreatic cancer as "low" primarily due to observational design and wide confidence intervals.]

2.2 Cancers of the urinary tract

See Table 2.2.

Four cohort and four case–control studies investigating the role of artificial sweetener consumption and cancers of the urinary tract are presented below according to the main type of exposure under study, and separately for kidney cancer, bladder cancer, and lower urinary tract cancers combined. Most studies focused on overall consumption of ASBs without specific assessment of aspartame (Hodge et al., 2018; Heath et al., 2021; McCullough et al., 2022; Ringel et al., 2023), and only two studies considered duration of use (Nomura et al., 1991; Andreatta et al., 2008). One small, hospital-based case–control study in men (54 cases only) was conducted in 2002–2008 in Lebanon and examined frequency of use of artificial sweeteners and bladder cancer risk (Kobeissi et al., 2013). [The Working Group noted that the study was subject to several potential biases, including selection bias (only 23% of the eligible cases participated), unclear exposure assessment, and a very small number of cases (n = 54), and was therefore considered to be uninformative (results were null).]

[The main development in the design of the studies was the evolution from case–control studies to cohort studies. There was little change, however, with regard to use of ASB consumption as a proxy for aspartame consumption (most studies), to more detailed assessments of surrogate definitions of aspartame use (i.e. ASB consumption, or consumption of sachets or tablets/week of artificial sweeteners) including duration of use, which was more common in the case–control studies (Nomura et al., 1991; Gallus et al., 2007; Andreatta et al., 2008).]

Four recent cohort studies, two from the USA (McCullough et al., 2022; Ringel et al., 2023), one from Australia (Hodge et al., 2018), and the other from Europe (Heath et al., 2021), provided information on ASB consumption and risk of kidney cancer. Both US studies (McCullough et al., 2022; Ringel et al., 2023) also assessed bladder cancer. Whereas McCullough et al. (2022) examined mortality only, Heath et al. (2021) presented results for both cancer mortality and incidence.

The cohort study from Australia, the MCCS, included 35 593 participants and 146 incident kidney cancer cases (Hodge et al., 2018). Baseline consumption of artificially sweetened soft drinks was assessed using FFQs. The results demonstrated lack of a positive association between consumption of artificially sweetened soft drinks and incident kidney cancer (linear model for increasing daily frequency of consumption, HR, 0.78; 95% CI, 0.40–1.55; P for trend, 0.48). [The Working Group noted that a weakness of the study was the small proportion of individuals consuming artificially sweetened soft drinks at least once daily (5.8%).]

The EPIC cohort study was conducted in 10 European countries and comprised 521 000 baseline participants and a mean follow-up of 15 years (for cancer incidence) and 16 years (for mortality) (Heath et al., 2021). Analyses on sugar-sweetened soft drinks and ASBs were restricted to 281 483 baseline participants with available information on types of soft drink, including 589 incident cases of renal cell carcinoma and 265 deaths from renal cell carcinoma (participants from Italy, Spain, and Umeå, Sweden

Table 2.2 Epidemiological studies on consumption of aspartame and cancers of the urinary tract

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hodge et al. (2018) Australia Enrolment, 1990–1994/ follow-up until 30 June 2013 Cohort	35 593; MCCS – a prospective cohort study of men and women aged 40–69 yr at recruitment and free of cancer, angina, heart attack, or diabetes at baseline; participants with extreme baseline energy intake were excluded Exposure assessment method: self-administered 121-item FFQ with separate questions on frequency of consumption in the past year of diet (artificially sweetened) soft drinks	Kidney, incidence	Artificially sweetened soft drink consumption (HR): Never or < 1/mo 1–3/mo 1–6/wk ≥ 1/day Continuous (per beverage/ day) Trend-test *P* value: 0.48	 114 11 12 9 146	 1 0.71 (0.38–1.36) 0.66 (0.36–1.22) 0.92 (0.46–1.84) 0.78 (0.40–1.55)	Age, sex, socioeconomic index, country of birth, alcohol intake, smoking status, physical activity, Mediterranean diet score, sugar-sweetened soft drink consumption, waist circumference	*Exposure assessment critique*: Key strengths were that it was a prospective study, that assessment was after aspartame introduction in diet soft drinks in Australia (1987), and that first half of follow-up largely overlapped with period of aspartame use in Australia. Key limitations were the FFQ assessment with no specific estimate of aspartame exposure, ASB as a proxy, and exposure data at baseline only; small number of consumers. *Other strengths*: adjustment for key confounders, including a measure of obesity (waist circumference). *Other limitations*: Likely bias from non-differential exposure misclassification given single baseline assessment and long follow-up.

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Heath et al. (2021) 10 European countries Enrolment, 1991–2000/ follow-up, mean, 15 yr (incidence), 16 yr (mortality) Cohort	281 483; members of the EPIC prospective cohort study (> 520 000 participants aged 30–70 yr) with complete data (389 220) and excluding participants from Italy, Spain, and Umeå (Sweden). Exposure assessment method: questionnaire; exposure to ASB soft drinks overall assessed once at baseline between 1991 and 2000 through country-specific validated tools (mainly FFQ) covering the usual diet over the past year; no specific assessment of aspartame or AS content of ASB	Kidney (RCC), incidence	Artificially sweetened soft drink intake (HR): Continuous (per 100 g/day) Trend-test *P* value: 0.61	589	1.02 (0.96–1.08)	Sex, country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption (several types of beverages) in several western Europe countries at a period relevant for aspartame exposure (between 1991 and 2000). Key limitations were that no other sources of aspartame were considered; uncertainty regarding aspartame content in ASBs in every country; and there was only one assessment at baseline. Other information: context of low ASB consumption in middle-aged adults. *Other strengths*: large cohort study; results adjusted for BMI; several relevant sensitivity analyses were considered. *Other limitations*: power was low in some sensitivity analyses.
		Kidney (RCC), incidence	Artificially sweetened soft drink intake (HR): Continuous (per 100 g/day) Trend-test *P* value: 0.59	589	1.02 (0.96–1.08)	Sex, country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake, fruit and vegetable intake	
		Kidney (RCC), incidence	Artificially sweetened soft drink intake, excluding the first 2 yr of follow-up (HR): Continuous (per 100 g/day) Trend-test *P* value: 0.87	528	0.99 (0.93–1.06)	Sex, country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Heath et al. (2021) (cont.)		Kidney (RCC), incidence	Artificially sweetened soft drink intake, excluding participants with self-reported diabetes at baseline (HR):			Sex, country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	
			Continuous (per 100 g/day)	562	1.02 (0.96–1.09)		
			Trend-test *P* value: 0.46				
		Kidney (RCC), incidence	Artificially sweetened soft drink intake, men (HR):			Country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	
			Continuous (per 100 g/day)	347	0.99 (0.91–1.08)		
			Trend-test *P* value: 0.85				
		Kidney (RCC), incidence	Artificially sweetened soft drink intake, women (HR):				
			Continuous (per 100 g/day)	242	1.05 (0.96–1.14)		
			Trend-test *P* value: 0.28				
		Kidney (RCC), mortality	Artificially sweetened soft drink intake (HR):			Sex, country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	
			Continuous (per 100 g/day)	265	1.06 (0.99–1.14)		
			Trend-test *P* value: 0.11				

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Heath et al. (2021) (cont.)		Kidney (RCC), mortality	Artificially sweetened soft drink intake (HR):			Sex, country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake, fruit and vegetable intake	
			Continuous (per 100 g/day)	265	1.06 (0.99–1.14)		
			Trend-test *P* value: 0.10				
		Kidney (RCC), mortality	Artificially sweetened soft drink intake, excluding the first 2 yr of follow-up (HR):			Sex, country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	
			Continuous (per 100 g/day)	256	1.05 (0.97–1.13)		
			Trend-test *P* value: 0.26				
		Kidney (RCC), mortality	Artificially sweetened soft drink intake, excluding participants with self-reported diabetes at baseline (HR):				
			Continuous (per 100 g/day)	256	1.06 (0.99–1.15)		
			Trend-test *P* value: 0.10				
		Kidney (RCC), mortality	Artificially sweetened soft drink intake, men (HR):			Country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	
			Continuous (per 100 g/day)	142	1.04 (0.93–1.15)		
			Trend-test *P* value: 0.49				

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Heath et al. (2021) (cont.)		Kidney (RCC), mortality	Artificially sweetened soft drink intake, women (HR):			Country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	
			Continuous (per 100 g/day)	123	1.08 (0.98–1.19)		
			Trend-test *P* value: 0.11				
		Kidney (RCC), incidence	Artificially sweetened soft drink intake (HR):			Sex, country, age, education, smoking status, alcohol consumption, physical activity, juice intake and sugar-sweetened soda intake, BMI, energy intake	
			0 g/day	NR	1		
			50 g/day	NR	1.18 (0.99–1.41)		
			100 g/day	NR	1.28 (0.98–1.66)		
			200 g/day	NR	1.29 (0.98–1.69)		
			300 g/day	NR	1.25 (0.95–1.63)		
			400 g/day	NR	1.21 (0.91–1.61)		
		Kidney (RCC), mortality	Artificially sweetened soft drink intake (HR):				
			0 g/day	NR	1		
			50 g/day	NR	1.13 (0.87–1.46)		
			100 g/day	NR	1.21 (0.82–1.78)		
			200 g/day	NR	1.28 (0.85–1.93)		
			300 g/day	NR	1.33 (0.90–1.96)		
			400 g/day	NR	1.38 (0.93–2.05)		

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) USA Enrolment, 1982/follow-up, through 2016 (median, 27.7 yr) Cohort	934 777 (416 313 men, 518 464 women); CPS-II prospective cohort; adults aged ≥ 28 yr; excluded participants with personal history at baseline of diabetes or cancer other than nonmelanoma skin cancer, men aged > 90 yr or women aged > 95 yr at enrolment, and those reporting only prior but not current consumption of either SSBs or ASBs Exposure assessment method: questionnaire; exposure to ASB assessed in 1982 through a question about the number of drinks/day of diet soda or ice teas (one pooled item) and potential changes over the past 10 yr; no specific assessment of aspartame content in ASB	Kidney, mortality	ASB consumption (HR): Never < 1 drink/day 1 drink/day ≥ 2 drinks/day Continuous (per drink/day) Trend-test *P* value: 0.412	 NR NR NR 3129	 1 0.98 (0.87–1.11) 1.08 (0.94–1.24) 1.04 (0.90–1.20) 1.01 (0.97–1.05)	Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption. Key limitations included a single dietary assessment at baseline in 1982 that was before the use of aspartame in ASBs, hence the relevance regarding aspartame exposure depends on the stability of ASB consumption over up to 34 yr of follow-up, but such information was not directly available; no other sources were considered (although these were more limited); and uncertainty regarding aspartame content in ASBs after the mid-2000s. Other comments: exclusion of participants who reported only prior but not current consumption of either SSBs or ASBs at baseline. *Other strengths*: large cohort with long follow-up; ability to examine multiple cancer types, stratify by sex or BMI, and limit to never-smokers; comprehensive adjustment for confounders, including SSB consumption. *Other limitations*: likely bias from non-differential exposure misclassification.
		Kidney, mortality	ASB consumption, BMI adjusted (HR): Never < 1 drink/day 1 drink/day ≥ 2 drinks/day Continuous (per drink/day) Trend-test *P* value: 0.468	 NR NR NR 3129	 1 0.92 (0.82–1.04) 1.01 (0.88–1.16) 0.95 (0.82–1.10) 0.99 (0.95–1.03)	Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Urinary bladder, mortality	ASB consumption (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	
			Never	NR	1		
			< 1 drink/day	NR	0.94 (0.83–1.06)		
			1 drink/day	NR	0.99 (0.86–1.14)		
			≥ 2 drinks/day	NR	1.04 (0.90–1.20)		
			Continuous (per drink/day)	3419	1.00 (0.95–1.04)		
			Trend-test *P* value: 0.794				
		Urinary bladder, mortality	ASB consumption, BMI adjusted (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.93 (0.82–1.05)		
			1 drink/day	NR	0.98 (0.85–1.13)		
			≥ 2 drinks/day	NR	1.02 (0.88–1.18)		
			Continuous (per drink/day)	3419	0.99 (0.95–1.03)		
			Trend-test *P* value: 0.922				

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Ringel et al. (2023) USA Enrolment, 1993–1998/ follow-up, until 28 February 2020 Cohort	80 388; Women's Health Initiative Observational Study participants (aged 50–79 yr at enrolment) who completed a questionnaire regarding ASBs and did not self-report a prior diagnosis of urinary tract cancer Exposure assessment method: questionnaire; exposure to ASB assessed in 1996–2001 through nine frequencies of consumption of diet drinks; no specific assessment of aspartame content in ASB	Kidney (RCC), incidence	ASB consumption (HR):			Age, race, ethnicity, neighbourhood SES (used as a proxy for the likelihood of environmental exposures), smoking, BMI, history of hypertension, and diet quality	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption at a relevant period for aspartame exposure from ASBs (the USA between 1996 and 2001). Key limitations were that no other sources were considered (although these were more limited); there was only one dietary assessment at baseline and no consideration of potential variations of ASB consumption over up to 24 yr of follow-up); and uncertainty regarding aspartame content in ASBs after the mid-2000s. *Other strengths*: prospective large cohort study, detailed outcome assessment, long follow-up period. *Other limitations*: potential for residual confounding.
			Never or < 1 serving/ wk	188	1		
			1–6 servings/wk	91	1.34 (1.03–1.75)		
			≥ 1 serving/ day	48	1.14 (0.80–1.62)		
		Urinary bladder, incidence	ASB consumption (HR):			Age, race, ethnicity, neighbourhood SES (used as a proxy for the likelihood of environmental exposures), smoking, water consumption	
			Never or < 1 serving/ wk	295	1		
			1–6 servings/wk	106	0.99 (0.78–1.26)		
			≥ 1 serving/ day	47	0.75 (0.53–1.06)		

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Nomura et al. (1991) USA, Hawaii (Oahu) 1977–1986 Case–control	Cases: 261; population-based, identified at seven largest community hospitals on Oahu that capture 96% of diagnosed cases (235 urinary bladder, 19 renal pelvis, 7 ureter) Controls: 522; population-based, identified from state survey; matched to cases by age (within 5 yr), sex, ethnic group (Caucasian [White] or Japanese), residence on Oahu; 1:2 ratio Exposure assessment method: questionnaire; diet history of 29 food items consumed during a usual week, usual diet 1 yr before diagnosis. assessing ASBs, such as diet or low-calorie sodas	Lower urinary tract (urinary bladder, renal pelvis, ureter), incidence	Use of ASs from diet beverages, men (OR):			Age, current residence on Oahu, ethnicity, pack-years of cigarette smoking	*Exposure assessment critique*: Key limitations were the retrospective recall; that there was no specific estimate of aspartame exposure, and ASBs as a proxy only (diet or low-calorie sodas). Limited value as exposure was collected in 1977–1986, but study participants could not have consumed aspartame before it was approved in 1983. *Other strengths*: captured almost all cases in catchment area (Oahu). *Other limitations*: adjustment only for pack-years of cigarette smoking (but used conditional logistic regression to account for matching factors);potential for bias from differential misclassification, as interviewers were not blinded to case–control status.
			Non-users	151	1		
			Users	44	1.4 (0.9–2.2)		
		Lower urinary tract (urinary bladder, renal pelvis, ureter), incidence	Use of ASs from diet beverages, men (OR):				
			Non-users	151	1		
			1–2 can-years	25	1.5 (0.8–2.6)		
			3+ can-years	19	1.3 (0.7–2.5)		
			Trend-test *P* value: 0.29				
		Lower urinary tract (urinary bladder, renal pelvis, ureter), incidence	Use of ASs from diet beverages, women (OR):				
			Non-users	52	1		
			Users	14	1.2 (0.5–2.7)		
		Lower urinary tract (urinary bladder, renal pelvis, ureter), incidence	Use of ASs from diet beverages, women (OR):				
			Non-users	52	1		
			1–2 can-years	10	2.0 (0.8–5.2)		
			3+ can-years	4	0.4 (0.1–1.7)		
			Trend-test *P* value: 0.31				

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gallus et al. (2007) Italy 1991–2004 Case–control	Cases: 767; multiple hospital-based case–control studies Controls: 1534; patients admitted to the same hospitals for acute, non-neoplastic disorders Exposure assessment method: questionnaire; assessment of use of tabletop sweeteners containing either saccharin or other sweeteners as sachets or tablets per week in the 2 yr before cancer diagnosis	Kidney (RCC), incidence	Consumption of ASs other than saccharin (OR): Non-consumers > 0 sachets or tablets/ day	 710 57	 1 1.03 (0.73–1.46)	Age, sex, study centre, education, alcohol use, tobacco smoking, BMI, total energy intake, consumption of hot beverages	*Exposure assessment critique*: Key limitations were that there was no specific assessment of aspartame, aspartame was considered as "other sweeteners" but with unclear actual contribution; only one source considered (tabletop sweeteners); retrospective assessment in a case–control study (potential for differential misclassification). Other information: according to the authors, there was low consumption of sources of sweeteners (including ASBs) in the study population, middle-aged adults in Italy between 1991 and 2004). *Other strengths*: high response rates (< 5% refusals) for cases and controls reduce the potential for selection bias; large sample size with large case numbers for rarer cancers; control for key potential confounders, including BMI. *Other limitations*: potential for recall bias and reverse causation; low statistical power.

Table 2.2 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Andreatta et al. (2008) Argentina 1999–2006 Case–control	Cases: 197; incident cases of histologically confirmed urinary tract tumours of transitional-cell types of the renal pelvis, ureter, and/or urinary bladder admitted to 10 public and private hospitals in the greater Cordoba region Controls: 397; with acute, non-neoplastic, and non-urinary tract diseases, selected from the respective hospital's admission registry at the same time as the case diagnosis Exposure assessment method: questionnaire; assessment of ever use and duration of use of AS in infusions (tea, coffee, mate) in Argentina between 1999 and 2006 (5 yr before cancer diagnosis)	Urinary tract (urinary bladder, renal pelvis, ureter), incidence	Duration of AS consumption (OR): Never Short-term (1–9 yr) Long-term (≥ 10 yr) Trend-test *P* value: < 0.02	 146 21 30	 1 1.10 (0.61–2.00) 2.18 (1.22–3.89)	Age, sex, BMI, social status, years of tobacco use	*Exposure assessment critique*: Key limitations were that there was no specific assessment of aspartame; only one source of exposure was considered (ASs added to infusions) and limited use of aspartame; no quantitative assessment (ever use and duration of use); retrospective assessment in a case–control study (potential for differential misclassification). *Other strengths*: both men and women enrolled; large catchment area in Argentina. *Other limitations*: AS varieties were classified into two subgroups (according to prevalence of sale at time of assessment period): saccharin/ cyclamate and aspartame/ acesulfame-K; results on duration of use were mainly driven by saccharin/cyclamate users and may not reflect exposure to aspartame.

AS, artificial sweetener; ASB, artificially sweetened beverage; BMI, body mass index; CI, confidence interval; CPS-II, Cancer Prevention Study II; EPIC, European Prospective Investigation into Cancer and Nutrition; FDA, Food and Drug Administration; FFQ, food frequency questionnaire; HR, hazard ratio; MCCS, Melbourne Collaborative Cohort Study; mo, month(s); NR, not reported; OR, odds ratio; RCC, renal cell carcinoma; SES, socioeconomic status; SSB, sugar-sweetened beverage; vs, versus; wk, week(s); yr, year(s).

were excluded). Statistical analyses were adjusted for a large number of potential confounders (age, sex, country, educational attainment, smoking status, alcohol consumption, physical activity, juice intake, sugar-sweetened soft drink consumption) and additionally for energy intake and BMI. Consumption of artificially sweetened soft drinks was not associated with the risk of either being diagnosed with renal cell carcinoma (HR, 1.02; 95% CI, 0.96–1.08) or dying from renal cell carcinoma (HR per 100 g/day, 1.06; 95% CI, 0.99–1.14). Analyses using restricted cubic splines that evaluated artificially sweetened soft drink consumption of 400 g/day versus 0 g/day did not suggest an elevated risk of renal cell carcinoma incidence (HR, 1.21; 95% CI, 0.91–1.61) or renal cell carcinoma mortality (HR, 1.38; 95% CI, 0.93–2.05). [The Working Group noted that power was low in some of the sensitivity analyses. Noted strengths were that sensitivity analyses were conducted, additionally adjusting for fruit and vegetable consumption, excluding the first 2 years of follow-up, and excluding participants with self-reported diabetes at baseline; none of these produced different results. The Working Group further noted that adjustment for BMI attenuated the findings, leaving the confidence intervals in the cubic spline graph (in the original publication, supplementary Fig. 2, bottom right) overlapping with 1. No distinction was made between aspartame and other types of artificial sweetener in ASBs, probably increasing non-differential exposure misclassification and leading to bias towards the null.]

A cohort study using data from the CPS-II in the USA included 934 777 participants and investigated the association between ASB consumption, measured at baseline via an FFQ, and subsequent cancer risk (McCullough et al., 2022). During follow-up (median, 27.7 years), 3129 kidney cancer deaths and 3419 bladder cancer deaths were recorded. Overall, the study found no association between ASB consumption and kidney cancer deaths. Results of multivariable-adjusted analyses (including age, sex, race/ethnicity, smoking, marital status, education, consumption of red and processed meat, fruit and vegetables, alcohol, sugar-sweetened beverage consumption) were presented without adjustment for BMI (RR for ≥ 2 drinks/day versus never, 1.04; 95% CI, 0.90–1.20) and with further adjustment for BMI (RR for ≥ 2 drinks/day versus never, 0.95; 95% CI, 0.82–1.10). Similar to the results for kidney cancer, no association was found between ASB consumption and risk of bladder cancer (HR for ≥ 2 ASBs/day versus never, 1.02; 95% CI, 0.88–1.18). [With only a single baseline assessment of exposure in 1982, 1 year before the approval of aspartame use in beverages, and a median follow-up of 27.7 years, the study had the potential for misclassification, which was likely to be non-differential and bias risk estimates towards the null. Although published data from more than 100 000 participants included in both the CPS-II and the CPS-II nutrition cohort (McCullough et al., 2014) suggested some decrease in ASB consumption over the 17 years between 1982 and 1999 in this population, the relative ranking of intake category was consistent during this period.]

The Women's Health Initiative Observational Study (WHI-OS) accrued a total of 804 cancers of the urinary tract among 80 388 eligible women in the USA (Ringel et al., 2023). Consumption of ASBs was assessed once, 3 years after enrolment, which took place between 1993 and 1998. Compared with no or < 1 serving/week, no increased risk for urinary bladder cancer (total of 448 cases) was noted for higher consumption of ASBs (HR for 1–6 servings/week, 0.99; 95% CI, 0.78–1.26; and HR for ≥ 1 serving/day, 0.75; 95% CI, 0.53–1.06), whereas for kidney cancer (total of 327 cases), an increased risk was observed in the 1–6 servings/week group (HR, 1.34; 95% CI, 1.03–1.75), but not in the ≥ 1 serving/day group (HR, 1.14; 95% CI, 0.80–1.62). [The Working Group noted as weaknesses of the study the one-time assessment of beverage consumption,

potential for residual confounding, and measurement error in the exposure assessment, which probably biased estimates towards the null. Strengths pertained to its large size and long follow-up.]

A case–control study conducted in Hawaii, USA, by Nomura et al. (1991) investigated the association between diet beverages and cancers of the lower urinary tract. In men, the odds ratio for any diet beverage consumption was 1.4 (95% CI, 0.9–2.2; 44 cases), compared with non-consumers, and an exposure–response relation (cumulative use) was not observed (*P* for trend, 0.29). Similarly, in women, an odds ratio of 1.2 (95% CI, 0.5–2.7; *P* for trend, 0.31; 14 cases) was reported when consumers were compared with non-consumers. [The Working Group noted that the time frame of this study (which included cancers diagnosed between 1977 and 1987) was of questionable relevance for aspartame exposure, considering that aspartame use in ASBs was authorized in the USA in 1983. Besides the retrospective assessment of diet after diagnosis, interviewers were not blinded to case–control status, which increased the potential for differential exposure misclassification.]

One hospital-based case–control study was conducted in 1991–2004 in four areas in Italy and combined cases of cancer at multiple organ sites with a selection of controls from the same overall pool of controls (Gallus et al., 2007). The study examined weekly consumption of saccharin and sweeteners other than saccharin, expressed in sachets or tablets/week. The authors stated that the consumption of "sweeteners other than saccharin" mainly comprised aspartame consumption. The study included 767 cases of kidney cancer and 1534 controls. After adjustment for a large number of potential confounders (including sex, age, alcohol, tobacco, BMI, energy intake, and hot beverages), there was no association between exposure to artificial sweeteners other than saccharin (> 0 sachets or tablets/day) and renal cell carcinoma risk (OR, 1.03; 95% CI, 0.73–1.46). Results were not presented separately by sex. [The Working Group noted as key limitations the uncertainty surrounding the authors' assumption with regard to aspartame intake, defined as "other sweeteners" (not distinguishing between aspartame and other artificial sweeteners other than saccharin); low power; and the low consumption of sweeteners in this Italian population. These limitations affected precision and probably introduced bias towards the null. A strength of this case–control study was the relatively high participation rates: fewer than 5% of both cases and controls who were contacted refused to participate.]

One case–control study, conducted in 1999–2006 in Argentina, examined the association between several definitions of aspartame use and (lower) urinary tract tumours (Andreatta et al., 2008). Compared with non-users, long-term (≥ 10 years) artificial sweetener consumption referring exclusively to use as an additive in infusions (tea, coffee, mate), without consideration of consumption from other sources such as soft drinks or dietetic foods, was associated with an increased risk of urinary tract tumours (OR, 2.18; 95% CI, 1.22–3.89). [The Working Group noted that the assessment of artificial sweetener consumption distinguished between saccharin/cyclamate and aspartame/acesulfame-K, but most artificial sweetener consumers were saccharin/cyclamate users, with 40 cases occurring in the saccharin/cyclamate group, and only 11 cases in the aspartame/acesulfame-K group. Results on duration of use, which did not distinguish between saccharin/cyclamate and aspartame/acesulfame-K, were therefore mainly driven by saccharin/cyclamate users and the results may not reflect exposure to aspartame.]

One meta-analysis on non-sugar sweeteners and health outcomes, which summarized eight studies (one of which was included twice) with a variety of types of non-sugar sweetener including but not limited to aspartame, reported a combined estimate (odds ratio) for bladder

cancer risk of 1.03 (95% CI, 0.84–1.25) (Toews et al., 2019). [The Working Group considered this meta-analysis to be uninformative, because the most recent study included was from 1994 (Momas et al., 1994), and the exposure assessment was unclear for the inclusion of any type of non-sugar sweetener.]

2.3 Cancers of the breast and prostate

See Table 2.3.

Six cohort studies (Hodge et al., 2018; Malik et al., 2019; Mullee et al., 2019; Romanos-Nanclares et al., 2021; Debras et al., 2022b; McCullough et al., 2022) investigating the association between consumption of aspartame and artificial sweeteners primarily containing aspartame and cancer of the breast are presented below according to main type of exposure under study. Results are presented separately for post- and premenopausal versus overall breast cancer. Five of these cohort studies also reported on prostate cancer outcomes (Hodge et al., 2018; Malik et al., 2019; Mullee et al., 2019; Debras et al., 2022b; McCullough et al., 2022). Whereas McCullough et al. (2022), Malik et al. (2019), and Mullee et al. (2019) examined mortality only, all other studies reported on breast or prostate cancer incidence.

Most studies focused on overall consumption of ASBs without separately assessing aspartame consumption. In addition to the cohort studies, three case–control studies on breast cancer (Ewertz and Gill, 1990; Gallus et al., 2007; Palomar-Cros et al., 2023) and two on prostate cancer (Gallus et al., 2007; Palomar-Cros et al., 2023) are described below.

[The main development in the design of the studies was the evolution from case–control studies to cohort studies. There has been little advancement, however, on the exposure assessment of aspartame intake, since most studies evaluated consumption of ASBs.]

The Australian MCCS cohort study included 946 incident postmenopausal breast cancer cases among 21 492 women and 433 aggressive prostate cancer cases among 14 101 men (Hodge et al., 2018). The study had up to 23 years of follow-up from 1990–1994 until 30 June 2013. Consumption of artificially sweetened soft drinks was classified into four categories, from < 1 drink/month to ≥ 1 drink/day. Overall, the study reported no association between consumption of artificially sweetened soft drinks and breast cancer risk (HR for ≥ 1 drink/day compared with < 1 drink/month, 0.95; 95% CI, 0.73–1.25). There was no association between consumption of artificially sweetened soft drinks and prostate cancer risk (HR for ≥ 1 drink/day compared with < 1 drink/month, 0.81; 95% CI, 0.49–1.33). [The Working Group noted that a potential limitation of this study was that a very large proportion of the study population reported no consumption of artificially sweetened soft drinks or < 1 drink/month (74.9%), and only 5.8% reported consuming artificially sweetened soft drinks at least daily. The single baseline assessment of beverage consumption, given the long follow-up, was likely to have introduced bias towards the null. The Working Group also noted that the same cohort was used to examine the association between consumption of artificially sweetened soft drinks and premenopausal breast cancer risk (181 cases) (Bassett et al., 2020). The results were presented combined for all "non-obesity-related cancers", but not for premenopausal breast cancer separately, and the study was therefore not informative for the evaluation of breast cancer risk.]

Malik et al. (2019) reported results from the NHS cohort, which included 121 700 women aged 30–55 years at study entry (1976), and found no association between consumption of ASBs and breast cancer mortality (HR for ≥ 2 servings/day versus < 1 serving/month, 1.14; 95% CI, 0.92–1.40). Additionally, Malik et al. (2019) reported results from the prospective HPFS cohort, which comprised 37 716 men

Table 2.3 Epidemiological studies on consumption of aspartame and cancers of the breast and prostate

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hodge et al. (2018) Australia Enrolment, 1990–1994/ follow-up until 30 June 2013 Cohort	35 593 (14 101 men, 21 492 women); MCCS – a prospective cohort study of men and women aged 40–69 yr at recruitment and free of cancer, angina, heart attack, or diabetes at baseline; participants with extreme baseline energy intake were excluded Exposure assessment method: self-administered 121-item FFQ with separate questions on frequency of consumption in the past year of diet (artificially sweetened) soft drinks	Breast (postmenopausal, invasive only), incidence	Artificially sweetened soft drink consumption (HR):			Age, socioeconomic index, country of birth, alcohol intake, smoking status, physical activity, Mediterranean diet score, sugar-sweetened soft drink consumption, waist circumference	*Exposure assessment critique*: Key strengths were that it was a prospective study, that assessment was after aspartame introduction in diet soft drinks in Australia (1987), and that first half of follow-up largely overlapped with period of aspartame use in Australia. Key limitations were the FFQ assessment with no specific estimate of aspartame exposure, ASBs as a proxy, and exposure data at baseline only; small number of consumers. *Other strengths*: adjustment for key confounders, including a measure of obesity (waist circumference). *Other limitations*: Likely bias from non-differential exposure misclassification given single baseline assessment and long follow-up.
			Never or < 1/mo	716	1		
			1–3/mo	69	0.94 (0.73–1.22)		
			1–6/wk	101	0.90 (0.72–1.12)		
			≥ 1/day	60	0.95 (0.73–1.25)		
			Continuous (per beverage/day)	946	0.92 (0.71–1.18)		
			Trend-test *P* value: 0.51				
		Prostate (aggressive/ advanced), incidence	Artificially sweetened soft drink consumption (HR):				
			Never or < 1/mo	333	1		
			1–3/mo	33	0.94 (0.65–1.36)		
			1–6/wk	50	1.09 (0.80–1.48)		
			≥ 1/day	17	0.81 (0.49–1.33)		
			Continuous (per beverage/day)	433	0.91 (0.60–1.38)		
			Trend-test *P* value: 0.66				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Malik et al. (2019) USA Enrolment, 1976 (NHS), 1986 (HPFS/follow-up, 1980–2014 (NHS), 1986–2014 (HPFS) Cohort	37 716 men and 80 647 women; female registered nurses aged 30–55 yr in the NHS and male health professionals aged 40–75 yr in the HPFS; excluding those with history of diabetes, cardiovascular disease, or cancer at baseline, or with implausible dietary intake Exposure assessment method: prospective assessment of ASB consumption through repeated FFQs between 1980–1986 and 2010	Breast, mortality	ASB intake, women (NHS) (HR): < 1 serving/mo 1–4 servings/mo 2–6 servings/wk 1 to < 2 servings/day ≥ 2 servings/day Continuous (per serving/day) Trend-test *P* value: 0.57	 NR NR NR NR NR	 1 1.06 (0.91–1.23) 0.90 (0.78–1.05) 0.92 (0.76–1.13) 1.14 (0.92–1.40) 1.01 (0.95–1.08)	Age, race, smoking, alcohol intake, postmenopausal hormone use, physical activity, family history of diabetes; family history of myocardial infarction, family history of cancer, multivitamin use, aspirin use, baseline history of hypertension and hypercholesterolemia, intake of whole grains, fruit, vegetables, or red and processed meat, total energy, BMI, SSB intake	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption from repeated, validated diet assessments every 4 yr, the majority at a very relevant period for aspartame exposure from ASBs (the USA between the 1980s and 2010) potentially capturing lifetime exposure to aspartame. Key limitations were that other sources of aspartame were not considered (although these were more limited); and uncertainty regarding aspartame content in ASBs after the mid-2000s. *Other strengths*: large cohort with long follow-up. *Other limitations*: likely bias from non-differential misclassification of exposure to aspartame; stratified numbers of deaths not provided for specific cancer sites.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Malik et al. (2019) (cont.)		Prostate, mortality	ASB intake, men (HPFS) (HR):			Age, race, smoking, alcohol intake, physical activity, family history of diabetes; family history of myocardial infarction, family history of cancer, multivitamin use, aspirin use, baseline history of hypertension and hypercholesterolemia, intake of whole grains, fruit, vegetables, or red and processed meat, total energy, BMI, SSB intake	
			< 1 serving/mo	NR	1		
			1–4 servings/mo	NR	0.80 (0.61–1.06)		
			2–6 servings/wk	NR	1.02 (0.84–1.25)		
			1 to < 2 servings/ day	NR	0.93 (0.66–1.32)		
			≥ 2 servings/day	NR	1.01 (0.67–1.52)		
			Continuous (per serving/day)	NR	1.02 (0.91–1.14)		
			Trend-test *P* value: 0.92				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mullee et al. (2019) Europe Enrolment, 1992–2000/ follow-up, through 2009–2013 (depending on study centre; mean, 16.4 yr) Cohort	451 743 (321 081 women, 130 662 men); EPIC cohort study participants from 10 European countries (Denmark, France, Greece, Germany, Italy, Netherlands, Norway, Spain, Sweden, and the United Kingdom); men and women excluding those with prevalent cancer, heart disease, stroke, or diabetes or with implausible dietary intake; for artificially sweetened soft drinks, participants from Italy, Spain, and Sweden were not included; 1402 breast cancer deaths; 907 prostate cancer deaths	Breast, mortality	Consumption of artificially sweetened soft drink (glass, 250 mL) (HR):			Age, centre, BMI, physical activity index, educational status, alcohol consumption, smoking status, smoking intensity, smoking duration, ever use of contraceptive pill, menopausal status, ever use of menopausal hormone therapy, intakes of total energy, red and processed meat, fruits and vegetables, coffee, fruit and vegetable juice, sugar-sweetened soft drinks	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption (several types of beverage) in several western Europe countries at a period relevant for aspartame exposure (between 1991 and 2000). Key limitations were that no other sources of aspartame were considered; uncertainty regarding aspartame content in ASBs in every country; and there was only one assessment at baseline. Other information: context of low ASB consumption in middle-aged adults. Other comments: in this study, total soft drink consumption (regardless of type of sweetener) was associated with higher (overall) mortality.
			< 1 glass/mo	NR	1		
			1–4 glasses/mo	NR	0.79 (0.63–0.98)		
			> 1 to 6 glasses/ wk	NR	0.90 (0.74–1.10)		
			≥ 1 glass/day	NR	0.85 (0.59–1.22)		
			Trend-test *P* value: 0.38				
		Prostate, mortality	Consumption of artificially sweetened soft drink (glass, 250 mL) (HR):			Age, centre, BMI, physical activity index, educational status, alcohol consumption, smoking status, smoking intensity, smoking duration, intakes of total energy, red and processed meat, fruits and vegetables, coffee, fruit and vegetable juice, sugar-sweetened soft drinks	
			< 1 glass/mo	NR	1		
			1–4 glasses/mo	NR	1.23 (0.95–1.60)		
			> 1 to 6 glasses/ wk	NR	1.36 (1.05–1.78)		
			≥ 1 glass/day	NR	1.05 (0.64–1.75)		
			Trend-test *P* value: 0.53				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mullee et al. (2019) (cont.)	Exposure assessment method: questionnaire; exposure to artificially sweetened soft drinks overall assessed once at baseline through country-specific validated tools (mainly FFQ) covering the usual diet over the past year; no specific assessment of aspartame or AS content of the artificially sweetened soft drinks						*Other strengths*: population-based cohort spanning multiple countries with different behaviours; large number of cases; results adjusted for appropriate potential confounders, including BMI.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Romanos-Nanclares et al. (2021) USA Enrolment, 1976 (NHS), 1989 (NHS-II)/follow-up, 1980–2016 (NHS), 1991–2017 (NHS-II) Cohort	82 713 (NHS) and 93 085 (NHS-II); NHS and NHS-II participants; female nurses aged 30–55 yr (NHS) or 25–42 yr (NHS-II) at enrolment; excluding women with prevalent cancer or implausible total energy intake Exposure assessment method: prospective and repeated FFQs assessing ASB consumption between 1980–1991 and 2016–2017	Breast, incidence	Cumulative average intake of ASBs (HR): < 1/mo ≥ 1 to ≤ 4/mo > 1 to < 7/wk ≥ 1/day Continuous (per 1 serving/day) Trend-test *P* value: 0.08	 3177 1531 4197 2474 11 379	 1 1.01 (0.95–1.07) 0.98 (0.94–1.03) 0.96 (0.91–1.02) 0.99 (0.97–1.01)	Age, calendar year, SSB intake, race, age at menarche, age at menopause, postmenopausal hormone use, oral contraceptive use, parity and age at first birth, breastfeeding history, family history of breast cancer, history of benign breast disease, height, cumulatively updated alcohol and total caloric intake, physical activity, BMI at age 18 yr, modified AHEI score (with SSBs and alcohol removed), SES, change in weight since age 18 yr, cohort	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption as a cumulative average from repeated, validated diet assessments every 4 yr, the majority at a very relevant period for aspartame exposure from ASBs, potentially capturing lifetime exposure to aspartame. Key limitations were that other sources of aspartame were not considered although these were more limited); and uncertainty regarding aspartame content in ASBs after the mid-2000s.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Romanos-Nanclares et al. (2021) (cont.)		Breast, incidence	Cumulative average intake of ASBs, NHS (HR):			Age, calendar year, SSB intake, race, age at menarche, age at menopause, postmenopausal hormone use, oral contraceptive use, parity and age at first birth, breastfeeding history, family history of breast cancer, history of benign breast disease, height, cumulatively updated alcohol and total caloric intake, physical activity, BMI at age 18 yr, modified AHEI score (with SSBs and alcohol removed), SES, change in weight since age 18 yr	*Other strengths*: long-running, large cohort studies, complete assessment and control for potential confounders, including weight changes. *Other limitations*: relative lack of statistical power in certain substrata; potential for bias from non-differential exposure misclassification.
			< 1/mo	2242	1		
			≥ 1 to ≤ 4/mo	1123	1.00 (0.93–1.08)		
			> 1 to < 7/wk	2870	0.97 (0.92–1.03)		
			≥ 1/day	1260	0.99 (0.92–1.07)		
			Continuous (per serving/day)	7495	1.00 (0.97–1.04)		
			Trend-test *P* value: 0.87				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Romanos-Nanclares et al. (2021) (cont.)		Breast, incidence	Cumulative average intake of ASBs, NHS-II, without adjustment for change in weight since age 18 yr (HR):			Age, calendar year, SSB intake, race, age at menarche, age at menopause, postmenopausal hormone use, oral contraceptive use, parity and age at first birth, breastfeeding history, family history of breast cancer, history of benign breast disease, height, cumulatively updated alcohol and total caloric intake, physical activity, BMI at age 18 yr, modified AHEI score (with SSBs and alcohol removed), SES	
			< 1/mo	935	1		
			≥ 1 to ≤ 4/mo	408	1.03 (0.91–1.16)		
			> 1 to < 7/wk	1327	0.99 (0.91–1.08)		
			≥ 1/day	1214	0.93 (0.84–1.01)		
			Continuous (per 1 serving/day)	3884	0.98 (0.95–1.01)		
			Trend-test *P* value: 0.04				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Romanos-Nanclares et al. (2021) (cont.)		Breast, incidence	Cumulative average intake of ASBs, NHS-II (HR):			Age, calendar year, SSB intake, race, age at menarche, age at menopause, postmenopausal hormone use, oral contraceptive use, parity and age at first birth, breastfeeding history, family history of breast cancer, history of benign breast disease, height, cumulatively updated alcohol and total caloric intake, physical activity, BMI at age 18 yr, modified AHEI score (with SSBs and alcohol removed), SES, change in weight since age 18 yr	
			< 1/mo	935	1		
			≥ 1 to ≤ 4/mo	408	1.02 (0.91–1.15)		
			> 1 to < 7/wk	1327	0.98 (0.89–1.07)		
			≥ 1/day	1214	0.91 (0.83–1.00)		
			Continuous (per 1 serving/day)	3884	0.98 (0.95–1.00)		
			Trend-test *P* value: 0.02				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) France Enrolment, 2009–2021/ follow-up until 22 January 2021 (median, 7.8 yr) Cohort	102 865 (80 711 women, 22 154 men); population-based cohort NutriNet-Santé (web-based); men and women aged ≥ 18 yr Exposure assessment method: questionnaire; participants are asked every 6 mo to complete a series of three validated web-based 24 h dietary records randomly assigned over a 2-wk period (2 weekdays, 1 weekend day); at least two 24 h dietary records during the first 2 yr of follow-up considered in analyses (mean ± SD, 5.6 ± 3.0)	Breast: incidence	Aspartame intake (HR): Non-consumers Lower consumers (< 15.39 mg/day) Higher consumers (≥ 15.39 mg/day) Trend-test *P* value: 0.036	 647 176 156	 1 1.09 (0.92–1.29) 1.22 (1.01–1.48)	Age, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, prevalent diabetes, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake, age at menarche, age at first childbirth, number of biological children, baseline menopausal status, and oral contraceptive use and hormonal treatment for menopause at baseline and during follow-up	*Exposure assessment critique:* A key strength was it was a prospective study using dietary records with detailed, quantitative assessment of aspartame based on food composition data updates for food supply changes over time. A key limitation was the baseline assessment in the main analysis, but a sensitivity analysis was conducted using all 24 h dietary records available during follow-up. *Other strengths*: large cohort; large number of cases; sensitivity analyses excluded prevalent diabetes or used all available 24 h dietary records throughout follow-up; explored menopause-related heterogeneity for breast cancer.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) (cont.)		Breast, incidence	Aspartame intake, participants without diabetes (HR):			Age, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake, age at menarche, age at first childbirth, number of biological children, baseline menopausal status, and oral contraceptive use and hormonal treatment for menopause at baseline and during follow-up	*Other limitations*: low aspartame use in the cohort (28%); self-selection may limit generalizability; potential for bias from residual confounding and reverse causation.
			Non-consumers	457	1		
			Lower consumers (< 15.39 mg/day)	170	1.09 (0.91–1.29)		
			Higher consumers (≥ 15.39 mg/day)	147	1.21 (0.99–1.46)		
			Trend-test *P* value: 0.052				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) (cont.)		Breast, incidence	Aspartame intake (time-dependent) (HR):			Age, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, prevalent diabetes, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake, age at menarche, age at first childbirth, number of biological children, baseline menopausal status, and oral contraceptive use and hormonal treatment for menopause at baseline and during follow-up	
			Non-consumers	647	1		
			Lower consumers (< 15.39 mg/day)	176	1.05 (0.89–1.23)		
			Higher consumers (≥ 15.39 mg/day)	156	1.18 (0.97–1.42)		
			Trend-test *P* value: 0.106				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) (cont.)		Breast (premenopausal), incidence	Aspartame intake (HR):			Age, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, prevalent diabetes, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake, age at menarche, age at first childbirth, number of biological children, baseline menopausal status, and oral contraceptive use and hormonal treatment for menopause at baseline and during follow-up	
			Non-consumers	261	1		
			Lower consumers (< 16.44 mg/day)	73	1.08 (0.83–1.42)		
			Higher consumers (≥ 16.44 mg/day)	61	1.07 (0.79–1.46)		
			Trend-test *P* value: 0.564				
		Breast (postmenopausal), incidence	Aspartame intake (HR):				
			Non-consumers	386	1		
			Lower consumers (< 12.16 mg/day)	100	1.13 (0.91–1.42)		
			Higher consumers (≥ 12.16 mg/day)	98	1.24 (0.98–1.57)		
			Trend-test *P* value: 0.060				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) (cont.)		Prostate, incidence	Aspartame intake (HR):			Age, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, prevalent diabetes, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake	
			Non-consumers	310	1		
			Lower consumers (< 14.45 mg/day)	49	0.95 (0.70–1.30)		
			Higher consumers (≥ 14.45 mg/day)	44	1.28 (0.91–1.79)		
			Trend-test *P* value: 0.280				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) (cont.)		Prostate, incidence	Aspartame intake, participants without diabetes (HR):			Age, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake	
			Non-consumers	297	1		
			Lower consumers (< 14.45 mg/day)	46	0.97 (0.71–1.33)		
			Higher consumers (≥ 14.45 mg/day)	37	1.24 (0.87–1.77)		
			Trend-test *P* value: 0.367				
		Prostate, incidence	Aspartame intake (time-dependent) (HR):				
			Non-consumers	310	1		
			Lower consumers (< 14.45 mg/day)	49	1.12 (0.87–1.45)		
			Higher consumers (≥ 14.45 mg/day)	44	1.21 (0.87–1.71)		
			Trend-test *P* value: 0.194				
McCullough et al. (2022) USA Enrolment, 1982/follow-up, through 2016 (median, 27.7 yr) Cohort	934 777 (416 313 men, 518 464 women); CPS-II prospective cohort; adults aged ≥ 28 yr; excluded participants with personal history at baseline of diabetes or cancer other than nonmelanoma skin cancer, men aged > 90 yr or	Breast (postmenopausal), mortality	ASB consumption (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, parity, age at menarche, estrogen use, oral contraceptive use, age at first live birth, menopausal status	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption. Key limitations were that only one dietary assessment was conducted at baseline in 1982 that was before the use of aspartame in ASBs, hence the relevance regarding aspartame exposure depends on the stability
			Never	NR	1		
			< 1 drink/day	NR	1.02 (0.94–1.10)		
			1 drink/day	NR	0.97 (0.88–1.06)		
			≥ 2 drinks/day	NR	1.10 (1.00–1.20)		
			Continuous (per drink/day)	6074	1.03 (1.00–1.06)		
			Trend-test *P* value: 0.153				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)	women aged > 95 yr at enrolment, and those reporting only prior but not current consumption of either SSBs or ASBs; breast cancer results limited to women postmenopausal at baseline (367 978) Exposure assessment method: questionnaire; exposure to ASB assessed in 1982 through a question about the number of drinks per day of diet soda or ice teas (one pooled item) and potential changes over the past 10 yr; no specific assessment of aspartame content in ASB	Breast (postmenopausal), mortality	ASB consumption, BMI adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, parity, age at menarche, estrogen use, oral contraceptive use, age at first live birth, menopausal status, BMI	of ASB consumption over up to 34 yr of follow-up, but such information was not directly available; no other sources were considered (although these were more limited); and uncertainty regarding aspartame content in ASBs after the mid-2000s. Other comments: exclusion of participants who reported only prior but not current consumption of either SSBs or ASBs at baseline. *Other strengths*: large cohort with long follow-up; ability to examine multiple cancer types, stratify by sex or BMI, and limit to never-smokers; comprehensive adjustment for confounders, including SSB consumption.
			Never	NR	1		
			< 1 drink/day	NR	0.97 (0.90–1.05)		
			1 drink/day	NR	0.92 (0.84–1.01)		
			≥ 2 drinks/day	NR	1.03 (0.94–1.13)		
			Continuous (per drink/day)	6074	1.01 (0.98–1.04)		
			Trend-test *P* value: 0.838				
		Prostate, mortality	ASB consumption (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	
			Never	NR	1		
			< 1 drink/day	NR	0.99 (0.92–1.07)		
			1 drink/day	NR	0.91 (0.83–1.00)		
			≥ 2 drinks/day	NR	0.98 (0.89–1.08)		
			Continuous (per drink/day)	9381	0.99 (0.96–1.02)		
			Trend-test *P* value: 0.258				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Prostate, mortality	ASB consumption, BMI adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	*Other limitations*: likely bias from non-differential exposure misclassification.
			Never	NR	1		
			< 1 drink/day	NR	0.99 (0.92–1.07)		
			1 drink/day	NR	0.91 (0.83–1.00)		
			≥ 2 drinks/day	NR	0.98 (0.89–1.08)		
			Continuous (per drink/day)	9381	0.99 (0.96–1.02)		
			Trend-test *P* value: 0.258				
		Prostate, mortality	ASB consumption, normal weight (BMI, 18.5 to < 25 kg/m^2) (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.94 (0.81–1.08)		
			1 drink/day	NR	0.78 (0.64–0.95)		
			≥ 2 drinks/day	NR	0.88 (0.71–1.08)		
			Continuous (per drink/day)	3461	0.96 (0.91–1.02)		
			Trend-test *P* value: 0.022				
		Prostate, mortality	ASB consumption, overweight (BMI, 25 to < 30 kg/m^2) (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.99 (0.90–1.10)		
			1 drink/day	NR	0.88 (0.78–1.00)		
			≥ 2 drinks/day	NR	0.92 (0.81–1.05)		
			Continuous (per drink/day)	4781	0.96 (0.93–1.00)		
			Trend-test *P* value: 0.062				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Prostate, mortality	ASB consumption, obese (BMI, ≥ 30 kg/m²) (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.01 (0.81–1.26)		
			1 drink/day	NR	1.15 (0.90–1.48)		
			≥ 2 drinks/day	NR	1.23 (0.98–1.53)		
			Continuous (per drink/day)	861	1.06 (1.00–1.12)		
			Trend-test *P* value: 0.052				
Ewertz and Gill (1990) Denmark 1983–1984 (1 yr) population-based Case–control	Cases: 1474; women aged < 70 yr with incident breast cancer identified from the Danish Cancer Registry and the nationwide clinical trial of the Danish Breast Cancer Cooperative Group Controls: 1322; age-stratified random sample of the general female population, selected from the Central Population Register, excluding those with a prior breast cancer diagnosis	Breast, incidence	AS intake in coffee or tea (OR):			Age at diagnosis and place of residence	*Exposure assessment critique*: Key limitations were the retrospective recall using an FFQ; no specific estimate of aspartame exposure, only sweetener use in tea and coffee as proxy; limited value due to timing of exposures; the assessment period was March 1982 to February 1983; aspartame was approved in Denmark in 1983 but whether it was available in January and February was questionable; saccharin and cyclamate were mainly used, therefore the likelihood that this reflects aspartame use was limited. *Other strengths*: large sample size.
			No	1216	1		
			Yes	147	0.94 (0.73–1.20)		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Ewertz and Gill (1990) (cont.)	Exposure assessment method: self-administered, semiquantitative FFQ with additional questions on consumption of tea, coffee, sugar, and AS						
Gallus et al. (2007) Italy 1991–2004 Case–control	Cases: 2569 (female breast), 1294 (prostate); hospital-based; median age, 55 yr (female breast) or 66 yr (prostate) Controls: 2588 (female breast), 1451 (prostate); patients admitted to the same hospitals as the cases, though for acute, non-neoplastic disorders; median age, 56 yr (female breast) or 63 yr (prostate)	Breast, incidence	Consumption of AS other than saccharin, women (OR):			Age, study centre, education, alcohol use, tobacco smoking, BMI, total energy intake, consumption of hot beverages, parity, and menopausal status/age at menopause	*Exposure assessment critique*: Key limitations were that there was no specific assessment of aspartame, aspartame was considered as "other sweeteners" but with unclear actual contribution; only one source was considered (tabletop sweeteners); the retrospective assessment in a case–control study (potential for differential misclassification). Other information: according to the authors, there was limited consumption of sources of sweeteners (including ASBs) in the study population (middle-aged adults in Italy between 1991 and 2004).
			Non-consumers	2350	1		
			> 0 sachets or tablets/day	219	0.80 (0.65–0.97)		
		Prostate, incidence	Consumption of AS other than saccharin (OR):			Age, study centre, education, alcohol use, tobacco smoking, BMI, total energy intake, consumption of hot beverages	
			Non-consumers	1217	1		
			> 0 sachets/ tablets per day	77	1.23 (0.86–1.76)		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gallus et al. (2007) (cont.)	Exposure assessment method: questionnaire; assessment of use of tabletop sweeteners containing either saccharin or other sweeteners as sachets or tablets per week in the 2 yr before cancer diagnosis						*Other strengths*: high response rates (< 5% refusals) for cases and controls reduced the potential for selection bias; large sample size with large case numbers for rarer cancers; control for key potential confounders, including BMI. *Other limitations*: somewhat limited adjustment for confounders as far as breast cancer risk factors are concerned (e.g. age at menarche, hormone use, family history of breast cancer); potential for recall bias and reverse causation.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) Spain 2008–2013 Case–control	Cases: 1510 (breast cancer), 972 (prostate cancer); aged 20–85 yr with newly diagnosed histologically confirmed cancer, resided in catchment area for at least 6 mo; no prior history of their cancer; enrolled as soon as possible after diagnosis; frequency-matched on age, sex, and region to population controls	Breast, incidence	Consumption of aspartame-containing products (OR):			Age, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, family history of breast cancer, night shift work, menopause, nulliparous, age at first child, use of contraceptive, other AS	*Exposure assessment critique*: A key strength was the categorization of intake of ASBs and tabletop sweeteners by type (aspartame vs others) using public data on ingredients in food supply, but it was unclear whether the assumption of aspartame content in products was correct. A key limitation was that the study assessed beverages and tabletop sweeteners but there was no consideration of ASs in the rest of the food supply.
			Non-consumers	1146	1		
			Medium intake (< 3rd quartile among controls)	265	0.82 (0.67–1.01)		
			High intake (≥ 3rd quartile among controls)	99	0.94 (0.69–1.28)		
			Trend-test *P* value: 0.2				
		Breast, incidence	Consumption of aspartame-containing products, participants without diabetes (OR):				
			Non-consumers	1065	1		
			Medium intake (< 3rd quartile among controls)	242	0.81 (0.66–1.01)		
			High intake (≥ 3rd quartile among controls)	91	1.04 (0.75–1.45)		
			Trend-test *P* value: 0.4				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)	Controls: 3629 (1674 for breast cancer, 1308 for prostate cancer); randomly selected from administrative records of selected primary health care centres within catchment area Exposure assessment method: self-administered, semiquantitative FFQ, 140 food items, assessing usual dietary intake during the previous year	Breast, incidence	Consumption of aspartame-containing products, participants with diabetes (OR):			Age, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, family history of breast cancer, night shift work, menopause, nulliparous, age at first child, use of contraceptive, other AS	*Other strengths*: large sample size for some outcomes; histopathological confirmation of cancer cases; extensive assessment of confounding, including from BMI (captured in a combined score based on WCRF/AICR evidence on lifestyle factors; Romaguera et al., 2017); stratification by diabetes status to evaluate heterogeneity of associations.
			Non-consumers	81	1		
			Medium intake (< 3rd quartile among controls)	23	0.73 (0.33–1.57)		
			High intake (≥ 3rd quartile among controls)	8	0.28 (0.08–0.83)		
			Trend-test *P* value: 0.03				
		Prostate, incidence	Consumption of aspartame-containing products (OR):			Age, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, family history of prostate cancer, night shift work, other AS	
			Non-consumers	814	1		
			Medium intake (< 3rd quartile among controls)	110	0.81 (0.61–1.07)		
			High intake (≥ 3rd quartile among controls)	48	0.96 (0.63–1.46)		
			Trend-test *P* value: 0.4				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)		Prostate, incidence	Consumption of aspartame-containing products, participants without diabetes (OR):			Age, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, family history of prostate cancer, night shift work, other AS	*Other limitations*: non-prospective study design (case–control); selection bias due to low participation among cases (participation rate was 71% for breast, 72% for prostate) and controls (mean participation rate, 53%); recall bias in exposure assessment; potential for exposure measurement error and residual confounding due to other correlates of AS use among those with diabetes; relatively low exposure contrasts for aspartame-containing products; potential for chance findings due to small number in some strata.
			Non-consumers	704	1		
			Medium intake (< 3rd quartile among controls)	87	0.84 (0.61–1.15)		
			High intake (≥ 3rd quartile among controls)	30	0.82 (0.48–1.36)		
			Trend-test *P* value: 0.2				
		Prostate, incidence	Consumption of aspartame-containing products, participants with diabetes (OR):				
			Non-consumers	110	1		
			Medium intake (< 3rd quartile among controls)	23	0.85 (0.45–1.56)		
			High intake (≥ 3rd quartile among controls)	18	1.91 (0.87–4.2)		
			Trend-test *P* value: 0.3				
		Prostate (low grade; Gleason score, < 7), incidence	Consumption of aspartame-containing products (OR):				
			Non-consumers	374	1		
			Medium intake (< 3rd quartile among controls)	49	0.8 (0.55–1.15)		
			High intake (≥ 3rd quartile among controls)	22	0.97 (0.55–1.65)		
			Trend-test *P* value: 0.4				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)		Prostate (low grade; Gleason score, < 7), incidence	Consumption of aspartame-containing products, participants without diabetes (OR):			Age, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, family history of prostate cancer, night shift work, other AS	
			Non-consumers	332	1		
			Medium intake (< 3rd quartile among controls)	44	0.95 (0.63–1.40)		
			High intake (≥ 3rd quartile among controls)	12	0.68 (0.32–1.35)		
			Trend-test *P* value: 0.3				
		Prostate (low grade, Gleason score, < 7), incidence	Consumption of aspartame-containing products, participants with diabetes (OR):				
			Non-consumers	42	1		
			Medium intake (< 3rd quartile among controls)	5	0.47 (0.15–1.24)		
			High intake (≥ 3rd quartile among controls)	10	2.46 (0.93–6.38)		
			Trend-test *P* value: 0.3				
		Prostate (high grade, Gleason score, ≥ 7), incidence	Consumption of aspartame-containing products (OR):				
			Non-consumers	[428]	1		
			Medium intake (< 3rd quartile among controls)	[58]	0.83 (0.59–1.17)		
			High intake (≥ 3rd quartile among controls)	[26]	1.07 (0.64–1.76)		
			Trend-test *P* value: 0.7				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)		Prostate (high grade; Gleason score, ≥ 7), incidence	Consumption of aspartame-containing products, participants without diabetes (OR):			Age, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, family history of prostate cancer, night shift work, other AS	
			Non-consumers	361	1		
			Medium intake (< 3rd quartile among controls)	41	0.77 (0.51–1.16)		
			High intake (≥ 3rd quartile among controls)	18	1.00 (0.53–1.81)		
			Trend-test *P* value: 0.5				
		Prostate (high grade, Gleason score, ≥ 7), incidence	Consumption of aspartame-containing products, participants with diabetes (OR):				
			Non-consumers	67	1		
			Medium intake (< 3rd quartile among controls)	17	1.11 (0.54–2.24)		
			High intake (≥ 3rd quartile among controls)	8	1.56 (0.54–4.19)		
			Trend-test *P* value: 0.4				

AHEI, Alternate Healthy Eating Index; AICR, American Institute of Cancer Research; AS, artificial sweetener; ASB, artificially sweetened beverage; BMI, body mass index; CI, confidence interval; CPS-II, Cancer Prevention Study II; EPIC, European Prospective Investigation into Cancer and Nutrition; FDA, Food and Drug Administration; FFQ, food frequency questionnaire; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; MCCS, Melbourne Collaborative Cohort Study; mo, month(s); NHS, Nurses' Health Study; NHS-II, Nurses' Health Study II; NR, not reported; OR, odds ratio; SD, standard deviation; SES, socioeconomic status; SSB, sugar-sweetened beverage; vs, versus; WCRF, World Cancer Research Fund; wk, week(s); yr, year(s).

aged 40–75 years at baseline in 1986, and found no association between consumption of ASBs and prostate cancer mortality (HR for ≥ 2 servings/day versus < 1 serving/month, 1.01; 95% CI, 0.67–1.52). [The Working Group noted that the main strengths of the study were the repeated exposure assessments during a time when aspartame had already been approved for use in ASBs (follow-up started between 1980 (NHS) and 1986 (HPFS), and continued through 2014); the focus on lethal prostate cancers; and the complete control for potential confounders. Nonetheless, the potential for residual confounding could not be ruled out. A weakness of note was the potential for measurement error, which would result in a bias towards the null.]

The EPIC cohort study was conducted in 10 European countries and at baseline included 321 081 female and 130 662 male participants, among whom 1402 breast cancer and 907 prostate cancer deaths were diagnosed during follow-up (mean follow-up, 16.4 years) (Mullee et al., 2019). The statistical analyses (which excluded participants from Italy, Spain, and Sweden because information on type of soft drink consumption was not collected in all participating centres) did not report a positive association between consumption of artificially sweetened soft drinks and breast cancer mortality (HR for ≥ 1 glass/day of artificially sweetened soft drink compared with < 1 glass/month, 0.85; 95% CI, 0.59–1.22; *P* for trend, 0.38). The findings were null for prostate cancer mortality. [The Working Group noted that a major strength of the cohort was its large size and that it spanned several European countries. A main limitation of the analyses was the single, baseline-only assessment of beverage consumption, which took place between 1992 and 2000. Exposure misclassification was likely to be non-differential, biasing results towards the null.]

Another study based in the USA was conducted in two long-running prospective cohort studies (NHS and NHS-II), with repeated diet exposure updates throughout follow-up, and reported on breast cancer incidence (Romanos-Nanclares et al., 2021). The NHS cohort comprises 121 700 registered nurses who have been followed for major disease outcomes via biennial questionnaires since study inception in 1976; the NHS-II cohort comprises 116 429 registered nurses who have been similarly followed since its establishment in 1986. In both cohort studies, diet was assessed with a validated FFQ (administered in the NHS in 1980, 1984, 1986, and every 4 years thereafter, and in the NHS-II in 1991 and every 4 years thereafter). ASBs were defined as caffeinated, noncaffeinated, and noncarbonated low-calorie or diet beverages. Questions included the frequency of consumption over the past year for a standard 355 mL (12 oz) serving (1 glass/can/bottle) of each beverage. Cumulative averages of dietary data were based on repeated measures from FFQs. During follow-up (1980–2016 for NHS and 1991–2017 for NHS-II), 11 379 incident breast cancer cases were documented across both cohorts (among 82 713 NHS and 93 085 NHS-II participants free of cancer at baseline and with valid data). The pooled hazard ratio comparing extreme categories of ASB consumption (≥ 1/day compared with < 1/month) was 0.96 (95% CI, 0.91–1.02). Although no association with ASB consumption was observed in the NHS, intake was inversely associated with the risk of breast cancer in the NHS-II (HR for ≥ 1/day compared with < 1/month, 0.93; 95% CI, 0.84–1.01; *P* for trend, 0.04; and HR with additional adjustment for weight change since age 18 years, 0.91; 95% CI, 0.83–1.00; *P* for trend, 0.02). Overall, the findings were not suggestive of an increase in breast cancer risk among women with higher consumption of ASBs. [The Working Group noted that the strengths of the NHS and NHS-II cohort studies outweighed their weaknesses; strengths included the cohorts' large size, repeated exposure assessment over a long follow-up period, and complete assessment and control for potential confounders. Main weaknesses pertained to

the relative lack of power in certain substrata. It was also noted that the findings from the breast cancer incidence analyses were consistent with those from the breast cancer mortality analyses (Malik et al., 2019). The potential for non-differential exposure misclassification also existed, probably attenuating results.]

NutriNet-Santé, a very detailed and large population-based cohort study in France, included 80 711 women and 979 total incident breast cancer cases (median follow-up, 7.8 years; 156 cases among the higher-consumers of aspartame) (Debras et al., 2022b; reported earlier in Chazelas et al., 2019 for ASB consumption). Among 22 154 men, a total of 403 incident prostate cancer cases (same follow-up; 44 cases among the higher-consumers of aspartame) were also included. The NutriNet-Santé cohort is a web-based cohort that started in 2009 and included 102 865 participants who are followed every 6 months by three non-consecutive web-based 24-hour dietary records, randomly assigned over 15 days (2 weekdays and 1 weekend day). Artificial sweetener intake was assessed through 24-hour dietary records, in which brands and commercial names of industrial products were routinely collected with the aim of assessing exposure to food additives (Chazelas et al., 2021). The study assessed exposure to several artificial sweeteners: acesulfame-K (European food additive identification number E950), aspartame (E951), cyclamates (E952), saccharin (E954), sucralose (E955), thaumatin (E957), dihydrochalcone (E959), steviol glycosides (E960), and salt of aspartame-acesulfame (E962); the quantities consumed of all these artificial sweeteners were summed to calculate the variable "total artificial sweeteners". The study reported on the association between cancer risk and exposure to total artificial sweeteners, aspartame, acesulfame-K and sucralose. In the main analysis, the study used the average of all available (up to 15) 24-hour dietary records during the first 2 years of follow-up (sensitivity analyses were also presented using all available dietary records during the whole follow-up period). The definition of aspartame intake accounted for all dietary sources, and the study also reported on other most frequently consumed artificial sweeteners. Among artificial sweetener consumers, higher-consumers (above the sex-specific median consumption for total sweeteners) had a mean aspartame intake of 47.42 mg/day versus only 3.24 mg/day for lower-consumers, illustrating the interindividual variability in intake in this cohort. Results were reported overall and for pre- and postmenopausal breast cancer separately. The results suggested an increased risk of breast cancer overall (HR, 1.22; 95% CI, 1.01–1.48; *P* for trend, 0.036) for women with higher consumption of aspartame, i.e. above the sex-specific median among aspartame users compared with non-users. Hazard ratios for pre- and postmenopausal women were as follows, with no statistical heterogeneity detected (formally tested): for premenopausal women, 1.07 (95% CI, 0.79–1.46; *P* for trend, 0.564); and for postmenopausal women, 1.24 (95% CI, 0.98–1.57; *P* for trend, 0.060). The study found no significantly increased risk of prostate cancer associated with higher consumption of aspartame, i.e. above the median among male aspartame users versus non-users (multivariable-adjusted HR, 1.28; 95% CI, 0.91–1.79; *P* for trend, 0.280) although the hazard ratio point estimate was quantitatively similar to that reported for breast cancer risk in the same cohort. Similar results were observed in sensitivity analyses of breast and prostate cancer excluding participants with a history of diabetes at baseline. No interaction between aspartame and BMI was detected for breast (*P* for interaction, 0.796) or prostate (*P* for interaction, 0.884) cancer. The study provided additional information, including whether differences in risk were observed between sugar or artificial sweeteners. [The Working Group noted that the investigators for the NutriNet-Santé cohort study had planned before the data-collection phase to investigate

several food additives and consequently made a special, and so far quite unique, investment in collecting detailed food consumption information on the type, and where possible the brand, of many foods and drinks, and building a database on the food additive content of hundreds of foods. To date, the NutriNet-Santé study seemed to be the only cohort study that had built the methodology required for separating with some level of precision the consumption of foods and drinks specifically containing aspartame. The main analysis also considered intake over the first 2 years of follow-up, and additional analysis used repeated assessments throughout the follow-up period. Nonetheless, the potential for non-differential exposure misclassification remained.]

The CPS-II cohort study in the USA assessed the association between ASB consumption evaluated at baseline in 1982 (median follow-up, 27.7 years) and mortality from postmenopausal breast cancer and prostate cancer (McCullough et al., 2022). For breast cancer, among the large cohort comprising 367 978 postmenopausal (at baseline) women and 6074 postmenopausal breast cancer deaths, multivariable-adjusted analyses were conducted considering BMI separately, and reported no association after adjustment for several potential confounders and BMI; the results were compatible with a 3% increase in risk (HR, 1.03; 95% CI, 1.00–1.06) of breast cancer mortality per 1 drink/day of ASBs, before adjusting for BMI. For prostate cancer, among the cohort comprising 416 313 men and 9381 prostate cancer deaths, multivariable-adjusted analyses were conducted considering BMI separately, and described a null finding before and after adjustment for BMI. There was a suggestive interaction with BMI and the consumption of ASBs (*P* for interaction, 0.013), with a positive association only among obese participants (≥ 2 drinks/day versus never: HR for individuals with BMI of 18.5 to < 25 kg/m^2, 0.88; 95% CI, 0.71–1.08; HR for BMI 25–30 kg/m^2, 0.92; 95% CI, 0.81–1.05; HR for BMI > 30 kg/m^2, 1.23; 95% CI, 0.98–1.53). [The Working Group noted that the dietary assessment preceded by 1 year the approval of aspartame for use in beverages in the USA; therefore, over the 27 years of follow-up, non-differential misclassification was likely, which would be expected to bias results towards the null. However, published data among participants included in both the CPS-II and the CPS-II nutrition cohort (McCullough et al., 2014) suggested that the relative ranking of intake category was consistent during the first 17-year period.]

Three case–control studies reported on the association between artificial sweetener use and breast cancer risk (two of these also reported on prostate cancer risk).

One population-based case–control study in Denmark examined associations between breast cancer and artificial sweetener consumption in coffee or tea (Ewertz and Gill, 1990). This study was fairly large, with 1474 breast cancer cases and 1322 controls, and reported a null finding. [The Working Group noted that the questionnaire assessment of artificial sweetener consumption referred to the period March 1982 to February 1983, and that aspartame was only approved for use in Denmark in 1983; therefore, this study was considered to be uninformative.]

One hospital-based case–control study, which combined multiple hospital-based case–control studies conducted in 1991–2004 across four areas in Italy, examined weekly consumption of sachets/tablets of "sweeteners other than saccharin" (assumed by the study authors to mainly comprise aspartame) and breast and prostate cancer risk (Gallus et al., 2007). This large study (2569 breast cancer cases and 2588 controls) reported an inverse association between breast cancer and consumption of artificial sweeteners other than saccharin. The overall association for prostate cancer was null in this study, which included 1294 prostate cases and 1451 controls. [The Working Group noted that a strength of this case–control study was the

relative high participation rates: fewer than 5% of both cases and controls contacted for participation refused; weaknesses pertained to the exposure assessment, which did not distinguish between aspartame and artificial sweeteners other than saccharin, probably leading to a bias towards the null; and recall bias, given its case–control design.]

The MCC-Spain study, a case–control study considering multiple cancer sites and conducted between 2008 and 2013 (Palomar-Cros et al., 2023), investigated associations between use of artificial sweeteners (assessed via FFQ), separated into aspartame and other artificial sweeteners both derived from ASBs, tabletop sweeteners, and consumption of "gaseosa" (an artificially sweetened soft drink commonly consumed in Spain), and their association with risk of breast (1510 cases) and prostate (972 cases) cancer, using 3629 population controls. Overall, no increased risk of breast or prostate cancer was observed among those with high aspartame consumption (defined as third quartile or above). For breast cancer, among the study participants with diabetes, compared with non-consumers, there was an inverse association(OR, 0.28; 95% CI, 0.08–0.83; *P* for trend, 0.03), whereas for prostate cancer, the odds ratio was 1.91 (95% CI, 0.87–4.20; *P* for trend, 0.3). [The Working Group noted that the study's strengths were its size and ability to adjust for a large variety of potential confounders, including BMI, captured in the WCFR/AICR score (Romaguera et al., 2017), and that it stratified by diabetes status. A further strength was that prostate cancer cases were stratified by low-grade (Gleason score, < 7) versus high-grade aggressive prostate cancer (Gleason score, ≥ 7). Limitations included the incomplete exposure assessment and the uncertainty of the assumption that all low-calorie or no-calorie sweetened beverages contained aspartame only, as well as the potential for recall bias that is inherent to case–control studies.]

A recent meta-analysis (Yin et al., 2022) included four studies on ASB use (Hodge et al., 2018; Chazelas et al., 2019; Romanos-Nanclares et al., 2021; Debras et al., 2022b) and breast cancer risk, with a combined estimate of 0.99 (95% CI, 0.90–1.08; $P = 0.75$; $I^2 = 50.3\%$) for highest versus lowest category of ASB consumption, although these four studies were deemed of very low certainty (using the risk of bias in non-randomized studies of exposures, ROBINS-E, grading scheme). This assessment was mainly based on the criterion "indirectness", the "inconsistency" of the results of these four studies, and a "moderate" risk of bias. No dose–response analyses were carried out for breast cancer separately. Two studies (Chazelas et al., 2019; Debras et al., 2022b), both with overlapping data from the NutriNet-Santé cohort, were included in a meta-analysis for prostate and breast cancer risk (Yin et al., 2022) that showed no association with ASB consumption. [One additional meta-analysis also reported on ASB consumption and prostate cancer risk (Llaha et al., 2021), but because of the incomplete summary of studies and small body of evidence considered, it was not considered informative by the Working Group.] The most recent meta-analysis, by Pan et al. (2023), examined the combined association between ASBs and breast and prostate cancer risk, reporting no association between higher consumption of ASBs (per increase of 250 mL/day) versus none, and breast cancer risk (HR, 0.95; 95% CI, 0.80–1.12) or prostate cancer risk (HR, 0.93; 95% CI, 0.69–1.26). [The Working Group noted that in this meta-analysis (Pan et al., 2023) only three studies contributed to the breast cancer findings and two studies to prostate cancer findings, and they determined that this study did not contribute additional information.]

2.4 Cancers of the brain, thyroid, and uterus, and other solid cancers

See Table 2.4.

Seven cohort studies (including eight cohorts) reported on aspartame or ASBs and cancers of the brain (Lim et al., 2006; McCullough et al., 2022), uterus (Inoue-Choi et al., 2013; Hodge et al., 2018; McCullough et al., 2022), thyroid (Zamora-Ros et al., 2022), ovary (Hodge et al., 2018; McCullough et al., 2022), or lung (Malik et al., 2019; McCullough et al., 2022; You et al., 2022).

Five case–control studies reported on ASBs or tabletop sweeteners and cancers of the brain (Gurney et al., 1997; Cabaniols et al., 2011), uterus (Bosetti et al., 2009), thyroid (Singh et al., 2020), larynx (Gallus et al., 2007), and ovary (Gallus et al., 2007).

One cohort (Lim et al., 2006) and two case–control studies (Gurney et al., 1997; Cabaniols et al., 2011) described their exposure assessment as being specific to aspartame intake, indicating that it was derived from data on aspartame contained in or added to drinks and foods. [Although the methodology described in the two publications left some uncertainty about the actual specificity of the reported exposure to aspartame, which could have resulted in exposure misclassification, the Working Group considered that the assessment of aspartame consumption from ASBs and tabletop sweeteners in Lim et al. (2006) covered a time period that was highly relevant for aspartame exposure from this source in the USA.] All other studies reported on the exposure to artificially sweetened drinks and foods and the use of sweeteners in sachet and tablet form, but without any further specification of the type of artificial sweetener to which the study participants had been exposed.

All studies estimated average daily consumption of aspartame or artificial sweeteners as part of usual diet. In cohort studies, the estimate referred to the time of participant enrolment in the study, whereas in case–control studies, it referred to a period of time preceding cancer diagnoses for cancer cases and to an equivalent period for controls. One study additionally considered age at start of consumption (Gurney et al., 1997).

Although most of the studies were not specifically designed to investigate the possible carcinogenic effect of aspartame, all the studies included in this section measured artificial sweetener consumption in the general context of measuring usual diet and included questions to separately identify sugar-sweetened and artificially sweetened drinks.

Within the USA-based NIH-AARP cohort, Lim et al. (2006) analysed data from 473 984 study participants with baseline validated 124-item FFQ and reported the association between aspartame consumption and brain cancer risk. In this study, particular attention was paid to improve the measurement of diet by requesting that all participants fill in an FFQ that included specific questions on the consumption of beverages, from which aspartame content was later estimated. The 124-item FFQ included questions on three beverages that potentially contained aspartame: soda ("soft drinks, soda, pop"), fruit drinks ("such as Hi-C, lemonade, and Kool-Aid"), and iced tea; it had additional questions on the consumption of artificial sweeteners, including aspartame, in the form of sachets and tablets added to cups of coffee or hot tea. From these responses, a "daily consumption of aspartame" was computed, taking into account aspartame content, portion size, and intake frequency of each beverage. The aspartame content per 100 g beverage was estimated to be 50 mg for diet soda (i.e. 180 mg/12 oz can), 14.95 mg for diet fruit drink, 25.55 mg for diet iced tea, and 35 mg per tabletop packet added to each cup of coffee/hot tea, based on the information provided by the Nutrition Data System for Research of the

Table 2.4 Epidemiological studies on consumption of aspartame and cancers of the brain, thyroid, and uterus, and other solid cancers

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Lim et al. (2006) USA Enrolment, 1995–1996/ follow-up, through 2000 Cohort	473 984 (285 079 men and 188 905 women); NIH-AARP Diet and Health Study: AARP members aged 50–71 yr residing in the study area (California, Florida, Pennsylvania, New Jersey, North Carolina, Louisiana, Atlanta, Detroit) excluding those with prevalent cancer, invalid BMI data, outliers on reported energy intake, and proxy responders; 315 gliomas including 231 glioblastomas Exposure assessment method: FFQ at baseline, 5 yr of follow-up; consumption frequency of three types of soft drink (soda, fruit drinks, and iced tea) + frequency of consumption of diet vs regular versions + three portion sizes; use of tabletop sweetener packets; standard doses of aspartame assigned to beverages and packets	Brain (glioma), incidence	Aspartame intake (RR):			Age at entry, sex, ethnicity, BMI, history of diabetes	*Exposure assessment critique*: A key strength was the prospective and quantitative assessment of aspartame exposure from various ASBs and tabletop packets in a relevant period for aspartame (1995–1996 in the USA). Key limitations were the sequential assessment of the frequency of consumption firstly of soft drinks and then of diet/sugar-free versions with imprecise frequencies inducing inaccuracies; other sources of aspartame were not considered (although these were more limited); exposure misclassification due to older age at enrolment and a lack of data before study entry (aspartame in beverages introduced in the USA 10 yr before baseline).
			None	162	1		
			> 0 to < 100 mg/day	83	0.99 (0.75–1.29)		
			≥ 100 to < 200 mg/day	32	0.70 (0.48–1.03)		
			≥ 200 to < 400 mg/day	16	0.66 (0.39–1.12)		
			≥ 400 mg/day	22	0.73 (0.46–1.15)		
			Trend-test *P* value: 0.05				
		Brain (glioma: glioblastoma), incidence	Aspartame intake (RR):			Age at entry, sex, ethnicity, BMI, history of diabetes	
			None	NR	1		
			> 0 to < 100 mg/day	NR	NR		
			≥ 100 to < 200 mg/day	NR	NR		
			≥ 200 to < 400 mg/day	NR	NR		
			≥ 400 mg/day	NR	0.64 (0.37–1.10)		
			Trend-test *P* value: 0.05				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Lim et al. (2006) (cont.)							*Other strengths*: large prospective cohort; case ascertainment based on cancer registries with high (> 90%) completeness; large numbers of cases. *Other limitations*: likely bias from non-differential exposure misclassification.
Inoue-Choi et al. (2013) USA (Iowa Women's Health Study) Enrolment, 1986/follow-up, through 2010 Cohort	23 039; women aged 55–69 yr randomly selected from Iowa drivers' licence list who responded to a mailed questionnaire in 1986 (42% response rate); women with a history of cancer except nonmelanoma skin cancer, who were not postmenopausal, or who reported extreme dietary intake were excluded Exposure assessment method: semiquantitative FFQ at baseline in 1986 assessing sugar-free beverage consumption with standard serving size	Uterus/uterine corpus (type I endometrial cancer), incidence	Consumption of sugar-free beverages (HR):			Age, smoking status, physical activity, estrogen use, alcohol use, age at menarche, age at menopause, number of live births, history of diabetes, coffee intake, BMI	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption at a very relevant period for aspartame exposure from ASBs (1986 in the USA). Key limitations were that no other sources were considered (although these were more limited); there was only one assessment at baseline, not considering potential variations in consumption over up to 24 yr of follow-up; and uncertainty of aspartame content in ASBs after the mid-2000s.
			0 servings/wk	152	1		
			> 0–0.0002 servings/wk	36	0.69 (0.48–1.00)		
			0.0003–0.4 servings/wk	93	0.85 (0.65–1.11)		
			0.5–2.8 servings/wk	125	1.03 (0.80–1.32)		
			2.8–64.1 servings/wk	100	0.77 (0.59–1.01)		
			Trend-test *P* value: 0.31				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Inoue-Choi et al. (2013) (cont.)		Uterus/uterine corpus (type I endometrial cancer), incidence	Consumption of sugar-free beverages, excluding women with a history of diabetes (HR):			Age, smoking status, physical activity, estrogen use, alcohol use, age at menarche, age at menopause, number of live births, coffee intake, BMI	*Other strengths*: sample size, prospective design, cancer registry linkage for outcome assessment, adjustment for BMI and other relevant confounders. *Other limitations*: likely bias from non-differential exposure misclassification.
			0 servings/wk	NR	1		
			> 0–0.0002 servings/wk	NR	0.73 (0.50–1.06)		
			0.0003–0.4 servings/wk	NR	0.86 (0.65–1.13)		
			0.5–2.8 servings/wk	NR	0.99 (0.76–1.28)		
			2.8–64.1 servings/wk	NR	0.80 (0.60–1.06)		
			Trend-test *P* value: 0.35				
		Uterus/uterine corpus (type II endometrial cancer), incidence	Consumption of sugar-free beverages (HR):			Age, smoking status, physical activity, estrogen use, alcohol use, age at menarche, age at menopause, number of live births, history of diabetes, coffee intake, BMI	
			0 servings/wk	27	1		
			> 0–0.0002 servings/wk	8	0.78 (0.34–1.79)		
			0.0003–0.4 servings/wk	13	0.66 (0.33–1.30)		
			0.5–2.8 servings/wk	21	1.09 (0.61–1.95)		
			2.8–64.1 servings/wk	17	0.89 (0.48–1.68)		
			Trend-test *P* value: 0.95				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Inoue-Choi et al. (2013) (cont.)		Uterus/uterine corpus (type II endometrial cancer), incidence	Consumption of sugar-free beverages (serving/week), excluding women with a history of diabetes (HR):			Age, smoking status, physical activity, estrogen use, alcohol use, age at menarche, age at menopause, number of live births, coffee intake, BMI	
			0 servings/wk	NR	1		
			> 0–0.0002 servings/wk	NR	0.81 (0.35–1.86)		
			0.0003–0.4 servings/wk	NR	0.63 (0.31–1.28)		
			0.5–2.8 servings/wk	NR	1.16 (0.64–2.09)		
			2.8–64.1 servings/wk	NR	0.87 (0.45–1.69)		
			Trend-test *P* value: 0.97				
Hodge et al. (2018) Australia Enrolment, 1990–1994/ follow-up until 30 June 2013 Cohort	35 593 (21 492 women); MCCS – a prospective cohort study of men and women aged 40–69 yr at recruitment and free of cancer, angina, heart attack, or diabetes at baseline; participants with extreme baseline energy intake were excluded	Uterus/ uterine corpus: endometrium, incidence	Artificially sweetened soft drink consumption (HR):			Age, socioeconomic index, country of birth, alcohol intake, smoking status, physical activity, Mediterranean diet score, sugar-sweetened soft drink consumption, waist circumference	*Exposure assessment critique*: Key strengths were that it was a prospective study, that assessment was after aspartame introduction in diet soft drinks in Australia (1987), and that first half of follow-up largely overlapped with period of aspartame use in Australia.
			Never or < 1/mo	125	1		
			1–3/mo	9	0.58 (0.29–1.16)		
			1–6/wk	23	1.11 (0.70–1.77)		
			≥ 1/day	10	0.81 (0.42–1.55)		
			Continuous (per beverage/day)	167	0.92 (0.52–1.65)		
			Trend-test *P* value: 0.78				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hodge et al. (2018) (cont.)	Exposure assessment method: self-administered 121-item FFQ with separate questions on frequency of consumption in the past year of diet (artificially sweetened) soft drinks	Ovary, incidence	Artificially sweetened soft drink consumption (HR):			Age, socioeconomic index, country of birth, alcohol intake, smoking status, physical activity, Mediterranean diet score, sugar-sweetened soft drink consumption, waist circumference	Key limitations were the FFQ assessment with no specific estimate of aspartame exposure, ASBs used as a proxy, and exposure data at baseline only; small number of consumers. *Other strengths*: adjustment for key confounders, including a measure of obesity (waist circumference). *Other limitations*: likely bias from non-differential exposure misclassification given single baseline assessment and long follow-up.
			Never or < 1/mo	75	1		
			1–3/mo	20	0.80 (0.38–1.69)		
			1–6/wk	24	1.39 (0.83–2.34)		
			≥ 1/day	11	1.37 (0.72–2.61)		
			Continuous (per beverage/day)	130	1.51 (0.84–2.73)		
			Trend-test *P* value: 0.17				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Malik et al. (2019) USA Enrolment, 1976 (NHS), 1986 (HPFS/ follow-up, 1980–2014 (NHS), 1986–2014 (HPFS) Cohort	37 716 men and 80 647 women; female registered nurses aged 30–55 yr in the NHS and male health professionals aged 40–75 yr in the HPFS; excluding those with history of diabetes, cardiovascular disease, or cancer at baseline, or with implausible dietary intake Exposure assessment method: prospective assessment of ASB consumption through repeated FFQs between 1980–1986 and 2010	Lung, mortality	ASB intake (HR): < 1 serving/mo 1–4 servings/mo 2–6 servings/wk 1 to < 2 servings/day ≥ 2 servings/day Continuous (per serving/day) Trend-test *P* value: 0.20	 NR NR NR NR NR 	 1 0.96 (0.85–1.08) 0.85 (0.76–0.95) 0.93 (0.80–1.08) 0.92 (0.77–1.09) 0.98 (0.92–1.08)	Age, race, smoking, alcohol intake, postmenopausal hormone use (NHS), physical activity, family history of diabetes; family history of myocardial infarction, family history of cancer, multivitamin use, aspirin use, baseline history of hypertension and hypercholesterolemia, intake of whole grains, fruit, vegetables, or red and processed meat, total energy, BMI, SSB intake	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption from repeated diet assessments every 4 yr, the majority at a very relevant period for aspartame exposure from ASBs (the USA between the 1980s and 2010) potentially capturing lifetime exposure to aspartame. A key limitation was that other sources of aspartame were not considered (although these were more limited); and uncertainty of aspartame content in ASBs after the mid-2000s. *Other strengths*: large, prospective cohort with long follow-up; detailed and validated assessments were updated at each survey round to account for changes in intake over time.

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Malik et al. (2019) (cont.)							*Other limitations*: bias from non-differential misclassifications of exposure to aspartame likely; stratified numbers of deaths not provided for specific cancer sites.
McCullough et al. (2022) USA Enrolment, 1982/follow-up, through 2016 (median, 27.7 yr) Cohort	934 777 (416 313 men, 518 464 women); CPS-II prospective cohort; adults aged ≥ 28 yr; excluded participants with personal history at baseline of diabetes or cancer other than nonmelanoma skin cancer, men aged > 90 yr or women aged > 95 yr at enrolment, and those reporting only prior but not current consumption of either SSBs or ASBs; uterine/ovarian cancer results exclude women with a history of hysterectomy, uterine surgery, surgically induced menopause, or oophorectomy (ovarian cancer only)	Uterus/uterine corpus, mortality	ASB consumption (HR): Never < 1 drink/day 1 drink/day ≥ 2 drinks/day Continuous (per drink/day) Trend-test *P* value: 0.049	 NR NR NR NR 1693	 1 1.04 (0.90–1.19) 1.07 (0.90–1.26) 1.18 (1.00–1.40) 1.04 (0.99–1.09)	Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, parity, age at menarche, estrogen use, oral contraceptive use, age at first live birth, menopausal status	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption. Key limitations were that only one dietary assessment was carried out at baseline in 1982 that was before the use of aspartame in ASBs, hence the relevance regarding aspartame exposure depends on the stability of ASB consumption over up to 34 yr of follow-up, but such information was not directly available; no other sources were considered (although these were more limited); and uncertainty of aspartame content in ASBs after the mid-2000s.

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)	Exposure assessment method: questionnaire; exposure to ASBs assessed in 1982 through a question about the number of drinks/day of diet soda or ice teas (one pooled item) and potential changes over the past 10 yr; no specific assessment of aspartame content in ASBs	Uterus/uterine corpus, mortality	ASB consumption, BMI-adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, parity, age at menarche, estrogen use, oral contraceptive use, age at first live birth, menopausal status, BMI	Other information: exclusion of participants who reported only prior but not current consumption of either SSBs or ASBs at baseline. *Other strengths*: large, prospective cohort with long follow-up; ability to examine multiple cancer types, stratify by sex or BMI, and limit to never-smokers; comprehensive adjustment for confounders, including SSB consumption. *Other limitations*: likely bias from non-differential exposure misclassification.
			Never	NR	1		
			< 1 drink/day	NR	0.93 (0.81–1.07)		
			1 drink/day	NR	0.95 (0.80–1.13)		
			≥ 2 drinks/day	NR	1.01 (0.85–1.21)		
			Continuous (per drink/day)	1693	0.99 (0.95–1.05)		
			Trend-test *P* value: 0.878				
		Uterus/uterine corpus, mortality	ASB consumption, never-smokers (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, parity, age at menarche, estrogen use, oral contraceptive use, age at first live birth, menopausal status	
			Never	NR	1		
			< 1 drink/day	NR	1.14 (0.96–1.36)		
			1 drink/day	NR	1.07 (0.85–1.33)		
			≥ 2 drinks/day	NR	1.15 (0.91–1.45)		
			Continuous (per drink/day)	1025	1.02 (0.95–1.05)		
			Trend-test *P* value: 0.167				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Uterus/uterine corpus, mortality	ASB consumption, never-smokers, BMI-adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, parity, age at menarche, estrogen use, oral contraceptive use, age at first live birth, menopausal status, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.03 (0.86–1.22)		
			1 drink/day	NR	0.95 (0.76–1.18)		
			≥ 2 drinks/day	NR	0.98 (0.77–1.24)		
			Continuous (per drink/day)	1025	0.97 (0.91–1.04)		
			Trend-test *P* value: 0.781				
		Uterus/uterine corpus, mortality	ASB consumption, BMI, 18.5 to < 25 kg/m^2 (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.89 (0.72–1.11)		
			1 drink/day	NR	0.93 (0.71–1.21)		
			≥ 2 drinks/day	NR	1.03 (0.78–1.37)		
			Continuous (per drink/day)	762	1.00 (0.93–1.09)		
			Trend-test *P* value: 0.883				
		Uterus/uterine corpus, mortality	ASB consumption, BMI, 25 to < 30 kg/m^2 (HR):				
			Never	NR	1		
			< 1 drink/day	NR	1.07 (0.85–1.36)		
			1 drink/day	NR	0.97 (0.72–1.32)		
			≥ 2 drinks/day	NR	1.19 (0.89–1.59)		
			Continuous (per drink/day)	520	1.00 (0.92–1.1)		
			Trend-test *P* value: 0.316				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Uterus/uterine corpus, mortality	ASB consumption, BMI, ≥ 30 kg/m² (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, parity, age at menarche, estrogen use, oral contraceptive use, age at first live birth, menopausal status, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.84 (0.61–1.14)		
			1 drink/day	NR	0.91 (0.64–1.29)		
			≥ 2 drinks/day	NR	0.78 (0.54–1.14)		
			Continuous (per drink/day)	325	0.96 (0.86–1.07)		
			Trend-test *P* value: 0.183				
		Brain, mortality	ASB consumption, BMI-adjusted (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.06 (0.96–1.18)		
			1 drink/day	NR	0.99 (0.87–1.12)		
			≥ 2 drinks/day	NR	0.97 (0.84–1.11)		
			Continuous (per drink/day)	3402	0.98 (0.95–1.02)		
			Trend-test *P* value: 0.737				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Brain, mortality	ASB consumption, men, BMI-adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.02 (0.86–1.21)		
			1 drink/day	NR	1.06 (0.87–1.29)		
			≥ 2 drinks/day	NR	0.96 (0.77–1.18)		
			Continuous (per drink/day)	1772	0.99 (0.93–1.05)		
			Trend-test *P* value: 0.928				
		Brain, mortality	ASB consumption, women, BMI-adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	1.10 (0.96–1.26)		
			1 drink/day	NR	0.95 (0.79–1.13)		
			≥ 2 drinks/day	NR	0.98 (0.82–1.18)		
			Continuous (per drink/day)	1630	0.98 (0.93–1.04)		
			Trend-test *P* value: 0.805				
		Lung, mortality	ASB consumption, BMI-adjusted (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.92 (0.88–0.95)		
			1 drink/day	NR	0.91 (0.86–0.95)		
			≥ 2 drinks/day	NR	0.90 (0.86–0.95)		
			Continuous (per drink/day)	34 381	0.97 (0.96–0.99)		
			Trend-test *P* value: < 0.0001				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Lung, mortality	ASB consumption, men, BMI-adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.93 (0.88–0.99)		
			1 drink/day	NR	0.91 (0.85–0.98)		
			≥ 2 drinks/day	NR	0.93 (0.87–1.00)		
			Continuous (per drink/day)	20 121	0.98 (0.96–1.00)		
			Trend-test *P* value: 0.002				
		Lung, mortality	ASB consumption, women, BMI-adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.89 (0.85–0.94)		
			1 drink/day	NR	0.88 (0.83–0.94)		
			≥ 2 drinks/day	NR	0.84 (0.79–0.90)		
			Continuous (per drink/day)	14 260	0.95 (0.94–0.97)		
			Trend-test *P* value: < 0.0001				
		Lung, mortality	ASB consumption, never-smokers, BMI-adjusted (HR):			Age, sex, race/ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.92 (0.82–1.03)		
			1 drink/day	NR	1.01 (0.89–1.16)		
			≥ 2 drinks/day	NR	0.99 (0.86–1.14)		
			Continuous (per drink/day)	3546	1.00 (0.96–1.04)		
			Trend-test *P* value: 0.775				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Lung, mortality	ASB consumption, men, never-smokers, BMI-adjusted (HR):			Age, race/ethnicity, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.95 (0.78–1.16)		
			1 drink/day	NR	1.06 (0.85–1.31)		
			≥ 2 drinks/day	NR	1.08 (0.86–1.36)		
			Continuous (per drink/day)	1545	1.02 (0.96–1.09)		
			Trend-test *P* value: 0.519				
		Lung, mortality	ASB consumption, women, never-smokers, BMI-adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.89 (0.78–1.02)		
			1 drink/day	NR	0.98 (0.83–1.15)		
			≥ 2 drinks/day	NR	0.92 (0.76–1.11)		
			Continuous (per drink/day)	2001	0.98 (0.93–1.04)		
			Trend-test *P* value: 0.276				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Ovary, mortality	ASB consumption, women, BMI-adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, parity, age at menarche, estrogen use, oral contraceptive use, age at first live birth, menopausal status, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.05 (0.95–1.16)		
			1 drink/day	NR	1.16 (1.03–1.30)		
			≥ 2 drinks/day	NR	1.01 (0.89–1.15)		
			Continuous (per drink/day)	3225	1.00 (0.97–1.04)		
			Trend-test *P* value: 0.284				
You et al. (2022) USA Enrolment, 1993–2001/ follow-up through 2009 (median, 11.3 yr) Cohort	92 997; PLCO cancer screening trial participants, men and women aged 55–74 yr in 10 study centres; participants with history of cancer or diabetes were excluded Exposure assessment method: diet history questionnaire: FFQ with 156 items	Lung, incidence	Type of soft drink consumption (HR):			Age, sex, race, study centre, arm, total energy intake, alcohol consumption, smoking status, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer	*Exposure assessment critique*: A key strength was the timing of exposure consistent for aspartame as major AS in beverages; ASBs were major source of aspartame in this country and time frame.
			No soft drink consumption	173	1		
			Regular only	657	0.87 (0.73–1.03)		
			Diet only	487	0.89 (0.75–1.07)		
			Both	200	0.79 (0.64–0.97)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
You et al. (2022) (cont.)		Lung, incidence	Type of soft drink consumption, men (HR):			Age, race, study centre, arm, total energy intake, alcohol consumption, smoking status, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer	Key limitations were that there was no specific estimate of aspartame exposure, ASBs were used as a proxy; only consumption vs non-consumption was considered (not dose), and exposure data were reported at baseline only. Other information: PLCO is a trial of lung cancer screening but in this study was considered a prospective cohort; allocation to control or intervention arms was not associated with soft drink choice. *Other strengths*: prospective analysis. *Other limitations*: likely bias from non-differential exposure misclassification.
			No soft drink consumption	77	1		
			Regular only	431	0.98 (0.76–1.26)		
			Diet only	234	1.08 (0.82–1.42)		
			Both	140	0.96 (0.72–1.28)		
		Lung, incidence	Type of soft drink consumption, women (HR):			Age, race, study centre, arm, total energy intake, alcohol consumption, smoking status, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer, estrogen use	
			No soft drink consumption	[96]	1		
			Regular only	[226]	0.80 (0.62–1.02)		
			Diet only	[253]	0.78 (0.61–0.99)		
			Both	[60]	0.62 (0.45–0.87)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
You et al. (2022) (cont.)		Lung, incidence	Type of soft drink consumption, never-smokers (HR):			Age, sex, race, study centre, arm, total energy intake, alcohol consumption, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer	
			No soft drink consumption	17	1		
			Regular only	47	0.79 (0.45–1.40)		
			Diet only	37	0.65 (0.36–1.18)		
			Both	24	0.85 (0.45–1.61)		
		Lung, incidence	Type of soft drink consumption, ever/current smokers (HR):				
			No soft drink consumption	156	1		
			Regular only	610	0.90 (0.75–1.08)		
			Diet only	450	0.82 (0.68–0.99)		
			Both	176	0.71 (0.57–0.89)		
		Lung (small cell/oat cell), incidence	Type of soft drink consumption (HR):			Age, sex, race, study centre, arm, total energy intake, alcohol consumption, smoking status, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer	
			No soft drink consumption	26	1		
			Regular only	91	0.78 (0.49–1.22)		
			Diet only	72	0.91 (0.57–1.46)		
			Both	25	0.71 (0.40–1.24)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Zamora-Ros et al. (2022) Europe Enrolment, 1992–2000/ follow-up, mean ± SD, 13.9 ± 4.0 yr Cohort	450 064; EPIC study participants, men and women, mostly aged 35–70 yr, from 9 European countries (Denmark, France, Germany, Italy, Netherlands, Norway, Spain, Sweden, and the United Kingdom); excluding participants with prevalent cancer other than nonmelanoma skin cancer at baseline or with extreme energy intake/expenditure; those with missing date of follow-up or lack information on lifestyle factors (Greece did not provided data for this study); data on ASBs was not available for participants from Florence, Ragusa, Turin, Umeå, and Varese; overall 712 thyroid cancers (573 papillary, 108 follicular, 31 otherwise specified)	Thyroid (differentiated: papillary, follicular, otherwise), incidence	ASB consumption (HR): Non-consumers (0 mL/day) 1st tertile (> 0–5.8 mL/day) 2nd tertile (5.9–42.9 mL/day) 3rd tertile (43.0–3389.5 mL/day) Continuous (per 100 mL/day) Trend-test *P* value: 0.26	 392 29 32 42 495	 1 0.88 (0.53–1.46) 0.83 (0.55–1.24) 1.16 (0.80–1.69) 1.00 (0.91–1.11)	Sex, centre, age at recruitment, BMI, smoking status, physical activity, educational level, alcohol and energy intake, and in women also for menopausal status, oral contraceptive use, and infertility problems	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption (several types of beverages) in several western European countries at a period relevant for aspartame exposure (between 1991 and 2000). Key limitations were that no other sources of aspartame were considered; uncertainty regarding the aspartame content in ASBs in every country; and there was only one assessment at baseline. Other information: context of low ASB consumption in middle-aged adults. *Other strengths*: prospective large study. *Other limitations*: likely bias from non-differential exposure misclassification.

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Zamora-Ros et al. (2022) (cont.)	Exposure assessment method: questionnaire; exposure to artificially sweetened soft drinks overall assessed once at baseline through country-specific validated tools (mainly FFQ) covering the usual diet over the past year; no specific assessment of aspartame or AS content of the artificially sweetened soft drinks	Thyroid (papillary), incidence	ASB consumption (HR):			Sex, centre, age at recruitment, BMI, smoking status, physical activity, educational level, alcohol and energy intake, and in women also for menopausal status, oral contraceptive use, and infertility problems	
			Non-consumers (0 mL/day)	385	1		
			1st tertile (> 0–6.6 mL/day)	22	0.95 (0.54–1.67)		
			2nd tertile (6.7–42.9 mL/day)	20	0.73 (0.44–1.19)		
			3rd tertile (43.0–3389.5 mL/day)	42	1.51 (1.04–2.19)		
			Continuous (per 100 mL/day)	469	1.05 (0.95–1.15)		
			Trend-test *P* value: 0.16				
Gurney et al. (1997) USA (Los Angeles, San Francisco) 1984–1991 Case–control	Cases: 56; age ≥ 19 yr; born in 1981 or after, primary brain tumour diagnosed in 1984–1991 Controls: 94; age ≥19 yr; born in 1981 or after, control subjects were recruited using random-digit dialling, frequency-matched on age at diagnosis, year of birth, sex, and study site	Brain, incidence	Consumption of aspartame in any food or drink (OR):			Sex, age at diagnosis, study site, birth year	*Exposure assessment critique*: A key strength was the lifetime exposure of children captured in a relevant period for aspartame (1981–1991 in the USA). Key limitations were the retrospective assessment in a case–control study; only frequency and duration of exposure were assessed (no dose); and unclear sources of aspartame.
			No consumption	39	1		
			Any consumption	17	1.1 (0.5–2.6)		
		Brain, incidence	Age at first consumption of aspartame in any food or drink (OR):			Sex, age at diagnosis, study site, birth year	
			No consumption	NR	1		
			< 3 yr	7	1.0 (0.3–3.1)		
			3–7 yr	10	1.2 (0.4–3.6)		
		Brain, incidence	Duration of consumption of aspartame in any food or drink (OR):			Sex, age at diagnosis, study site, birth year	
			No consumption	NR	1		
			< 2 yr	9	1.2 (0.4–3.3)		
			≥ 2 yr	8	1.1 (0.3–3.4)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gurney et al. (1997) (cont.)	Exposure assessment method: questionnaire; retrospective assessment through in-person interviews of mothers regarding the period and frequency of consumption by their children/during their pregnancy of several sources of aspartame	Brain, incidence	Frequency of consumption of aspartame in any food or drink (OR):			Sex, age at diagnosis, study site, birth year	*Other strengths*: data from food questionnaire; dose–response analysis; in utero exposure. *Other limitations*: small sample size; potential for bias from differential exposure misclassification.
			No consumption	NR	1		
			< 1 time/wk	7	1.6 (0.5–5.2)		
			≥ 1 time/wk	10	0.9 (0.3–2.4)		
		Brain, incidence	Consumption of aspartame in diet drinks (OR):				
			No consumption	47	1		
			Any consumption	9	0.9 (0.3–2.4)		
		Brain, incidence	Age at first consumption of aspartame in diet drinks (OR):				
			No consumption	NR	1		
			< 3 yr	4	0.8 (0.2–3.1)		
			≥ 3 yr	5	1.0 (0.3–3.4)		
		Brain, incidence	Duration of consumption of aspartame in diet drinks (OR):				
			No consumption	NR	1		
			< 2 yr	4	0.8 (0.2–3.1)		
			≥ 2 yr	5	0.9 (0.3–3.4)		
		Brain, incidence	Frequency of consumption of aspartame in diet drinks (OR):				
			No consumption	NR	1		
			< 1 time/wk	5	1.2 (0.3–4.5)		
			≥ 1 time/wk	4	0.6 (0.2–2.3)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gurney et al. (1997) (cont.)		Brain, incidence	Consumption of aspartame in any food or drink during pregnancy or breastfeeding (effect in offspring, OR):			Sex, age at diagnosis, study site, birth year	
			No consumption	40	1		
			Any consumption	9	0.7 (0.3–1.7)		
		Brain, incidence	Consumption of aspartame in any food or drink during pregnancy (effect in offspring, OR):				
			No consumption	NR	1		
			Any consumption	7	0.6 (0.2–1.7)		
		Brain, incidence	Consumption of aspartame in any food or drink during breastfeeding (effect in offspring, OR):				
			No consumption	NR	1		
			Any consumption	5	0.7 (0.2–2.0)		
		Brain, incidence	Consumption of aspartame in diet drinks during pregnancy or breastfeeding (effect in offspring, OR):				
			No consumption	44	1		
			Any consumption	5	0.9 (0.3–2.8)		
		Brain, incidence	Consumption of aspartame in diet drinks during pregnancy (effect in offspring, OR):				
			No consumption	NR	1		
			Any consumption	3	0.7 (0.2–2.7)		
		Brain, incidence	Consumption of aspartame in diet drinks during breastfeeding (effect in offspring, OR):				
			No consumption	NR	1		
			Any consumption	4	1.1 (0.3–4.0)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gallus et al. (2007) Italy 1991–2004 Case–control	Cases: 460 (larynx), 1031 (ovary); histologically confirmed cancers of larynx (415 men, 45 women; median age, 61 yr), or ovary (1031; median age, 55 yr); > 95% participation Controls: 1088 (larynx), 2411 (ovary); controls admitted to same network of general and teaching hospitals as cases for acute, non-neoplastic disorders; total of 7028 patients (3301 men and 3727 women; 4838 included in more than one study); 24% trauma, 31% other nontraumatic orthopaedic conditions, 17% acute surgical disorders, 28% miscellaneous other diseases; > 95% participation	Larynx, incidence	Consumption of ASs other than saccharin (OR):			Age, sex, study centre, education, tobacco smoking, alcohol drinking, BMI, total energy intake, consumption of hot beverages	*Exposure assessment critique*: Key limitations were that there was no specific assessment of aspartame, aspartame was considered as "other sweeteners" but with unclear actual contribution; only one source considered (tabletop sweeteners); retrospective assessment in a case–control study. Other information: according to the authors, limited consumption of sources of sweeteners (including ASBs) in the study population (middle-aged adults in Italy between 1991 and 2004). *Other strengths*: high response rates (< 5% refusals) for cases and controls reduce the potential for selection bias; large sample size with large case numbers for rarer cancers; control for key potential confounders, including BMI.
			Non-consumers	439	1		
			> 0 sachets or tablets/day	21	1.62 (0.84–3.14)		
		Ovary, incidence	Consumption of ASs other than saccharin (OR):			Age, study centre, education, tobacco smoking, alcohol drinking, BMI, total energy intake, consumption of hot beverages, parity, menopausal status/ age at menopause	
			Non-consumers	958	1		
			> 0 sachets or tablets/day	73	0.75 (0.56–1.00)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gallus et al. (2007) (cont.)	Exposure assessment method: questionnaire; assessment of use of tabletop sweeteners containing either saccharin or other sweeteners as sachets or tablets per week in the 2 yr before cancer diagnosis						*Other limitations*: low AS use; potential for recall bias and reverse causation.
Bosetti et al. (2009) Italy 1997–2007 Case–control	Cases: 454; hospital-based, greater Milan area; cases had histologically confirmed endometrial cancer (median age, 60 yr) Controls: 908; controls selected from same network of general and teaching hospitals as cases for acute, non-neoplastic disorders, frequency-matched on age, sex, and study centre; 25% traumas, 32% nontraumatic orthopaedic conditions, 15% acute surgical disorders, 27% miscellaneous other diseases; > 95% control participation	Uterus/ uterine corpus (endometrium), incidence	Consumption of low-calorie sweeteners other than saccharin (OR): Non-users Users	 394 58	 1 1.37 (0.96–1.95)	Age, study centre	*Exposure assessment critique*: Key limitations were that there was no specific assessment of aspartame, aspartame was included "other sweeteners" but with unclear actual contribution; only one source considered (tabletop sweeteners); retrospective assessment in a case–control study (potential for differential misclassification). Other information: according to the authors, limited consumption of sources of sweeteners (including ASBs) in the study population (middle-aged adults in Italy between 1991 and 2007).
		Uterus/ uterine corpus (endometrium), incidence	Consumption of low-calorie sweeteners other than saccharin (OR): Non-users Users	 394 58	 1 1.07 (0.71–1.61)	Age, study centre, year of interview, education, BMI, tobacco smoking, history of diabetes, consumption of hot beverages, total energy intake	

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bosetti et al. (2009) (cont.)	Exposure assessment method: questionnaire; assessment of use of tabletop sweeteners containing either saccharin or other sweeteners as several sachets or tablets per week in the 2 yr before cancer diagnosis; exposure to "other sweeteners" considered as ever users vs non-users only						*Other strengths*: > 95% participation rate among controls; adjustment for several key confounders, including BMI and diabetes status. *Other limitations*: exposure assessment limited to users vs non-users which increases the potential for exposure misclassification; low frequency of consumers; potential for reverse causation, recall bias.

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Cabaniols et al. (2011) France (Marseille and Toulon) 2005 Case–control	Cases: 122; recruitment of consecutive new cases of primary malignant brain (glioma grades II–IV) tumour in patients aged > 18 yr Controls: 122; controls, matched on age (± 5 yr) and sex, randomly selected from patients hospitalized for reasons unrelated to cancer in the neurosurgery department Exposure assessment method: self-administered questionnaire with question on aspartame consumption frequency for the past 5 yr	Brain (glioma, grades II–IV), incidence	Aspartame consumption (OR): < 1 time/wk ≥ 1 time/wk	 92 30	 1 1.02 (0.57–1.85)	Age, sex	*Exposure assessment critique*: Key limitations were the retrospective recall using an unknown question on frequency of aspartame use (question and validity not reported); unclear sources considered. *Other limitations*: small sample size, recall bias; study methodology not well described.

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Singh et al. (2020) USA 2004–2014 Case–control	Cases: 50; hospital-based study (Mount Sinai, Queens hospital centre, Elmhurst hospital centre); cases were patients undergoing total thyroidectomy for well-differentiated thyroid cancer; all were aged > 18 yr Controls: 50; controls (aged > 18 yr) chosen among patients diagnosed with thyroid nodules through needle aspiration; matching and control recruitment not mentioned Exposure assessment method: telephone based, self-report questionnaire on use of ASs before diagnosis	Thyroid (well-differentiated), incidence	AS consumption (OR): None Any consumption	 12 38	 1 10.1 (4.01–25.10)	None	*Exposure assessment critique*: Key limitations were the retrospective recall using a questionnaire. no specific assessment of aspartame, only total ASs and only in beverages, multiple sweeteners in products, with aspartame a major contributor but contribution could not be quantified). *Other limitations*: small sample size, the exposure period was unclear with respect to the diagnosis, unclear control selection, control for confounders not reported, control group participants had thyroid nodules; study methodology not well described; statistical methods appear to be non-standard.

AS, artificial sweetener; ASB, artificially sweetened beverage; BMI, body mass index; CI, confidence interval; CPS-II, Cancer Prevention Study-II; EPIC, European Prospective Investigation into Cancer and Nutrition; FDA, Food and Drug Administration; FFQ, food frequency questionnaire; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; MCCS, Melbourne Collaborative Cohort Study; mo, month(s); NHS, Nurses' Health Study; NIH-AARP, National Institutes of Health-American Association of Retired Persons; NR, not reported; OR, odds ratio; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer screening trial; SD, standard deviation; SSB, sugar-sweetened beverage; vs, versus; wk, week(s); yr, year(s).

University of Minnesota (Nutrition Coordinating Center, 2023). The multivariable data analyses on incident cancer cases accumulated during follow-up (1995–1996 to 2000) found that aspartame was not positively associated with glioma overall (RR for ≥ 400 mg/day versus none, 0.73; 95% CI, 0.46–1.15; *P* for trend, 0.05) or with glioblastoma, the largest subtype of brain tumours (RR for ≥ 400 mg/day versus none, 0.64; 95% CI, 0.37–1.10; *P* for trend, 0.05; 231 cases). The associations were null when the lowest level of aspartame intake was set as the reference category (data not reported in Table 2.4). [The Working Group noted that, although the researchers applied exposure assessment methods aimed at more specifically estimating consumption of aspartame (separately from that of other artificial sweeteners) and the period of assessment coincided with a relevant period of aspartame exposure from ASBs in the USA, aspartame in beverages in the USA was introduced 10 years before the baseline questionnaire administration; therefore, a certain degree of exposure misclassification may have been introduced by the lack of data before study entry periods. As in other prospective studies, participants were potentially exposed to various and changing types of artificial sweetener during adulthood in the 1980s to 2000s, depending on the evolution of drinks and food products on the market, and these variations were not measured. Therefore, some random measurement error leading to underestimation of a potential association between aspartame and cancer risk was likely. Additionally, this study was limited by a rather short follow-up (5 years).]

The Iowa Women's Health Study Cohort included 23 039 women aged 55–69 years in 1986, who were randomly selected from driver's licence lists in Iowa, USA, and who responded to a mailed questionnaire (Inoue-Choi et al., 2013). During follow-up (1986–2010), 592 cases of endometrial cancer (type I, 506; type II, 86) were diagnosed. The article reported the BMI-adjusted association between quintiles of sugar-free beverage consumption and risk of endometrial cancer (type I and II separately), before and after exclusion of participants with a history of diabetes. There was no association for endometrial cancer risk in all women or in women without history of diabetes. [The Working Group noted that there was no specific assessment of aspartame, since only ASB consumption was measured. The assessment of ASB consumption (1986) covered a relevant period in the USA for the use of aspartame in beverages. In addition, ASB consumption may have varied at the individual level over time, thus the study was limited by the single baseline assessment. All these uncertainties in exposure assessment were likely to result in non-differential misclassification of aspartame exposure.]

The MCCS included 35 593 participants (14 101 men and 21 492 women) aged 40–69 years (Hodge et al., 2018). The baseline diet questionnaire, with 121 drinks and food items, included questions about consumption of diet soft drinks (artificially sweetened) by number of times per day or per week. During follow-up (1990–1994 to 2013), 167 cancers of the endometrium and 130 cancers of the ovary were identified. No association was found between frequency of consumption of artificially sweetened soft drinks and risk of endometrial cancer (HR for ≥ 1/day versus < 1/month, 0.81; 95% CI, 0.42–1.55). Risk of ovarian cancer was positively associated with artificially sweetened soft drink consumption; however, with a wide confidence interval (HR for ≥ 1/day versus < 1/month, 1.37; 95% CI, 0.72–2.61). In the combined cohort of the NHS (follow-up, 1980–2014) and the HPFS (follow-up, 1986–2014), there was no association between ASB consumption and lung cancer mortality (HR per 1 serving/day increment, 0.98; 95% CI, 0.92–1.08) (Malik et al., 2019). [The Working Group noted that the study provided no specific estimate of aspartame exposure. Only ASB consumption was investigated, which was considered by the Working Group as a proxy for aspartame. Malik et al. (2019) assessed ASB exposure during a time

period that was relevant for aspartame being used as the main artificial sweetener in the USA, although there was more uncertainty about the use of aspartame as the only artificial sweetener in beverages between the mid-2000s and 2010. The dietary questionnaire collected information on consumption frequency only, without portion sizes. Therefore, non-differential misclassification of the exposure to aspartame was likely.]

The CPS-II cohort study included 416 313 men and 518 464 women aged ≥ 28 years at baseline (follow-up, 1982–2016) (McCullough et al., 2022). The baseline questionnaire included a grid that asked how many cups, glasses, or drinks of ASBs were usually drunk per day and for how many years, with write-in reporting by frequency and duration. Diet soda and diet iced teas were considered as ASB, whereas "non-diet colas" and "other non-diet soft drinks" were considered sugar-sweetened beverages. The CPS-II cohort study used cancer mortality as the end-point. The multivariable-adjusted analysis additionally adjusting for BMI found no association between intake of artificial sweeteners and risk of dying from cancer of the brain (HR for ≥ 2 drinks/day versus none, 0.97; 95% CI, 0.84–1.11) or the lung (for the entire cohort, HR per drink of ASB consumed per day, 0.97; 95% CI, 0.96–0.99; and for never-smokers, HR per drink per day, 1.00; 95% CI, 0.96–1.04). The cohort also included 1693 deaths from cancer of the uterus and 3225 deaths from cancer of the ovary. No association was found with the consumption of ASBs after adjustment for BMI. For uterine cancer, the HR for ≥ 2 drinks/day versus never was 1.18 (95% CI, 1.00–1.40; *P* for trend, 0.049) in the model without adjustment for BMI, and 1.01 (95% CI, 0.85–1.21; *P* for trend, 0.878) in the model controlling for BMI. The BMI-adjusted overall relative risk expressed per drink per day was 0.99 (95% CI, 0.95–1.05) for endometrial cancer and 1.00 (95% CI, 0.97–1.04) for ovarian cancer.

[The Working Group noted that the exposure assessment took place in 1982, 1 year before the approval of aspartame use in food or beverages in the USA. The Working Group considered that this study was relevant for aspartame exposure, given the published data on the participants of the CPS-II cohort who were also in the CPS-II nutrition cohort, which showed consistent relative ranking of consumption of ASBs over 17 years of follow-up (McCullough et al., 2014). However, there was large potential for random error in the exposure measurement and in confounders, with only one exposure measurement at baseline and a median follow-up of 27.7 years.]

The PLCO cohort (follow-up, 1993–2001 to 2009) reported on diet soft drink consumption without further specification of the type of artificial sweetener or type of drink, using an FFQ containing 156 items (You et al., 2022). The overall analyses (including both men and women, and both smokers and never-smokers, adjusted for smoking, sex, and other potential confounders) found a hazard ratio of 0.89 (95% CI, 0.75–1.07) for consuming only diet soft drink compared with no consumption of any soft drink. [The Working Group noted that the relative risk for consumption of only regular soft drinks versus no consumption of any soft drinks was very similar (RR, 0.87; 95% CI, 0.73–1.03).] Separate analyses restricted to never-smokers, to men only, and to women only, found similar estimates of relative risk, all close to or below 1 (see Table 2.4), suggesting no association between lung cancer risk and the consumption of diet soft drinks compared with no soft drink consumption. [The Working Group noted that the assessment of consumption of ASBs in this study was consistent with a period when aspartame was used as the major sweetener in ASBs in the USA; however, the lack of information on intake before baseline may result in potential non-differential exposure misclassification. The lack of assessment of consumption of drinks and foods potentially containing aspartame and other artificial

sweeteners may also result in non-differential exposure misclassification.]

The EPIC study examined the association between artificial sweeteners and thyroid cancer (Zamora-Ros et al., 2022). The investigation included 450 064 adults from 10 European countries. Dietary intake was assessed using validated country-specific dietary questionnaires. After a mean follow-up time of 14 years, 712 first primary differentiated thyroid cancers were diagnosed, but the analysis on ASBs included 495 thyroid cancer cases, because data on ASB consumption were not available in some centres. The main purpose of this study was to analyse the association between dietary patterns and thyroid cancer risk. In addition, results were presented for specific food groups, including sugar-sweetened beverages and ASBs. Overall, the study found little evidence of an association between consumption of ASBs and thyroid cancer risk. The hazard ratio comparing the higher tertile of consumption of ASBs with no consumption was 1.16 (95% CI, 0.80–1.69).

[The Working Group noted that the study measured ASBs, but there was no specific methodology aimed at the assessment of aspartame consumption. However, despite uncertainty regarding the aspartame content of artificially sweetened soft drinks, the study was conducted during a time period that was relevant for aspartame exposure from ASBs. Potential variations in the aspartame content of available artificially sweetened soft drinks over time was not measured, since the assessment was performed at baseline only; therefore, there was potential for non-differential misclassification of aspartame exposure.]

One case–control study from USA investigated the risk of brain tumours in childhood in relation to consumption of aspartame during infancy and childhood (Gurney et al., 1997). Information was collected from the biological mothers of the cases and the matched controls. The study also investigated the association between consumption of aspartame during pregnancy and subsequent risk of brain tumours in the offspring. Overall, the results indicated no association between aspartame consumption and brain tumour risk in childhood (OR, 1.1; 95% CI, 0.5–2.6), or between aspartame consumption during pregnancy and brain tumour risk in the offspring (OR, 0.7; 95% CI, 0.3–1.7). [The Working Group noted that only information on frequency of consumption and duration of exposure was ascertained, but portion sizes were not collected. Exposure referred to the period between 1981 and 1991 in the USA, potentially capturing lifetime exposure to aspartame in the USA. However, the lack of specificity regarding food source and the retrospective assessment may have resulted in measurement errors. Therefore, there was potential for inadequate extrapolation to exposure to aspartame, and possible differential misclassification because of retrospective assessment in a case–control study.]

A case–control study of 460 cases of laryngeal cancer and 1031 cases of ovarian cancer were conducted in 1991–2004 in Italy, with controls selected from the same network of general and teaching hospitals (Gallus et al., 2007). After adjustment for age, sex, study centre, education, tobacco smoking, alcohol drinking, BMI, total energy intake, and consumption of hot beverages, the study found a positive but imprecise association between the consumption of artificial sweeteners other than saccharin and laryngeal cancer risk (OR, 1.62; 95% CI, 0.84–3.14), and an inverse association with ovarian cancer risk (OR, 0.75; 95% CI, 0.56–1.00) in models further adjusted for parity, menopausal status, and age at menopause).

Another case–control study was conducted in Italy (Bosetti et al., 2009), similarly to the hospital-based case–control research programme described above (Gallus et al., 2007). The study included 454 cases of histologically confirmed endometrial cancer and 908 controls with acute, non-neoplastic disorders and selected from same

network of general and teaching hospitals as the cases. Statistical analyses adjusting for age, study centre, year of interview, education, BMI, tobacco smoking, history of diabetes, consumption of hot beverages, and total energy intake found no association between use of artificial sweeteners other than saccharin and endometrial cancer risk (OR, 1.07; 95% CI, 0.71–1.61). [The Working Group noted that for both studies, Bosetti et al. (2009) and Gallus et al. (2007), aspartame intake was estimated on the basis of use of tabletop sweetener sachets or tablets other than saccharin. The assumption that these sweeteners were actually aspartame was based on data from teenagers in 2000 (Arcella et al., 2004) and 1996 (Leclercq et al., 1999) that may not be valid, since the consumption of foods by adults and teenagers may differ. In addition, it was not clear whether aspartame was widely used in Italy over the whole data collection period, and there was no consideration of exposure from ASBs, although the authors claimed that such exposure was limited in middle-aged elderly Italian people at the time of study assessment.]

A small case–control study on brain cancer, conducted in France in 2005, was primarily focused on personal habits and psychological stress (Cabaniols et al., 2011). The study reported no association with aspartame. [The Working Group noted that the overall methodology of the study was only very briefly described in the article, and it did not specify how aspartame exposure was measured or how it was separated from generic exposure to artificial sweeteners.]

A small case–control study carried out in 2004–2014 in the USA included 50 patients diagnosed with well-differentiated thyroid cancer and 50 control patients diagnosed with benign thyroid nodules (Singh et al., 2020). Artificial sweetener consumption was assessed by a telephone interview and included questions on amount and duration of artificial sweetener consumption. A crude odds ratio was reported (OR, 10.1; 95% CI, 4.01–25.10) based on 38 cases and 12 controls who reported consumption of artificial sweetener. [The Working Group noted that the description of the study methodology was very limited, and it appeared that it was not analysed according to the epidemiological standards for case–control studies, i.e. the covariates used in the analysis were not specified and controls were not matched.]

Yin et al. (2022) reported a meta-analysis of studies evaluating ASBs (without restriction to time during which aspartame was used) and risk of glioma or cancers of the endometrium, ovary, and thyroid. [The Working Group determined that this meta-analysis did not contribute useful information, since its aim was to study the association between ASBs and cancer, without specific consideration of aspartame. Also, only one study on glioma and ovarian and thyroid cancers was included, and only two studies on endometrial cancer (Inoue-Choi et al., 2013; Hodge et al., 2018).]

2.5 Cancers of lymphatic and haematopoietic tissues

See Table 2.5.

A total of four original prospective cohort studies conducted in the USA (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014, 2022), one case–control study in Spain (Palomar-Cros et al., 2023), and four meta-analyses (Toews et al., 2019; WHO et al., 2022; Yin et al., 2022; Pan et al., 2023) reported results on the association between proxies of aspartame exposure and risk of lymphatic and haematopoietic cancers.

The NIH-AARP Diet and Health Study cohort included 285 079 men and 188 905 women in the USA (Lim et al., 2006). During follow-up (1995–2000), 1888 haematopoietic cancers were ascertained by cancer registries. Estimated daily aspartame intake was derived from responses to a limited baseline self-administered FFQ on

Table 2.5 Epidemiological studies on consumption of aspartame and cancers of lymphatic and haematopoietic tissues

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Lim et al. (2006) USA Enrolment, 1995–1996/ follow-up, through 2000 Cohort	473 984 (285 079 men and 188 905 women); NIH-AARP Diet and Health Study: AARP members aged 50–71 yr residing in the study area (California, Florida, Pennsylvania, New Jersey, North Carolina, Louisiana, Atlanta, Detroit) excluding those with prevalent cancer, invalid BMI data, outliers on reported energy intake, and proxy responders Exposure assessment method: FFQ at baseline, 5 yr of follow-up; consumption frequency of three types of soft drinks (soda, fruit drinks, and iced tea) + frequency of consumption of diet vs regular versions + three portion sizes; use of tabletop sweetener packets; standard doses of aspartame assigned to beverages and packets	All haematopoietic cancers, incidence	Aspartame intake (RR):			Age at entry, sex, ethnicity, BMI, history of diabetes	*Exposure assessment critique*: A key strength was the prospective and quantitative assessment of aspartame exposure from various ASBs and tabletop packets in a relevant period for aspartame (1995–1996 in the USA). Key limitations were the sequential assessment of first the frequency of consumption of soft drinks and then of diet/sugar-free versions with imprecise frequencies inducing inaccuracies; other sources of aspartame were not considered (although these were more limited). *Other strengths*: large cohort; case ascertainment based on cancer registries with high (> 90%) completeness; large numbers of cases. *Other limitations*: potential for bias from non-differential exposure misclassification due to older age at enrolment and a lack of data before study entry (aspartame in beverages introduced in the USA 10 yr before baseline).
			None	869	1		
			> 0, < 100 mg/day	432	0.91 (0.81–1.03)		
			≥ 100, < 200 mg/day	280	1.10 (0.96–1.26)		
			≥ 200, < 400 mg/day	137	1.01 (0.84–1.21)		
			≥ 400, < 600 mg/day	104	1.05 (0.85–1.29)		
			≥ 600 mg/day	66	0.98 (0.76–1.27)		
			Trend-test *P* value: 0.56				
		All haematopoietic cancer, incidence	Aspartame intake, men (RR):			Age at entry, ethnicity, BMI, history of diabetes	
			None	615	1		
			> 0, < 100 mg/day	294	0.95 (0.83–1.10)		
			≥ 100, < 200 mg/day	187	1.09 (0.92–1.29)		
			≥ 200, < 400 mg/day	95	1.03 (0.83–1.29)		
			≥ 400, < 600 mg/day	76	1.07 (0.84–1.36)		
			≥ 600 mg/day	51	1.06 (0.79–1.42)		
			Trend-test *P* value: 0.42				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Lim et al. (2006) (cont.)		All haematopoietic cancers, incidence	Aspartame intake, women (RR):			Age at entry, ethnicity, BMI, history of diabetes	
			None	254	1		
			> 0, < 100 mg/day	138	0.83 (0.67–1.02)		
			≥ 100, < 200 mg/day	93	1.11 (0.87–1.41)		
			≥ 200, < 400 mg/day	42	0.95 (0.68–1.32)		
			≥ 400, < 600 mg/day	28	1.02 (0.69–1.51)		
			≥ 600 mg/day	15	0.80 (0.47–1.35)		
			Trend-test *P* value: 0.87				
		Hodgkin lymphoma, incidence	Aspartame intake (RR):				
			None	29	1		
			> 0 mg/day	28	0.77 (0.44–1.32)		
			Trend-test *P* value: 0.34				
		Multiple myeloma, incidence	Aspartame intake (RR):				
			None	127	1		
			> 0, < 100 mg/day	57	0.85 (0.62–1.17)		
			≥ 100, < 200 mg/day	48	1.39 (0.99–1.96)		
			≥ 200, < 400 mg/day	20	1.13 (0.70–1.83)		
			≥ 400 mg/day	21	1.03 (0.64–1.66)		
			Trend-test *P* value: 0.40				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Lim et al. (2006) (cont.)		NHL, overall, incidence	Aspartame intake (RR):			Age at entry, sex, ethnicity, BMI, history of diabetes	
			None	592	1		
			> 0, < 100 mg/day	300	0.93 (0.81–1.07)		
			≥ 100, < 200 mg/day	177	1.01 (0.85–1.19)		
			≥ 200, < 400 mg/day	92	0.98 (0.78–1.22)		
			≥ 400, < 600 mg/day	73	1.06 (0.82–1.35)		
			≥ 600 mg/day	45	0.95 (0.70–1.29)		
			Trend-test *P* value: 0.91				
		NHL (SLL and CLL), incidence	Aspartame intake (RR):				
			None	109	1		
			> 0, < 100 mg/day	60	1.03 (0.75–1.41)		
			≥ 100, < 200 mg/day	36	1.16 (0.79–1.71)		
			≥ 200 mg/day	36	1.02 (0.69–1.52)		
			Trend-test *P* value: 0.84				
		NHL (immunoblastic lymphoma and lymphoblastic lymphoma/ leukaemia), incidence	Aspartame intake (RR):				
			None	23	1		
			> 0 mg/day	22	0.77 (0.42–1.42)		
			Trend-test *P* value: 0.40				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Lim et al. (2006) (cont.)		Non-lymphoid leukaemia), incidence	Aspartame intake (RR):			Age at entry, sex, ethnicity, BMI, history of diabetes	
			None	121	1		
			> 0, < 100 mg/day	62	0.93 (0.68–1.27)		
			≥ 100, < 200 mg/day	47	1.31 (0.93–1.85)		
			≥ 200, < 400 mg/day	20	1.05 (0.65–1.70)		
			≥ 400 mg/day	29	1.25 (0.82–1.91)		
			Trend-test *P* value: 0.19				
Schernhammer et al. (2012) USA Enrolment, 1976 (NHS), 1986 (HPFS)/ follow-up, 1984–2006 (NHS), 1986–2006 (HPFS Cohort	125 028 (77 218 women and 47 810 men). 1324 NHL, 285 multiple myeloma, and 339 leukaemia; Women of the NHS were included in 1976: registered nurses, aged 30–55 yr; men of the HPFS were included in 1986: dentists, veterinarians, pharmacists, optometrists, podiatrists, and osteopaths, aged 40–75 yr; participants reporting any previous diagnosis of cancer were excluded	NHL, incidence	Frequency of diet soda consumption, men (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy; race, BMI, height, discretionary physical activity, smoking history	*Exposure assessment critique*: A key strength was the prospective quantitative assessment of aspartame exposure as a cumulative average from repeated diet assessments every 4 yr; aspartame was assessed from diet sodas and aspartame packets using assigned aspartame content at a very relevant period for aspartame exposure from ASBs (the USA between 1984 and 2006) potentially capturing lifetime exposure to aspartame. A key limitation was that other sources of aspartame including non-soda ASBs were not considered (although these were more limited).
			None	172	1		
			< 1 serving/wk	122	1.12 (0.88–1.43)		
			1–3.9 servings/ wk	124	1.06 (0.83–1.34)		
			4–6.9 servings/ wk	53	0.96 (0.69–1.32)		
			≥ 1 serving/day	100	1.31 (1.01–1.72)		
			Trend-test *P* value: 0.11				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)	Exposure assessment method: assessment of aspartame exposure from diet soda and sachets of aspartame through repeated FFQ (every 4 yr); assigned values of aspartame for packets and sodas (weighted average of representative sodas)	NHL, incidence	Frequency of diet soda consumption, women (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy; race, BMI, height, discretionary physical activity, smoking history, menopausal status and use of HRT (women only)	*Other strengths*: length of follow-up; large number of haematopoietic cancer cases overall. *Other limitations*: potential for bias from non-differential exposure misclassification.
			None	189	1		
			< 1 serving/wk	167	0.98 (0.79–1.22)		
			1–3.9 servings/wk	173	0.90 (0.72–1.11)		
			4–6.9 servings/wk	87	0.85 (0.65–1.10)		
			≥ 1 serving/day	137	1.00 (0.78–1.26)		
			Trend-test *P* value: 0.999				
		NHL, incidence	Frequency of diet soda consumption (RR):				
			None	361	1		
			< 1 serving/wk	289	1.04 (0.89–1.22)		
			1–3.9 servings/wk	297	0.96 (0.82–1.13)		
			4–6.9 servings/wk	140	0.89 (0.72–1.09)		
			≥ 1 serving/day	237	1.13 (0.94–1.34)		
			Trend-test *P* value: 0.28				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		NHL, incidence	Aspartame intake, men, follow-up 1994–2006 (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI; height; discretionary physical activity, smoking history	
			None	95	1		
			< 19 mg/day	55	0.92 (0.65–1.29)		
			19–59 mg/day	65	1.13 (0.82–1.57)		
			60–142 mg/day	49	0.98 (0.68–1.40)		
			≥ 143 mg/day	69	1.64 (1.17–2.29)		
			Trend-test *P* value: 0.002				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		NHL, incidence	Aspartame intake, women, follow-up 1994–2006 (RR):			Age, questionnaire cycle, total sugar intake, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI, height, discretionary physical activity, smoking history, menopausal status and use of HRT (women only)	
			None	172	1		
			< 19 mg/day	114	0.94 (0.74–1.20)		
			19–55 mg/day	110	0.96 (0.75–1.22)		
			56–128 mg/day	91	0.83 (0.64–1.08)		
			≥ 129 mg/day	86	0.91 (0.69–1.20)		
			Trend-test *P* value: 0.48				
		NHL, incidence	Aspartame intake, follow-up 1994–2006 (RR):				
			None	267	1		
			< 19 mg/day	169	0.93 (0.76–1.13)		
			19–59 mg/day (men), 19–55 mg/day (women)	175	1.02 (0.83–1.24)		
			60–142 mg/day (men), 56–128 mg/day (women)	140	0.88 (0.71–1.09)		
			≥ 143 mg/day (men), ≥ 129 mg/day (women)	155	1.16 (0.93–1.43)		
			Trend-test *P* value: 0.12				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		NHL, incidence	Frequency of diet soda consumption, men, low alcohol intake (< 6 g/day) (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein and total energy, race, BMI, height, discretional physical activity, and smoking history	
			None	82	1		
			< 1 serving/wk	51	0.99 (0.69–1.43)		
			1–3.9 servings/ wk	66	1.27 (0.90–1.79)		
			4–6.9 servings/ wk	22	0.85 (0.52–1.39)		
			1–1.9 servings/ day	27	1.02 (0.64–1.61)		
			≥ 2 servings/day	29	2.34 (1.46–3.76)		
			Trend-test *P* value: 0.004				
		NHL, incidence	Frequency of diet soda consumption, men, high alcohol intake (≥ 6 g/day) (RR):				
			None	90	1		
			< 1 serving/wk	71	1.20 (0.86–1.67)		
			1–3.9 servings/ wk	58	0.84 (0.59–1.2)		
			4–6.9 serving/ wk	31	0.99 (0.64–1.52)		
			1–1.9 servings/ day	34	1.19 (0.77–1.82)		
			≥ 2 serving/day	10	0.96 (0.48–1.90)		
			Trend-test *P* value: 0.98				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Multiple myeloma, incidence	Frequency of diet soda consumption, men (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI, height, discretionary physical activity, smoking history	
			None	40	1		
			< 1 serving/wk	27	1.17 (0.70–1.96)		
			1–3.9 servings/wk	23	1.04 (0.61–1.78)		
			4–6.9 servings/wk	12	1.08 (0.55–2.12)		
			≥ 1 serving/day	29	2.02 (1.20–3.40)		
			Trend-test *P* value: 0.01				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Multiple myeloma, incidence	Frequency of diet soda consumption, women (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI, height, discretionary physical activity, smoking history, menopausal status and use of HRT (women only)	
			None	39	1		
			< 1 serving/wk	28	0.71 (0.43–1.17)		
			1–3.9 servings/wk	40	0.86 (0.54–1.37)		
			4–6.9 servings/wk	23	0.95 (0.55–1.63)		
			≥ 1 serving/day	24	0.79 (0.45–1.36)		
			Trend-test *P* value: 0.79				
		Multiple myeloma, incidence	Frequency of diet soda consumption (RR):				
			None	79	1		
			< 1 serving/wk	55	0.91 (0.63–1.30)		
			1–3.9 servings/wk	63	0.94 (0.66–1.33)		
			4–6.9 servings/wk	35	1.00 (0.65–1.52)		
			≥ 1 serving/day	53	1.29 (0.89–1.89)		
			Trend-test *P* value: 0.10				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Multiple myeloma, incidence	Aspartame intake, men, follow-up 1994–2006: (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI, height, discretionary physical activity, smoking history	
			None	10	1		
			< 19 mg/day	17	3.33 (1.48–7.49)		
			19–59 mg/day	11	1.70 (0.68–4.23)		
			60–142 mg/day	14	2.96 (1.25–6.96)		
			≥ 143 mg/day	13	3.36 (1.38–8.19)		
			Trend-test *P* value: 0.05				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Multiple myeloma, incidence	Aspartame intake, women, follow-up 1994–2006 (RR):			Age, questionnaire cycle, total sugar intake, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI, height, discretionary physical activity, smoking history, menopausal status and use of HRT (women only)	
			None	45	1		
			< 19 mg/day	14	0.40 (0.22–0.74)		
			19–55 mg/day	25	0.76 (0.46–1.27)		
			56–128 mg/day	25	0.83 (0.50–1.39)		
			≥ 129 mg/day	15	0.59 (0.32–1.09)		
			Trend-test *P* value: 0.48				
		Multiple myeloma, incidence	Aspartame intake, follow-up 1994–2006 (RR):				
			None	55	1		
			< 19 mg/day	31	0.86 (0.53–1.41)		
			19–59 mg/day (men), 19–55 mg/day (women)	36	0.92 (0.59–1.44)		
			60–142 mg/day (men), 56–128 mg/day (women)	39	1.16 (0.75–1.81)		
			≥ 143 mg/day (men), ≥ 129 mg/day (women)	28	1.03 (0.62–1.72)		
			Trend-test *P* value: 0.44				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Multiple myeloma, incidence	Frequency of diet soda consumption, men, low alcohol intake (< 6 g/day) (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein and total energy, race, BMI, height, discretional physical activity, and smoking history	
			None	17	1		
			< 1 servings/wk	17	1.98 (0.95–4.11)		
			1–3.9 servings/wk	10	1.38 (0.60–3.17)		
			4–6.9 servings/wk	6	1.51 (0.56–4.08)		
			≥ 1 serving/day	19	3.79 (1.80–8.00)		
			Trend-test *P* value: 0.002				
		Multiple myeloma, incidence	Frequency of diet soda consumption, men, high alcohol intake (≥ 6 g/day) (RR):				
			None	23	1		
			< 1 serving/wk	10	0.63 (0.27–1.48)		
			1–3.9 servings/wk	13	0.86 (0.41–1.79)		
			4–6.9 servings/wk	6	0.73 (0.28–1.89)		
			≥ 1 serving/day	10	0.98 (0.43–2.23)		
			Trend-test *P* value: 0.85				
		Leukaemia, incidence	Frequency of diet soda consumption, men (RR):				
			None	52	1		
			< 1 serving/wk	33	1.07 (0.68–1.68)		
			1–3.9 servings/wk	49	1.51 (1.00–2.28)		
			4–6.9 servings/wk	19	1.29 (0.75–2.24)		
			≥ 1 serving/day	33	1.47 (0.92–2.35)		
			Trend-test *P* value: 0.13				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Leukaemia, incidence	Frequency of diet soda consumption, women (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI, height, discretionary physical activity, smoking history, menopausal status, and use of HRT (women only)	
			None	33	1		
			< 1 serving/wk	31	1.04 (0.63–1.73)		
			1–3.9 servings/wk	37	1.05 (0.64–1.72)		
			4–6.9 servings/wk	21	1.21 (0.68–2.17)		
			≥ 1 serving/day	31	1.36 (0.80–2.31)		
			Trend-test *P* value: 0.20				
		Leukaemia, incidence	Frequency of diet soda consumption (RR):				
			None	85	1		
			< 1 serving/wk	64	1.06 (0.75–1.48)		
			1–3.9 servings/wk	86	1.30 (0.95–1.78)		
			4–6.9 servings/wk	40	1.26 (0.84–1.87)		
			≥ 1 serving/day	64	1.42 (1.00–2.02)		
			Trend-test *P* value: 0.05				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Leukaemia, incidence	Aspartame intake, men, follow-up 1994–2006 (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI, height, discretionary physical activity, smoking history	
			None	23	1		
			< 19 mg/day	14	0.89 (0.45–1.77)		
			19–59 mg/day	23	1.69 (0.91–3.12)		
			60–142 mg/day	19	1.55 (0.81–2.94)		
			≥ 143 mg/day	18	1.56 (0.79–3.06)		
			Trend-test *P* value: 0.17				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Leukaemia, incidence	Aspartame intake, women, follow-up 1994–2006 (RR):			Age, questionnaire cycle, total sugar intake, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein, and total energy, race, BMI, height, discretionary physical activity, smoking history, menopausal status and use of HRT (women only)	
			None	34	1		
			< 19 mg/day	21	0.85 (0.48–1.58)		
			19–55 mg/day	32	1.34 (0.81–2.21)		
			56–128 mg/day	21	0.95 (0.54–1.66)		
			≥ 129 mg/day	21	1.04 (0.58–1.85)		
			Trend-test *P* value: 0.94				
		Leukaemia, incidence	Aspartame intake, follow-up 1994–2006 (RR):				
			None	57	1		
			< 19 mg/day	35	0.86 (0.56–1.33)		
			19–59 mg/day (men), 19–55 mg/day (women)	55	1.47 (1.00–2.17)		
			60–142 mg/day (men), 56–128 mg/day (women)	40	1.17 (0.77–1.79)		
			≥ 143 mg/day (men), ≥ 129 mg/day (women)	39	1.23 (0.80–1.91)		
			Trend-test *P* value: 0.31				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Schernhammer et al. (2012) (cont.)		Leukaemia, incidence	Frequency of diet soda consumption, men, low alcohol intake (< 6 g/day) (RR):			Age, questionnaire cycle, sugar-sweetened soda consumption, fruit and vegetable consumption, multivitamin use, intakes of alcohol, saturated fat, animal protein and total energy, race, BMI, height, discretional physical activity, and smoking history	
			None	20	1		
			< 1 serving/wk	21	1.76 (0.91–3.37)		
			1–3.9 servings/wk	25	2.14 (1.13–4.07)		
			4–6.9 servings/wk	6	1.09 (0.41–2.88)		
			≥ 1 serving/day	20	1.83 (0.91–3.67)		
			Trend-test *P* value: 0.38				
		Leukaemia, incidence	Frequency of diet soda consumption, men, high alcohol intake (≥ 6 g/day) (RR):				
			None	32	1		
			< 1 serving/wk	12	0.58 (0.29–1.17)		
			1–3.9 servings/wk	24	1.11 (0.63–1.97)		
			4–6.9 servings/wk	13	1.44 (0.72–1.90)		
			≥ 1 serving/day	13	1.05 (0.52–2.10)		
			Trend-test *P* value: 0.40				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) USA Enrolment, 1999/follow-up, until 30 June 2009 Cohort	100 442 men and women; participants were adults (aged 47–95 yr) from the CPS-II nutrition cohort who returned the 1999 FFQ Exposure assessment method: aspartame exposure assessed quantitatively through an FFQ in 1999 and updated in 2003 for consumption of artificially sweetened carbonated beverage and aspartame packet; aspartame content values assigned by type for all carbonated beverages	NHL, all (including multiple myeloma), incidence	Aspartame intake quintile (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	*Exposure assessment critique*: Key strengths were the prospective quantitative assessment of aspartame intake from carbonated ASBs (aspartame values assigned by type of ASB) and aspartame packets; and that exposure was assessed twice at a very relevant period for aspartame exposure through ASBs (the USA in 1999 and 2003). A key limitation was that other sources were not considered (although these were more limited). *Other strengths*: large number of NHL cases. *Other limitations*: potential for bias from non-differential exposure misclassification.
			1st quintile (median, 0 mg/day)	230	1		
			2nd quintile (median, 3.6 mg/day)	266	1.29 (1.08–1.54)		
			3rd quintile (median, 12.6 mg/day)	260	1.27 (1.06–1.52)		
			4th quintile (median, 35.8 mg/day)	234	1.10 (0.91–1.32)		
			5th quintile (median, 145 mg/day)	206	1.02 (0.84–1.24)		
			Continuous (per 50 mg/day)	1196	0.99 (0.95–1.03)		
			Trend-test *P* value: 0.69				
		NHL, all (including multiple myeloma), incidence	Aspartame intake quintile, men (RR):			Age at baseline, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			1st quintile (median, 0 mg/day)	114	1		
			2nd quintile (median, 4.9 mg/day)	141	1.36 (1.05–1.74)		
			3rd quintile (median, 14.4 mg/day)	136	1.43 (1.11–1.84)		
			4th quintile (median, 47.6 mg/day)	122	1.17 (0.90–1.52)		

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)			5th quintile (median, 155 mg/day)	120	1.23 (0.94–1.61)		
			Continuous (per 50 mg/day)	633	1.01 (0.97–1.06)		
			Trend-test *P* value: 0.38				
		NHL, all (including multiple myeloma), incidence	Aspartame intake quintile, women (RR):			Age at baseline, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			1st quintile (median, 0 mg/day)	116	1		
			2nd quintile (median, 3.6 mg/day)	125	1.21 (0.93–1.56)		
			3rd quintile (median, 9.8 mg/day)	124	1.11 (0.86–1.44)		
			4th quintile (median, 31.9 mg/day)	112	1.02 (0.78–1.33)		
			5th quintile (median, 127 mg/day)	86	0.82 (0.61–1.10)		
			Continuous (per 50 mg/day)	563	0.94 (0.88–1.01)		
			Trend-test *P* value: 0.12				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)		NHL, all (including multiple myeloma), incidence	Artificially sweetened carbonated beverage intake (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			Nondrinkers	331	1		
			> 0–3 cans/mo	424	1.14 (0.98–1.32)		
			1–4 cans/wk	303	0.97 (0.82–1.14)		
			5–6 cans/wk	40	0.77 (0.56–1.08)		
			≥ 1 can/day	98	0.92 (0.73–1.17)		
			Continuous (per 1 can/day)	1196	1.00 (0.98–1.01)		
			Trend-test *P* value: 0.14				
		NHL, incidence	Long-term artificially sweetened carbonated beverage intake, men (RR):			Age at baseline, sex, smoking status, BMI, weight change from 1982 to 1999, history of diabetes, energy, long-term intake of sugar-sweetened carbonated beverage	
			No past & no current	61	1		
			Low past & low current	135	1.08 (0.79–1.48)		
			High past & low current	56	1.25 (0.84–1.87)		
			Low past & high current	25	0.87 (0.54–1.41)		
			High past & high current	37	1.17 (0.74–1.83)		

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)		NHL, incidence	Long-term artificially sweetened carbonated beverage intake, women (RR):			Age at baseline, sex, smoking status, BMI, weight change from 1982 to 1999, history of diabetes, energy, long-term intake of sugar-sweetened carbonated beverage	
			No past & no current	68	1		
			Low past & low current	141	0.82 (0.61–1.11)		
			High past & low current	90	0.94 (0.67–1.33)		
			Low past & high current	21	0.77 (0.46–1.27)		
			High past & high current	24	0.53 (0.32–0.87)		
		NHL, excluding multiple myeloma, incidence	Aspartame intake quintile (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			1st quintile (median, 0 mg/day)	187	1		
			2nd quintile (median, 3.6 mg/day)	221	1.31 (1.08–1.60)		
			3rd quintile (median, 12.6 mg/day)	215	1.29 (1.06–1.58)		
			4th quintile (median, 35.8 mg/day)	203	1.18 (0.96–1.45)		
			5th quintile (median, 145 mg/day)	172	1.07 (0.86–1.33)		
			Continuous (per 50 mg/day)	998	1.00 (0.96–1.04)		
			Trend-test *P* value: 0.83				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)		NHL, excluding multiple myeloma, incidence	Artificially sweetened carbonated beverage intake (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			Nondrinkers	272	1		
			> 0–3 cans/mo	349	1.13 (0.96–1.33)		
			1–4 cans/wk	261	1.02 (0.86–1.22)		
			5–6 cans/wk	33	0.79 (0.55–1.14)		
			≥ 1 can/day	83	0.97 (0.75–1.26)		
			Continuous (per 1 can/day)	998	1.00 (0.98–1.02)		
			Trend-test *P* value: 0.45				
		Multiple myeloma, incidence	Aspartame intake quintile (RR):				
			1st quintile (median, 0 mg/day)	43	1		
			2nd quintile (median, 3.6 mg/day)	45	1.18 (0.77–1.81)		
			3rd quintile (median, 12.6 mg/day)	45	1.18 (0.77–1.81)		
			4th quintile (median, 35.8 mg/day)	31	0.74 (0.46–1.20)		
			5th quintile (median, 145 mg/day)	34	0.83 (0.51–1.33)		
			Continuous (per 50 mg/day)	198	0.93 (0.84–1.04)		
			Trend-test *P* value: 0.14				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)		Multiple myeloma, incidence	Artificially sweetened carbonated beverage intake (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			Nondrinkers	59	1		
			> 0–3 cans/mo	75	1.15 (0.81–1.63)		
			1–4 cans/wk	42	0.71 (0.47–1.07)		
			≥ 5 cans/wk	22	0.70 (0.42–1.17)		
			Continuous (per 1 can/day)	198	0.97 (0.92–1.01)		
			Trend-test *P* value: 0.05				
		NHL (DLBCL), incidence	Aspartame intake quintile (RR):				
			1st quintile (median, 0 mg/day)	40	1		
			2nd quintile (median, 3.6 mg/day)	63	1.82 (1.22–2.72)		
			3rd quintile (median, 12.6 mg/day)	56	1.62 (1.07–2.45)		
			4th quintile (median, 35.8 mg/day)	50	1.38 (0.90–2.11)		
			5th quintile (median, 145 mg/day)	49	1.39 (0.90–2.16)		
			Continuous (per 50 mg/day)	258	1.02 (0.94–1.10)		
			Trend-test *P* value: 0.51				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)		NHL (DLBCL), incidence	Artificially sweetened carbonated beverage intake (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			Nondrinkers	67	1		
			> 0–3 cans/mo	90	1.23 (0.89–1.70)		
			1–4 cans/wk	71	1.12 (0.80–1.59)		
			≥ 5 cans/wk	30	0.92 (0.59–1.45)		
			Continuous (per 1 can/day)	258	1.01 (0.98–1.04)		
			Trend-test *P* value: 0.89				
		NHL (CLL and SLL), incidence	Aspartame intake quintile (RR):				
			1st quintile (median, 0 mg/day)	59	1		
			2nd quintile (median, 3.6 mg/day)	58	1.05 (0.73–1.52)		
			3rd quintile (median, 12.6 mg/day)	55	1.02 (0.70–1.48)		
			4th quintile (median, 35.8 mg/day)	52	0.95 (0.65–1.39)		
			5th quintile (median, 145 mg/day)	43	0.85 (0.56–1.29)		
			Continuous (per 50 mg/day)	267	0.96 (0.88–1.05)		
			Trend-test *P* value: 0.40				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)		NHL (CLL and SLL), incidence	Artificially sweetened carbonated beverage intake (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			Nondrinkers	80	1		
			> 0–3 cans/mo	92	0.97 (0.72–1.32)		
			1–4 cans/wk	68	0.89 (0.64–1.23)		
			≥ 5 cans/wk	27	0.71 (0.45–1.12)		
			Continuous (per 1 can/day)	267	0.99 (0.95–1.02)		
			Trend-test *P* value: 0.15				
		NHL (follicular lymphoma), incidence	Aspartame intake quintile (RR):				
			1st quintile (median, 0 mg/day)	27	1		
			2nd quintile (median, 3.6 mg/day)	31	1.41 (0.84–2.39)		
			3rd quintile (median, 12.6 mg/day)	27	1.22 (0.71–2.10)		
			4th quintile (median, 35.8 mg/day)	29	1.21 (0.71–2.09)		
			5th quintile (median, 145 mg/day)	28	1.20 (0.69–2.11)		
			Continuous (per 50 mg/day)	142	1.03 (0.94–1.13)		
			Trend-test *P* value: 0.72				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)		NHL (follicular lymphoma), incidence	Artificially sweetened carbonated beverage intake (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			Nondrinkers	41	1		
			> 0–3 cans/mo	46	1.08 (0.70–1.66)		
			1–4 cans/wk	36	0.97 (0.61–1.54)		
			≥ 5 cans/wk	19	0.98 (0.55–1.74)		
			Continuous (per 1 can/day)	142	1.00 (0.96–1.04)		
			Trend-test *P* value: 0.85				
		NHL (other B-cell lymphoma), incidence	Aspartame intake quintile (RR):				
			1st quintile (0 mg/day)	35	1		
			2nd quintile (median, 3.6 mg/day)	37	1.18 (0.74–1.89)		
			3rd quintile (median, 12.6 mg/day)	48	1.58 (1.01–2.46)		
			4th quintile (median, 35.8 mg/day)	43	1.39 (0.88–2.20)		
			5th quintile (median, 145 mg/day)	29	1.01 (0.60–1.68)		
			Continuous (per 50 mg/day)	192	0.94 (0.85–1.05)		
			Trend-test *P* value: 0.63				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2014) (cont.)		NHL (other B-cell lymphoma), incidence	Artificially sweetened carbonated beverage intake (RR):			Age at baseline, sex, history of diabetes, BMI, smoking status, energy intake, sugar-sweetened carbonated beverage intake	
			Nondrinkers	46	1		
			> 0–3 cans/mo	72	1.41 (0.97–2.06)		
			1–4 cans/wk	51	1.24 (0.82–1.88)		
			≥ 5 cans/wk	23	1.13 (0.67–1.91)		
			Continuous (per 1 can/day)	192	1.00 (0.96–1.04)		
			Trend-test *P* value: 0.59				
McCullough et al. (2022) USA Enrolment, 1982/follow-up, through 2016 (median, 27.7 yr) Cohort	934 777 (416 313 men, 518 464 women); CPS-II prospective cohort; adults aged ≥ 28 yr; excluded participants with personal history at baseline of diabetes or cancer other than nonmelanoma skin cancer, men aged >90 yr or women aged >95 yr at enrolment, and those reporting only prior but not current consumption of either SSBs or ASBs	NHL, mortality	ASB consumption (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption. Key limitations were that there was only one dietary assessment at baseline in 1982 that was before the use of aspartame in ASB, hence the relevance to aspartame exposure depends on the stability of ASB consumption over up to 34 yr of follow-up, but such information was not directly available; no other sources were considered (although these were more limited); and uncertainty regarding aspartame content in ASBs after the mid-2000s. Other information: exclusion of participants who reported only prior but not current consumption of either SSBs or ASBs at baseline.
			Never	NR	1		
			< 1 drink/day	NR	0.97 (0.89–1.05)		
			1 drink/day	NR	0.97 (0.88–1.07)		
			≥ 2 drinks/day	NR	1.01 (0.92–1.12)		
			Continuous (per drink/day)	6600	1.00 (0.98–1.03)		
			Trend-test *P* value: 0.870				
		NHL, mortality	ASB consumption, men (HR):				
			Never	NR	1		
			< 1 drink/day	NR	1.01 (0.89–1.15)		
			1 drink/day	NR	0.99 (0.85–1.14)		
			≥ 2 drinks/day	NR	0.97 (0.83–1.13)		
			Continuous (per drink/day)	3409	1.00 (0.95–1.04)		
			Trend-test *P* value: 0.714				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)	Exposure assessment method: questionnaire; exposure to ASBs assessed in 1982 through a question about the number of drinks/day of diet soda or ice teas (one pooled item) and potential changes over the past 10 yr; no specific assessment of aspartame content in ASB	NHL, mortality	ASB consumption, women (HR):			Age, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	*Other strengths*: large cohort with long follow-up; ability to examine multiple cancer types, stratify by sex or BMI, and limit to never-smokers; comprehensive adjustment for confounders, including SSB consumption. *Other limitations*: potential for bias from non-differential exposure misclassification.
			Never	NR	1		
			< 1 drink/day	NR	0.94 (0.85–1.04)		
			1 drink/day	NR	0.95 (0.84–1.08)		
			≥ 2 drinks/day	NR	1.04 (0.91–1.18)		
			Continuous (per drink/day)	3191	1.01 (0.97–1.05)		
			Trend-test *P* value: 0.916				
		NHL, mortality	ASB consumption, BMI, 18.5 to < 25 kg/m² (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.96 (0.85–1.08)		
			1 drink/day	NR	0.84 (0.71–0.98)		
			≥ 2 drinks/day	NR	0.89 (0.75–1.06)		
			Continuous (per drink/day)	2970	0.96 (0.91–1.01)		
			Trend-test *P* value: 0.035				
		NHL, mortality	ASB consumption, BMI, 25 to < 30 kg/m² (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.00 (0.88–1.13)		
			1 drink/day	NR	1.05 (0.91–1.21)		
			≥ 2 drinks/day	NR	1.08 (0.93–1.25)		
			Continuous (per drink/day)	2689	1.02 (0.98–1.06)		
			Trend-test *P* value: 0.274				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		NHL, mortality	ASB consumption, BMI, ≥ 30 kg/m² (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.89 (0.71–1.11)		
			1 drink/day	NR	1.06 (0.83–1.36)		
			≥ 2 drinks/day	NR	1.00 (0.79–1.27)		
			Continuous (per drink/day)	721	1.02 (0.95–1.08)		
			Trend-test *P* value: 0.896				
		Multiple myeloma, mortality	ASB consumption (HR):				
			Never	NR	1		
			< 1 drink/day	NR	1.00 (0.90–1.11)		
			1 drink/day	NR	1.01 (0.89–1.15)		
			≥ 2 drink/day	NR	1.03 (0.90–1.17)		
			Continuous (per drink/day)	3511	1.02 (0.98–1.06)		
			Trend-test *P* value: 0.707				
		Multiple myeloma, mortality	ASB consumption, men (HR):			Age, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.02 (0.86–1.22)		
			1 drink/day	NR	0.98 (0.80–1.20)		
			≥ 2 drinks/day	NR	1.04 (0.84–1.27)		
			Continuous (per drink/day)	1791	1.02 (0.96–1.07)		
			Trend-test *P* value: 0.802				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Multiple myeloma, mortality	ASB consumption, women (HR):			Age, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.98 (0.85–1.12)		
			1 drink/day	NR	1.03 (0.87–1.21)		
			≥ 2 drinks/day	NR	1.01 (0.85–1.21)		
			Continuous (per drink/day)	1720	1.02 (0.97–1.07)		
			Trend-test *P* value: 0.841				
		Multiple myeloma, mortality	ASB consumption, BMI, 18.5 to < 25 kg/m^2 (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.12 (0.95–1.31)		
			1 drink/day	NR	1.10 (0.91–1.34)		
			≥ 2 drink/day	NR	1.16 (0.94–1.43)		
			Continuous (per drink/day)	1603	1.07 (1.01–1.13)		
			Trend-test *P* value: 0.079				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Multiple myeloma, mortality	ASB consumption, BMI, 25 to < 30 kg/m² (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.92 (0.77–1.09)		
			1 drink/day	NR	0.95 (0.77–1.16)		
			≥ 2 drinks/day	NR	1.04 (0.85–1.27)		
			Continuous (per drink/day)	1402	1.00 (0.95–1.06)		
			Trend-test *P* value: 0.997				
		Multiple myeloma, mortality	ASB consumption, BMI, ≥ 30 kg/m² (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.93 (0.70–1.26)		
			1 drink/day	NR	1.01 (0.72–1.41)		
			≥ 2 drinks/day	NR	0.76 (0.53–1.09)		
			Continuous (per drink/day)	391	0.94 (0.85–1.04)		
			Trend-test *P* value: 0.181				
		Leukaemia, mortality	ASB consumption (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.95 (0.88–1.04)		
			1 drink/day	NR	0.99 (0.90–1.10)		
			≥ 2 drinks/day	NR	0.97 (0.87–1.07)		
			Continuous (per drink/day)	6319	0.99 (0.96–1.02)		
			Trend-test *P* value: 0.444				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Leukaemia, mortality	ASB consumption, men (HR):			Age, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.94 (0.83–1.07)		
			1 drink/day	NR	1.06 (0.92–1.21)		
			≥ 2 drink/day	NR	0.96 (0.83–1.12)		
			Continuous (per drink/day)	3551	0.99 (0.95–1.04)		
			Trend-test *P* value: 0.797				
		Leukaemia, mortality	ASB consumption, women (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.96 (0.86–1.07)		
			1 drink/day	NR	0.94 (0.82–1.08)		
			≥ 2 drinks/day	NR	0.96 (0.84–1.11)		
			Continuous (per drink/day)	2768	0.98 (0.94–1.03)		
			Trend-test *P* value: 0.417				
		Leukaemia, mortality	ASB consumption, BMI, 18.5 to < 25 kg/m^2 (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	1.05 (0.93–1.19)		
			1 drink/day	NR	0.99 (0.85–1.16)		
			≥ 2 drinks/day	NR	0.90 (0.75–1.08)		
			Continuous (per drink/day)	2769	0.96 (0.91–1.02)		
			Trend-test *P* value: 0.373				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Leukaemia, mortality	ASB consumption, BMI, 25 to < 30 kg/m^2 (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.88 (0.77–1.00)		
			1 drink/day	NR	1.01 (0.87–1.16)		
			≥ 2 drink/day	NR	1.04 (0.90–1.21)		
			Continuous (per drink/day)	2688	1.01 (0.96–1.05)		
			Trend-test *P* value: 0.783				
		Leukaemia, mortality	ASB consumption, BMI ≥ 30 kg/m^2) (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.92 (0.73–1.16)		
			1 drink/day	NR	0.97 (0.74–1.26)		
			≥ 2 drinks/day	NR	0.79 (0.60–1.04)		
			Continuous (per drink/day)	656	0.96 (0.89–1.04)		
			Trend-test *P* value: 0.108				

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) Spain 2008–2013 Case–control	Cases: 109; aged 20–85 yr with newly diagnosed histologically confirmed cancer (prevalent CLL cases were also recruited and were retained for this analysis if diagnosis was done 1 yr before the interview), resided in catchment area for at least 6 mo; no prior history of their cancer; enrolled as soon as possible after diagnosis; frequency-matched on age, sex, and region to population controls. Controls: 3629; randomly selected from administrative records of selected primary health care centres within catchment area. Exposure assessment method: self-administered, semiquantitative FFQ, 140 food items, assessing usual dietary intake during the previous year	NHL (CLL, ICD-10 C91.1), incidence	Consumption of aspartame-containing products (OR): Non-consumers Medium intake (< 3rd quartile among controls) High intake (≥ 3rd quartile among controls) Trend-test *P* value: 0.6	 89 9 11	 1 0.56 (0.25–1.08) 1.76 (0.84–3.41)	Age, sex, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, other ASs	*Exposure assessment critique*: A key strength was the categorization of intake of ASBs and tabletop sweeteners by type (aspartame vs others) using public data on ingredients in food supply, but it was unclear whether the assumption of aspartame content in products was correct. A key limitation was that beverages and tabletop sweeteners were assessed, but there was no consideration of ASs in the rest of the food supply. *Other strengths*: large sample size for some outcomes; histopathological confirmation of cancer cases; extensive assessment of confounding, including from BMI (captured in a combined score based on WCRF/AICR evidence on lifestyle factors; Romaguera et al., 2017); stratification by diabetes status to evaluate heterogeneity of associations.

Table 2.5 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Palomar-Cros et al. (2023) (cont.)		NHL (CLL, ICD-10 C91.1), incidence	Consumption of aspartame-containing products, participants without diabetes (OR):			Age, sex, study centre, education, smoking, radiation exposure, total WCRF score continuous, total energy intake, total sugar intake, other ASs	*Other limitations*: non-prospective study design (case–control); selection bias due to low participation among controls (mean participation rate, 53%); recall bias in exposure assessment; potential for exposure measurement error and residual confounding due to other correlates of AS use among those with diabetes; relatively low exposure contrasts for aspartame-containing products; potential for chance findings due to small number in some strata.
			Non-consumers	75	1		
			Medium intake (< 3rd quartile among controls)	7	0.56 (0.23–1.18)		
			High intake (≥ 3rd quartile among controls)	9	2.15 (0.93–4.51)		
			Trend-test *P* value: 0.4				
		NHL (CLL, ICD-10 C91.1), incidence	Consumption of aspartame-containing products, participants with diabetes (OR):				
			Non-consumers	14	1		
			Medium intake (< 3rd quartile among controls)	2	0.55 (0.08–2.37)		
			High intake (≥ 3rd quartile among controls)	2	0.63 (0.06–3.41)		
			Trend-test *P* value: 0.6				

AICR, American Institute of Cancer Research; AS, artificial sweetener; ASB, artificially sweetened beverage; BMI, body mass index; CI, confidence interval; CLL, chronic lymphocytic leukaemia; CPS-II, Cancer Prevention Study-II; DLBCL, diffuse large B-cell lymphoma; FDA, Food and Drug Administration; FFQ, food frequency questionnaire; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; HRT, hormone replacement therapy; ICD-10, International Classification of Diseases 10th revision; mo, month(s); NHL, non-Hodgkin lymphoma; NHS, Nurses' Health Study; NIH-AARP, National Institutes of Health-American Association of Retired Persons; NR, not reported; OR, odds ratio; RR, relative risk; SLL, small lymphocytic lymphoma; SSB, sugar-sweetened beverage; vs, versus; WCRF, World Cancer Research Fund; wk, week(s); yr, year(s).

four aspartame-containing beverages and aspartame added to hot coffee and tea during the past year. No association with aspartame intake was detected for all haematopoietic cancers, overall or in either men or women, or for Hodgkin lymphoma, multiple myeloma, non-Hodgkin lymphoma (NHL) overall or two of its subtypes (small lymphocytic lymphoma and CLL; immunoblastic lymphoma and lymphoblastic lymphoma/leukaemia) and non-lymphoid leukaemia. [This prospective cohort included a large sample of participants and a substantial number of incident cases ascertained by registries. Another strength was the prospective and quantitative assessment of aspartame exposure from various ASBs and tabletop packets between 1995 and 1996, a relevant period for aspartame use in beverages in the USA. However, the length of follow-up was relatively limited, and the age at enrolment was relatively high (average age of cohort at baseline, 62 years, with no assessment of aspartame intake from younger ages). Additional weaknesses were that the questionnaire used to estimate aspartame exposure was limited and prone to exposure misclassification, only focusing on beverage and tabletop sweetener sources (there was no information on other dietary sources of aspartame – although these would be expected to be less common); that the question on the frequency of the type of sweetener in a beverage (regular versus diet) followed the question on the frequency of consumption by type of beverage (soda, fruit drinks, or iced tea); and the minimally specific assessment of the type of sweetener ingested (the questionnaire asked about whether sweetener added to coffee or tea was "Equal or aspartame" or "Sweet'N Low or saccharin"). All these factors may have limited the ability of the authors to detect an association.]

The association between aspartame-containing soda and incidence of haematopoietic cancers was investigated in the NHS (77 218 women) and the HPFS (47 810 men) (Schernhammer et al., 2012). Over 22 years of follow-up (1984–2006 for the NHS and 1986–2006 for the HPFS), 1324 NHL, 285 multiple myeloma, and 339 leukaemia cases were ascertained. In men, ≥ 1 daily serving of diet soda was associated with increased risk of NHL (RR, 1.31; 95% CI, 1.01–1.72; *P* for trend, 0.11) and multiple myeloma (RR, 2.02; 95% CI, 1.20–3.40; *P* for trend, 0.01) compared with men who did not consume diet soda. No association was observed for women for NHL (RR, 1.00; 95% CI, 0.78–1.26; *P* for trend, 0.999) or for multiple myeloma (RR, 0.79; 95% CI, 0.45–1.36; *P* for trend, 0.79). When data from the two cohorts were combined in a pooled analysis to maximize statistical precision, diet soda intake was associated with increased risk of leukaemia (RR, 1.42; 95% CI, 1.00–2.02; *P* for trend, 0.05). Since aspartame became most broadly used in the USA in soda in 1992 when its patent expired and the price dropped substantially (so the aspartame measure using diet soda consumption probably became more precise after that date), and since the questionnaires started to include tabletop sweeteners after that date, a secondary analysis was conducted, starting follow-up from 1994. In that analysis, among men, an association was observed between aspartame exposure (estimated from diet soda and aspartame packets consumed) and increased risk of NHL (RR for ≥ 143 mg/day versus none, 1.64; 95% CI, 1.17–2.29; *P* for trend, 0.002) and multiple myeloma (RR for ≥ 143 mg/day versus none, 3.36; 95% CI, 1.38–8.19; *P* for trend, 0.05); these associations were not observed in women. For leukaemia, the RR for ≥ 143 mg/day versus none was 1.56 (95% CI, 0.79–3.06; *P* for trend, 0.17) in men. For NHL, an interaction ($P = 0.03$) with alcohol was detected among men: compared with no consumption, ≥ 2 servings of diet soda/day was associated with increased risk (RR, 2.34; 95% CI, 1.46–3.76; *P* for trend, 0.004) in men who consumed < 6 g alcohol/day (median intake) but not in men with a higher alcohol consumption (RR, 0.96; 95% CI, 0.48–1.90; *P* for trend, 0.98). Risks of multiple myeloma and leukaemia

associated with diet soda at ≥ 1 serving/day were also higher in men with a lower alcohol intake. In contrast, for women, risks associated with diet soda did not differ by alcohol consumption for any of the outcomes [data not provided], although few women in this cohort consumed high amounts of alcohol. The authors hypothesized that these sex-specific differences may have been due to the recognized higher enzymatic activity of alcohol dehydrogenase type I in men, which possibly induced higher conversion rates from methanol to the carcinogenic substrate formaldehyde. Because the concurrent ingestion of ethanol inhibits methanol metabolism (Lee et al., 2011), they conducted analyses stratified by alcohol intake. [The Working Group noted that these results supported the authors' initial hypothesis that men with lower regular alcohol consumption would have more unbound alcohol dehydrogenase activity (Frezza et al., 1990) and thus higher formaldehyde conversion rates if they consumed large amounts of diet soda and, consequently, higher cancer risk. The length of follow-up, large number of haematopoietic cancer cases, prospective design, and updating of dietary intake data every 4 years at a relevant period for aspartame exposure from ASBs in the USA (between the early to mid-1980s and mid-2000s) constituted important strengths of this study. The main limitation related to potential measurement error in aspartame exposure, which was derived only from diet soda and packets used at the table assessed by FFQ (other sources of aspartame including non-soda ASBs were not considered, although these would be expected to be less common), without brand information for products other than tabletop sweeteners, and thus no data on the specific type of sweetener ingested. The secondary analyses starting in 1994 suggested that more precise measures might strengthen the results.]

Two studies were conducted within the CPS-II prospective cohort to investigate the association between ASBs and aspartame and risk of lymphoid neoplasm incidence (McCullough et al., 2014) and mortality (McCullough et al., 2022). The first study (McCullough et al., 2014), in the CPS-II nutrition cohort (a subcohort of the parent CPS-II mortality cohort), included 100 442 men and women who completed a modified Willett FFQ in 1999 (which was re-administered in 2003). Mean consumption of artificially sweetened carbonated beverages ["1 glass, bottle, or can (355 mL)"] during the past year was queried (categories ranging from "never" to "≥ 4 per day"). Reported intake of specific artificially and sugar-sweetened carbonated beverage types listed (i.e. cola with caffeine, other carbonated beverages with or without caffeine) were summed. Participants were asked about "use of NutraSweet or Equal (1 packet) (not Sweet'N Low)" (from "never" to "≥ 6 per day"). Total aspartame intake was calculated using the following values: 180 mg/355 mL (1 serving) of low-calorie cola with caffeine, 90 mg/355 mL of other low-calorie soda with caffeine, and 70 mg/355 mL of other low-calorie soda without caffeine; and 20 mg of aspartame per packet of artificial sweetener reported. Over a 10-year follow-up, 1196 cases of NHL were ascertained and verified by medical records or by linkage with cancer registries. No association was detected between consumption of artificially sweetened carbonated beverages or aspartame and NHL risk overall or by subtype (multiple myeloma, diffuse large B-cell lymphoma, CLL/small lymphocytic lymphoma, and follicular and other B-cell lymphoma). No heterogeneity in the association was suggested by sex. A secondary analysis was conducted to examine "long-term" past consumption patterns by incorporating participant responses from a questionnaire completed 10 years before baseline (as part of the parent cohort). In 1982, participants were asked about usual consumption of artificially sweetened carbonated beverages per day as "cups, glasses, or drinks" (responses ranged from none to 10 servings/day, assuming 355 mL per serving).

Aspartame consumption was not specifically calculated for this exposure assessment. This analysis suggested an inverse association between "long-term" regular consumption of artificially sweetened carbonated beverages and NHL in women (RR versus long-term non-consumers, 0.53; 95% CI, 0.32–0.87). These inverse associations were however interpreted by the authors as potentially caused by chance or confounding by other unknown exposures. [This study benefited from the large number of haematopoietic cancer cases, the prospective design, and the quantitative assessment of aspartame intake from carbonated ASBs (aspartame values assigned by type of ASB – although not brand-specific) and aspartame packets. Exposure was assessed twice in 1999 and 2003, at a relevant period for aspartame exposure through ASBs in the USA. Limitations pertained to the level of detail in the aspartame exposure assessment and the fact that no other sources were considered (although these would be expected to be less important), thus resulting in potential misclassification. Although the direction of the potential bias cannot be definitively determined, these non-differential classification errors were more likely to bias observed associations towards the null. Regarding the "long-term" analysis, although aspartame was not quantified, the consumption of ASBs was considered to be an informative proxy for aspartame exposure, since aspartame had just been authorized in the USA in 1983, i.e. 1 year after the measurement of exposure in 1982.]

In the second investigation in the CPS-II cohort, analyses were conducted on 934 777 cancer-free men and women who provided answers to the 1982 questionnaire about their usual consumption of ASBs (McCullough et al., 2022). From 1982 through 2016 (follow-up, 34 years), 135 093 cancer deaths occurred, of which 6600 were from NHL, 6319 from leukaemia, and 3511 from multiple myeloma (ascertained by personal inquiries to participants or their relatives and verified by death certificates, and from 1989 onwards by linkage to the National Death Index). No association was detected for any of these outcomes in men, women, or sex-combined analyses. An interaction with BMI was detected for NHL (*P* for interaction, 0.029): the hazard ratio estimates for ≥ 2 drinks/day of ASBs versus non-consumers were 0.89 (95% CI, 0.75, 1.06) for normal weight (18.5 to < 25 kg/m^2), 1.08 (95% CI, 0.93, 1.25) for overweight (25 to < 30 kg/m^2), and 1.00 (95% CI, 0.79, 1.27) for obese (≥ 30 kg/m^2) participants. [This study was based on a very large number of cases, long follow-up, and prospective design. The main limitation lay in the evaluation of the exposure, which was based on a unique baseline frequency question, "How many cups, glasses, or drinks of diet soda or diet iced teas do you usually drink a day, and for how many years?", focused only on beverage sources, with no specific assessment of the type of sweetener ingested and no brand-specific data. As mentioned above, aspartame had only been authorized in the USA in 1983, i.e. 1 year after exposure was assessed. However, combining data from McCullough et al. (2014, 2022) indicated some stability in ASB consumption ranking over years; hence, the Working Group concluded that ASB consumption assessed in 1982 may reflect later consumption, when aspartame became the predominant sweetener in ASBs. These misclassification errors probably limited the ability to detect associations (although not systematically, so that non-differential classification errors probably biased associations towards the null).]

The MCC-Spain study conducted in 2008–2013 included 109 cases of CLL with no prior history of disease and a total of 3629 controls (1631 controls for CLL-specific analyses) (Palomar-Cros et al., 2023). Consumption of products containing artificial sweeteners was estimated for tabletop sweeteners and ASB, assessed through a self-administered validated FFQ. No association was observed between exposure to "aspartame-containing products" and CLL risk either in all participants or in analyses stratified

by diabetes status. [The main limitation of this study related to its non-prospective design (case–control). Another limitation related to the precision of the aspartame exposure estimate, which was derived only from ASBs and tabletop sweeteners assessed by FFQ, without brand information, and thus with few data on the specific type of sweetener ingested and relying on the assumption that all low- or no-calorie beverages contained aspartame, which may not be realistic considering the time period for this case–control study in the Spanish context. In addition, the numbers of cases overall and in diabetes strata were small, and results should be interpreted cautiously.]

Four meta-analyses presented results on aspartame and/or ASBs and haematopoietic cancer risk.

In 2019, Toews et al. performed a meta-analysis of randomized and non-randomized controlled trials and observational studies on the association between intake of non-sugar sweeteners and health outcomes, following a PRISMA flow chart of included studies and the Cochrane Collaboration methodology for the systematic literature review and meta-analysis (Toews et al., 2019). Of individual studies finally included in this review, two related to haematopoietic cancers (i.e. Lim et al., 2006, and McCullough et al., 2014, presented above). Results of the meta-analyses showed no association between higher consumption of aspartame and incidence of the main subtypes of lymphoid cancers, NHL subtypes, or non-lymphoid leukaemia. However, the study by Schernhammer et al. (2012) was not included in the review [no specific explanation found in the article], nor was the study by McCullough et al. (2022) (which was published later).

In 2022, WHO published a report (coordinated by Rios-Leyvraz and Montez) on the health effects of use of non-sugar sweeteners that included a systematic review and meta-analyses of studies published through July 2021 (WHO et al., 2022). Although this overall report did not focus only on aspartame, for the specific cancer location studied here (haematopoietic cancers) the four prospective cohort studies (NIH-AARP, NHS, HPFS, CPS-II) from the three articles published at that time (i.e. all those described previously in this section except for the study by McCullough et al., 2022) were those deemed relevant for the present monograph (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014). The results of these meta-analyses found little evidence of positive associations, but the number of included studies remained low: leukaemia, three cohorts, RR, 1.24; 95% CI, 0.92–1.69, I^2 = 0%; multiple myeloma, four cohorts, RR, 1.05; 95% CI, 0.70–1.59, I^2 = 70%; Hodgkin lymphoma, one cohort, RR, 0.77; 95% CI, 0.44–1.33; NHL, four cohorts, RR, 1.08; 95% CI, 0.87–1.34, I^2 = 64%.

Also in 2022, Yin et al. published a meta-analysis of prospective studies on ASB consumption and cancer risk (Yin et al., 2022). The same studies as in the WHO report (WHO et al., 2022) were those included for haematopoietic cancers (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014) (although the overall publication did not focus only on aspartame and also included studies conducted before 1980). In contrast to the WHO meta-analysis, Yin et al. found that high intake of ASBs was associated with elevated risk of non-lymphoid leukaemia (for three studies, RR, 1.35; 95% CI, 1.03–1.77; P = 0.030, with evidence of low heterogeneity across studies). Dose–response analysis indicated a positive linear association between ASB intake and the risk of leukaemia (P for a linear relation, 0.027). The risk associated with daily ASB intake increased by 15% per increment of one serving (355 mL) (RR, 1.15; 95% CI, 1.02–1.30). [The Working Group noted that the three cohorts included in the WHO report (WHO et al., 2022) and in the study by Yin et al. (2022) for the analysis of leukaemia were the same, i.e. Lim et al. (2006) for NIH-AARP, and Schernhammer et al. (2012) for NHS and HPFS. However, the estimates used

for NHS and HPFS differed, since Yin et al. used estimates from models of ASB intake, whereas WHO used models of "total aspartame".] The results of Yin et al. were consistent with those of the WHO report in that no association was found between ASB intake and multiple myeloma (RR for highest versus lowest category, 1.18; 95% CI, 0.69–2.02; P = 0.537; I^2 = 68.9%), NHL (RR for highest versus lowest category, 1.05; 95% CI, 0.91–1.21; P = 0.506; I^2 = 16.3%), or Hodgkin lymphoma (RR for highest versus lowest category, 0.77; 95% CI, 0.45–1.33; P = 0.351; I^2 = 0.0%).

Pan et al. (2023) published a dose–response meta-analysis of prospective cohort studies (from inception to June 2022) on ASB intake and several cancer types, including haematopoietic cancers. The publications by Schernhammer et al. (2012) and McCullough et al. (2014) were included, but that by Lim et al. (2006) was omitted. The authors reported that an increment of ASBs of 250 mL/day was associated with a 16% increase in risk of leukaemia (RR, 1.16; 95% CI, 1.00–1.35; I^2 = 0%), but only the publication of Schernhammer et al. (2012) (considering two estimates, one from the US NHS cohort and one from the US HPFS cohort) was included in this meta-analysis. No association was observed for NHL or multiple myeloma, but the number of included studies was very small (Schernhammer et al., 2012; McCullough et al., 2014).

2.6 Obesity-related cancers and other groupings

See Table 2.6.

Overall, three cohort studies reported on aspartame or ASB consumption and the risk of developing or dying from obesity-related cancers. A separate analysis in one of these cohorts evaluated the association between artificially sweetened soft drinks and non-obesity-related cancers.

The MCCS included 35 593 men and women aged 40–69 years and enrolled between 1990 and 1994 (Hodge et al., 2018). The baseline diet questionnaire, with 121 drink and food items, included questions about the number of times that diet soft drinks (artificially sweetened) were consumed per day, per week or per month. During follow-up, 3283 cases of obesity-related cancers were diagnosed, including cancers of the liver, prostate (aggressive), ovary, gallbladder, kidney, colorectum, oesophagus (adenocarcinoma), breast (postmenopausal), pancreas, endometrium, and gastric cardia. Proportional hazard models were adjusted for age and other potential confounders, including socioeconomic position, alcohol intake, country of birth, Mediterranean diet index, physical activity, sex, smoking, frequency of sugar-sweetened soft drink consumption, and waist circumference. Using no consumption or consumption of < 1 time/month as the reference category, the study did not find a positive association with increasing consumption of artificial sweeteners. The hazard ratios were 0.87 (95% CI, 0.76–1.00) for consumption of 1–3 times/month, 1.04 (95% CI, 0.93–1.17) for 1–6 times/week, 0.81 (95% CI, 0.66–0.99) for 1 time/day, and 1.00 (95% CI, 0.79–1.27) for > 1 time/day. The analyses of the association using soft drink consumption expressed as a continuous variable using the median daily equivalent frequency to each of the five categories of consumption also found no positive association with the risk of developing obesity-related cancers (HR, 0.98; 95% CI, 0.90–1.07).

Data from the MCCS were also used to evaluate the associations between consumption of artificially sweetened soft drinks and non-obesity-related cancers (Bassett at al., 2020). Over 19 years of follow-up, 4789 cancers not related to obesity occurred among 35 109 eligible participants. After adjusting for age, alcohol intake, country of birth, Mediterranean diet score, physical activity, socioeconomic position, sex,

Table 2.6 Epidemiological studies on consumption of aspartame and obesity-related cancers and other groupings

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hodge et al. (2018) Australia Enrolment, 1990–1994/ follow-up until 30 June 2013 Cohort	35 593; MCCS – a prospective cohort study of men and women aged 40–69 yr at recruitment and free of cancer, angina, heart attack, and diabetes at baseline; participants with extreme baseline energy intake were excluded; obesity-related cancers according to the WCRF/ AICR (WCRF/AICR, 2018) Exposure assessment method: self-administered 121-item FFQ with separate questions on frequency of consumption in the past year of diet (artificially sweetened) soft drinks	Obesity-related cancers: liver, prostate (aggressive), ovary, gallbladder, kidney, colorectum, oesophagus (adenocarcinoma), breast (postmenopausal), pancreas, uterus/ uterine corpus (endometrium), stomach (gastric cardia), incidence	Artificially sweetened soft drink consumption (HR): Never or < 1/mo 1–3/mo 1–6/wk 1/day > 1/day Continuous (per beverage/ day) Trend-test *P* value: 0.61	 2498 233 376 102 74 3283	 1 0.87 (0.76–1.00) 1.04 (0.93–1.17) 0.81 (0.66–0.99) 1.00 (0.79–1.27) 0.98 (0.90–1.07)	Age, sex, socioeconomic index, country of birth, alcohol intake, smoking status, physical activity, Mediterranean diet score, sugar-sweetened soft drink consumption, waist circumference	*Exposure assessment critique*: Key strengths were that it was a prospective study, that assessment was after aspartame introduction in diet soft drinks in Australia (1987), and that first half of follow-up largely overlapped with period of aspartame use in Australia. A key limitation was the FFQ assessment with no specific estimate of aspartame exposure, ASBs as a proxy and exposure data at baseline only; small number of consumers. *Other strengths*: adjustment for key confounders, including a measure of obesity (waist circumference). *Other limitations*: likely bias from non-differential exposure misclassification given single baseline assessment and long follow-up.

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bassett et al. (2020) Australia Enrolment, 1990–1994/ follow-up, 30 June 2015 Cohort	35 109; MCCS – a prospective cohort study of men and women aged 27–76 yr at recruitment (99% aged between 40 and 70 yr) and free of cancer, heart attack, angina and diabetes at baseline; participants with extreme energy intake were excluded Exposure assessment method: self-administered 121-item FFQ with separate questions on frequency of consumption in the past year of diet (artificially sweetened) soft drinks	Non-obesity-related cancers: sites other than oesophagus (adenocarcinoma), pancreas, colorectum, breast (postmenopausal), uterus/ uterine corpus (endometrium), kidney, ovary, gallbladder, liver, stomach (gastric cardia), meningioma, thyroid, multiple myeloma, incidence	Artificially sweetened soft drink consumption (HR): Never or < 1/mo 1–3/mo 1–6/wk 1/day > 1/day Continuous (per 1 serving/ day) Trend-test *P* value: 0.006	 3625 371 490 189 114 4789	 1 0.96 (0.86–1.07) 0.96 (0.87–1.06) 1.23 (1.06–1.43) 1.23 (1.02–1.48) 1.10 (1.03–1.17)	Age, sex, alcohol intake, country of birth, Mediterranean diet score, physical activity, socioeconomic position, smoking status, sugar-sweetened soft drink consumption	*Exposure assessment critique*: A key strength was that it was prospective study. A key limitation was the FFQ assessment with no specific estimate of aspartame exposure, ASBs were used as a proxy, and exposure data were reported at baseline only. *Other strengths*: large cohort. *Other limitations*: heterogeneous group (combined different cancer types with different etiology, hence difficult controlling for all potential confounders); likely bias from non-differential exposure misclassification.

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) France Enrolment, 2009–2021/ follow-up until 22 January 2021 (median, 7.8 yr) Cohort	102 865; population-based cohort NutriNet-Santé (web-based); men and women aged ≥ 18 yr Exposure assessment method: records; participants are asked every 6 mo to complete a series of three validated web-based 24 h dietary records randomly assigned over a 2-wk period (2 weekdays, 1 weekend day); at least two 24 h dietary records during the first 2 yr of follow-up considered in analyses (mean ± SD, 5.6 ±3.0)	Obesity-related cancers: colorectum, stomach, liver, mouth, pharynx, larynx, oesophagus, breast, ovary, uterus/ uterine corpus (endometrium), and prostate, incidence	Aspartame intake (HR): Non-consumers Lower consumers (men, < 14.45 mg/day; women, < 15.39 mg/day) Higher consumers (men, ≥ 14.45 mg/day; women, ≥ 15.39 mg/day) Trend-test *P* value: 0.026	 1401 337 285	 1 1.08 (0.96–1.22) 1.15 (1.01–1.32)	Age, sex, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, prevalent diabetes, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake	*Exposure assessment critique*: A key strength was that it was a prospective study using dietary records and quantitative assessment of aspartame based on food composition data updates for food supply changes over time. A key limitation was the baseline assessment in the main analysis, but sensitivity analysis conducted using all 24 h dietary records available during follow-up. *Other strengths*: large cohort; large number of cases; sensitivity analyses excluded prevalent diabetes or used all available 24 h dietary records throughout follow-up. *Other limitations*: low aspartame use in the cohort (28%); self-selection may limit generalizability; potential for residual confounding and reverse causation.

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) (cont.)		Obesity-related cancers colorectum, stomach, liver, mouth, pharynx, larynx, oesophagus, breast, ovary, uterus/ uterine corpus (endometrium), and prostate, incidence	Aspartame intake, participants without diabetes (HR):			Age, sex, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake	
			Non-consumers	1360	1		
			Lower consumers (men, < 14.45 mg/day; women, < 15.39 mg/day)	318	1.09 (0.96–1.23)		
			Higher consumers (men, ≥ 14.45 mg/day; women, ≥ 15.39 mg/day)	260	1.16 (1.01–1.34)		
			Trend-test *P* value: 0.024				

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) (cont.)		Obesity-related cancers: colorectum, stomach, liver, mouth, pharynx, larynx, oesophagus, breast, ovary, uterus/ uterine corpus (endometrium), and prostate, incidence	Aspartame intake (time-dependent) (HR):			Age, sex, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, prevalent diabetes, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake	
			Non-consumers	1401	1		
			Lower consumers (men, < 14.45 mg/day; women, < 15.39 mg/day)	337	1.07 (0.96–1.20)		
			Higher consumers (men, ≥ 14.45 mg/day; women, ≥ 15.39 mg/day)	285	1.13 (0.99–1.30)		
			Trend-test *P* value: 0.046				

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) USA Enrolment, 1982/follow-up, through 2016 (median, 27.7 yr) Cohort	934 777 (416 313 men, 518 464 women); CPS-II prospective cohort; adults 28 yr and older; excluded participants with personal history at baseline of diabetes or cancer other than nonmelanoma skin cancer, men aged > 90 yr or women aged > 95 yr at enrolment, and those reporting only prior but not current consumption of either SSBs or ASBs Exposure assessment method: questionnaire; exposure to ASBs assessed in 1982 through a question about the number of drinks/day of diet soda or ice teas (one pooled item) and potential changes over the past 10 yr; no specific assessment of aspartame content in ASB	Obesity-related cancers: oesophagus, stomach, colorectum, liver, gallbladder, pancreas, breast (postmenopausal), uterus/ uterine corpus (endometrium), ovary, kidney, and multiple myeloma, mortality	ASB consumption (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption. Key limitations were that there was only one dietary assessment at baseline in 1982 that was before the use of aspartame in ASB, hence the relevance to aspartame exposure depends on the stability of ASB consumption over up to 34 yr of follow-up, but such information was not available; no other sources were considered (although these were more limited); and uncertainty of aspartame content in ASBs after the mid-2000s. Other information: exclusion of participants who reported only prior but not current consumption of either SSBs or ASBs at baseline.
			Never	NR	1		
			< 1 drink/day	NR	1.02 (0.99–1.05)		
			1 drink/day	NR	1.04 (1.01–1.08)		
			≥ 2 drinks/day	NR	1.05 (1.01–1.08)		
			Continuous (per drink/ day)	50 613	1.02 (1.01–1.03)		
			Trend-test *P* value: 0.001				
		Obesity-related cancers: oesophagus, stomach, colorectum, liver, gallbladder, pancreas, kidney, and multiple myeloma, mortality	ASB consumption, men (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	
			Never	NR	1		
			< 1 drink/day	NR	1.02 (0.97–1.07)		
			1 drink/day	NR	1.06 (1.00–1.13)		
			≥ 2 drinks/day	NR	1.08 (1.02–1.15)		
			Continuous (per drink/ day)	20 595	1.03 (1.01–1.04)		
			Trend-test *P* value: 0.002				

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Obesity-related cancers: oesophagus, stomach, colorectum, liver, gallbladder, pancreas, breast (postmenopausal), uterus/ uterine corpus (endometrium), ovary, kidney, and multiple myeloma, mortality	ASB consumption, women (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	*Other strengths*: large cohort with long follow-up; ability to examine multiple cancer types, stratify by sex or BMI, and limit to never-smokers; comprehensive adjustment for confounders, including SSB consumption. *Other limitations*: likely non-differential exposure misclassification.
			Never	NR	1		
			< 1 drink/day	NR	1.02 (0.99–1.05)		
			1 drink/day	NR	1.04 (0.99–1.08)		
			≥ 2 drinks/day	NR	1.03 (0.99–1.08)		
			Continuous (per drink/ day)	30 018	1.01 (1.00–1.03)		
			Trend-test *P* value: 0.038				
		Obesity-related cancers: oesophagus, stomach, colorectum, liver, gallbladder, pancreas, breast (postmenopausal), uterus/ uterine corpus (endometrium), ovary, kidney, and multiple myeloma, mortality	ASB consumption, BMI-adjusted (HR):			Age, sex, race/ ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.98 (0.95–1.01)		
			1 drink/day	NR	1.00 (0.97–1.03)		
			≥ 2 drinks/day	NR	0.99 (0.95–1.02)		
			Continuous (per drink/ day)	50 613	1.00 (0.99–1.01)		
			Trend-test *P* value: 0.469				

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		Obesity-related cancers: oesophagus, stomach, colorectum, liver, gallbladder, pancreas, kidney, and multiple myeloma, mortality	ASB consumption, men, BMI-adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.98 (0.93–1.03)		
			1 drink/day	NR	1.01 (0.96–1.08)		
			≥ 2 drinks/day	NR	1.02 (0.96–1.08)		
			Continuous (per drink/ day)	20 595	1.01 (0.99–1.03)		
			Trend-test *P* value: 0.599				
		Obesity-related cancers: oesophagus, stomach, colorectum, liver, gallbladder, pancreas, breast (postmenopausal), uterus/ uterine corpus (endometrium), ovary, kidney, and multiple myeloma, mortality	ASB consumption, women, BMI-adjusted (HR):				
			Never	NR	1		
			< 1 drink/day	NR	0.98 (0.95–1.01)		
			1 drink/day	NR	0.99 (0.95–1.03)		
			≥ 2 drinks/day	NR	0.98 (0.94–1.02)		
			Continuous (per drink/ day)	30 018	1.00 (0.98–1.01)		
			Trend-test *P* value: 0.213				

AICR, American Institute of Cancer Research; AS, artificial sweetener; ASB, artificially sweetened beverage; BMI, body mass index; CI, confidence interval; CPS-II, Cancer Prevention Study-II; FDA, Food and Drug Administration; FFQ, food frequency questionnaire; HR, hazard ratio; MCCS, Melbourne Collaborative Cohort Study; mo, month(s); NR, not reported; RR, relative risk; standard deviation; SSB, sugar-sweetened beverage; WCRF, World Cancer Research Fund; wk, week(s); yr, year(s).

smoking status, and sugar-sweetened soft drink consumption, intake of > 1 serving/day of artificially sweetened soft drinks relative to none or < 1 time/month was associated with an increased risk of non-obesity-related cancers (HR, 1.23; 95% CI, 1.02–1.48; *P* for trend, 0.006), with a hazard ratio of 1.10 (95% CI, 1.03–1.17) for each extra serving per day.

[The Working Group noted that the study provided no specific estimate of aspartame exposure, using only ASBs as a proxy, and information only on consumption frequency; therefore, non-differential misclassification of exposure to aspartame was likely.]

The NutriNet-Santé cohort study investigated the association between aspartame and risk of developing obesity-related cancers (Debras et al., 2022b). The cohort was followed up from 2009 until 22 January 2021, and included 102 865 adults, among whom 2023 incident obesity-related cancers were diagnosed. In this cohort study, individual diet was measured every 6 months by three non-consecutive web-based 24-hour dietary records, randomly assigned over 15 days (2 weekdays and 1 weekend day). Baseline dietary intakes were evaluated by averaging all 24-hour dietary records provided during the first 2 years of follow-up, with a minimum of 2 and up to 15 dietary records per study participant. Detailed, brand-specific consumption of a wide variety of individual artificial sweeteners was assessed. Obesity-related cancers were selected on the basis of the report by WCRF/AICR in 2018 (WCRF/AICR, 2018), in which the scientific evidence supporting a link between obesity and cancer risk was classified as "probable" or "convincing". [The Working Group noted that, according to the WCRF, "probable" is the level of evidence that should trigger public health action (WCRF/AICR, 2018).] The statistical model adjusted for all potential confounders considered in this study (age, sex, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24-hour dietary records, family history of cancer, history of diabetes at baseline, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, and intake of other artificial sweeteners), used "non-consumers" as the reference category, and estimated that the relative risk (hazard ratio) of developing one of the obesity-related cancers considered in their analyses was 1.08 (95% CI, 0.96–1.22) for lower-consumers of aspartame and 1.15 (95% CI, 1.01–1.32) for higher-consumers (i.e. above the sex-specific median of exposure among aspartame users), with a test for trend over the three exposure categories of $P = 0.026$. Similar results were observed in sensitivity analyses that excluded participants with a history of diabetes at baseline or accounted for all available dietary records completed during follow-up. No interaction between aspartame and BMI was detected for obesity-related cancer (*P* for interaction, 0.925). [The Working Group noted that a major strength of this study was the assessment and separation of the consumption of foods and drinks specifically containing aspartame and the careful incorporation of this information into the epidemiological analysis.]

The CPS-II study included 934 777 men and women aged ≥ 28 years at baseline (McCullough et al., 2022). The study started in 1982, and follow-up was extended through 2016, with a median follow-up of 27.7 years. The baseline questionnaire included a grid that asked how many cups, glasses, or drinks of ASBs were usually consumed a day and for how many years, with write-in reporting by frequency and duration. Diet soda and diet iced teas were considered as ASBs, whereas "non-diet colas" and "other non-diet soft drinks" were considered as sugar-sweetened beverages. The CPS-II study used cancer mortality as the end-point. All Cox proportional hazards models used age as the timescale and adjusted for several

potential confounders that included sex, race/ethnicity, smoking, marital status, education, red and processed meat intake, fruit and vegetable intake, alcohol, and sugar-sweetened beverage intake. The authors presented the analyses of the association between ASB consumption and obesity-related cancers risk separately with non-BMI-adjusted and BMI-adjusted statistical models. When not adjusted for BMI, the estimated trends for increasing levels of consumption of ASBs reached statistical significance. Considering non-consumers as the reference category, the hazard ratios were 1.02 (95% CI, 0.99–1.05) for consumption of < 1 drink/day; 1.04 (95% CI, 1.01–1.08) for 1 drink/day; and 1.05 (95% CI, 1.01–1.08) for ≥ 2 drinks/day; with a *P* for trend across groups of 0.001, for men and women combined. In the statistical analyses additionally adjusted for BMI, the hazard ratio for the same increasing levels of exposure were all close to null (HR for < 1 drink/day, 0.98; 95% CI, 0.95–1.01; HR for 1 drink/day, 1.00; 95% CI, 0.97–1.03; and HR for ≥ 2 drinks/day, 0.99; 95% CI, 0.95–1.02). In analyses stratified by sex, the BMI-adjusted hazard ratio for consumption of ≥ 2 drinks/day versus none was 1.02 (95% CI, 0.96–1.08; *P* for trend, 0.599) for men and 0.98 (95% CI, 0.94–1.02; *P* for trend, 0.213) for women (*P* for interaction, 0.425). [The Working Group noted that, from a quantitative point of view, the hazard ratios reported by the CPS-II study were rather modest, in fact just slightly higher than 1, and they were statistically precise because of the extremely large sample size of the CPS-II study, which has more than 900 000 participants. The adjustment for BMI had a negligible impact on the point estimate and only slightly increased the confidence interval width.]

[The Working Group noted that these three cohort studies (Hodge et al., 2018; Debras et al., 2022b; McCullough et al., 2022) shared the aim of investigating the association between ASBs, more specifically aspartame in one study (Debras et al., 2022b), and the risk of obesity-related cancers; however, the cancer sites included in each study's case definition had some overlap but also some differences. Table 2.7 summarizes and compares the cancer end-points included in the obesity-related group in each study.]

A single meta-analysis reported on cancers related to obesity and on cancers not related to obesity, finding no association for either (Yin et al., 2022). [The Working Group noted that only the studies of Hodge et al. (2018) and Bassett et al. (2020) specifically looked at these cancer groupings, so the results included in the summary estimates came from studies with results for various individual cancer sites but were not reported as a group of "obesity-related cancers". Furthermore, the study by Debras et al. (2022b) was included in the non-obesity-related cancer meta-analysis, despite not reporting results for this cancer group. Additionally, some of the studies involved exposure to other artificial sweeteners and were not specific for aspartame.]

2.7 Cancer of all sites combined

See Table 2.8.

Eight studies including seven cohorts evaluated overall cancer incidence (NutriNet-Santé, Chazelas et al., 2019 and Debras et al., 2022b; and PLCO, You et al., 2022) or mortality (EPIC, Mullee et al., 2019; NHS, HPFS, Malik et al., 2019; National Health and Nutrition Examination Survey, NHANES, Zhang et al., 2021 and Fulgoni and Drewnowski, 2022; and CPS-II, McCullough et al., 2022) in relation to different estimates of aspartame consumption. Most studies used artificially sweetened soft drink intake or frequency as the exposure, but an analysis from NutriNet-Santé (Debras et al., 2022b) investigated specific estimates of aspartame intake derived from different sources using detailed information from multiple dietary records. [The Working Group noted that this study also included longer follow-up of the NutriNet-Santé cohort; hence, the study by Chazelas et al. (2019) was not further

Table 2.7 Cancers included in the "obesity-related" category used in the three cohort studies reported in Section 2.6

Cancer	Cohort study		
	NutriNet-Santé (Debras et al., 2022b)[a]	MCCS (Hodge et al., 2018)[a]	CPS-II mortality cohort (McCullough et al., 2022)[b]
Colorectum	✓	✓	✓
Stomach	✓	Gastric cardia	✓
Liver	✓	✓	✓
Mouth	✓	–	–
Pharynx	✓	–	–
Larynx	✓	–	–
Oesophagus	✓	Adenocarcinoma	✓
Breast, postmenopausal	✓	✓	✓
Breast, premenopausal	✓	–	–
Ovary	✓	✓	✓
Endometrium	✓	✓	✓
Prostate	✓	Aggressive	–
Pancreas	–	✓	✓
Kidney	–	✓	✓
Gallbladder	–	✓	✓
Multiple myeloma	–	–	✓
Meningioma	–	–	–

✓, Included; –, not included; CPS-II, Cancer Prevention Study-II; MCCS, Melbourne Collaborative Cohort Study.
[a] Based on the definition in WCRF/AICR (2018).
[b] This study evaluated mortality. No reference was provided regarding the definition of the obesity-related cancer grouping.

considered.] A ninth study (Liu et al., 2022b) examined consumption of coffee with artificial sweetener in the United Kingdom Biobank. [The Working Group considered this study to be uninformative since the exposure was not an adequate proxy for aspartame intake. An additional limitation of this study was that, in the United Kingdom Biobank and similarly in the results on coffee from the AARP-NIH and EPIC studies, coffee was associated with reduced risk of deaths both from all causes and from cancer, which would confound any association between artificial sweetener use and cancer.]

A prospective study of 102 865 adults followed for a median of 7.8 years in the French population-based NutriNet-Santé cohort examined aspartame consumption in relation to the incidence of all cancers combined (Debras et al., 2022b). The study observed 3358 cancers that were verified using medical and anatomopathological reports. In this analysis, aspartame intake from all possible dietary sources was specifically assessed. Intake estimates were based on all records available (at least two or more) during the first 2 years of follow-up. Individuals classified as having a higher intake of aspartame (i.e. above the sex-specific median among aspartame consumers, from an average of 5.6 dietary records per participant) had an elevated cancer risk compared with non-consumers (HR for higher-consumers, 1.15; 95% CI, 1.03–1.28; *P* for trend, 0.002). Similar results were observed in sensitivity analyses that excluded participants with a history of diabetes at baseline or accounted for all available dietary records completed during follow-up. No interaction between aspartame and BMI was detected for overall cancer ($P = 0.893$). [An important strength of this study

Table 2.8 Epidemiological studies on consumption of aspartame and cancer of all sites combined

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Malik et al. (2019) USA Enrolment, 1976 (NHS), 1986 (HPFS/follow-up, 1980–2014 (NHS), 1986–2014 (HPFS) Cohort	37 716 men; 80 647 women; female registered nurses aged 30–55 yr in the NHS and male health professionals aged 40–75 yr in the HPFS; excluding those with history of diabetes, cardiovascular disease, or cancer at baseline, or with implausible dietary intake Exposure assessment method: questionnaire; prospective assessment of ASB consumption through repeated FFQs between 1980–1986 and 2010	All cancers combined, mortality	ASB intake (HR):			Age, race, smoking, alcohol intake, postmenopausal hormone use (NHS), physical activity, family history of diabetes; family history of myocardial infarction, family history of cancer, multivitamin use, aspirin use, baseline history of hypertension and hypercholesterolaemia, intake of whole grains, fruit, vegetables, or red and processed meat, total energy, BMI, SSB intake	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption as a cumulative average from repeated, validated diet assessments every 4 yr, the majority at a very relevant period for aspartame exposure from ASBs (the USA between the 1980s and 2010) potentially capturing lifetime exposure to aspartame. A key limitation was that other sources of aspartame were not considered (although these were more limited); and uncertainty regarding aspartame content in ASBs after the mid-2000s. *Other strengths*: large cohort with long follow-up.
			< 1 serving/mo	6272	1		
			1–4 servings/mo	1803	1.01 (0.96–1.07)		
			2–6 servings/wk	2516	0.99 (0.94–1.04)		
			1 to < 2 servings/day	1013	1.00 (0.93–1.07)		
			≥ 2 servings/day	776	1.04 (0.96–1.12)		
			Continuous (per serving/day)	12 380	1.01 (0.98–1.03)		
			Trend-test *P* value: 0.58				
		All cancers combined, mortality	ASB intake estimated using cumulative average intake (HR):				
			< 1 serving/mo	NR	1		
			1–4 servings/mo	NR	0.90 (0.85–0.95)		
			2–6 servings/wk	NR	0.88 (0.84–0.92)		
			1 to < 2 servings/day	NR	0.89 (0.83–0.95)		
			≥ 2 servings/day	NR	0.91 (0.84–1.00)		
			Continuous (per serving/day)	NR	0.98 (0.96–1.01)		
			Trend-test *P* value: 0.03				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Malik et al. (2019) (cont.)		All cancers combined, mortality	ASB intake, 8-yr lag (HR):			Age, race, smoking, alcohol intake, postmenopausal hormone use (NHS), physical activity, family history of diabetes; family history of myocardial infarction, family history of cancer, multivitamin use, aspirin use, baseline history of hypertension and hypercholesterolaemia, intake of whole grains, fruit, vegetables, or red and processed meat, total energy, BMI, SSB intake	*Other limitations*: likely bias from non-differential misclassifications of exposure to aspartame; stratified numbers of deaths not provided for specific cancer sites.
			< 1 serving/mo	NR	1		
			1–4 servings/mo	NR	0.96 (0.90–1.02)		
			2–6 servings/wk	NR	0.93 (0.88–0.98)		
			1 to < 2 servings/day	NR	0.91 (0.84–0.98)		
			≥ 2 servings/day	NR	1.01 (0.93–1.10)		
			Continuous (per serving/day)	NR	1.01 (0.99–1.04)		
			Trend-test *P* value: 0.83				
		All cancers combined, mortality	ASB intake, men (HPFS) (HR):			Age, race, smoking, alcohol intake, physical activity, family history of diabetes; family history of myocardial infarction, family history of cancer, multivitamin use, aspirin use, baseline history of hypertension and hypercholesterolaemia, intake of whole grains, fruit, vegetables, or red and processed meat, total energy, BMI, SSB intake	
			< 1 serving/mo	2118	1		
			1–4 servings/mo	469	1.01 (0.92–1.12)		
			2–6 servings/wk	996	1.03 (0.95–1.11)		
			1 to < 2 servings/day	283	0.99 (0.87–1.13)		
			≥ 2 servings/day	196	0.95 (0.82–1.11)		
			Continuous (per serving/day)	4062	0.99 (0.95–1.03)		
			Trend-test *P* value: 0.51				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Malik et al. (2019) (cont.)		All cancers combined, mortality	ASB intake, women (NHS) (HR):			Age, race, smoking, alcohol intake, postmenopausal hormone use (NHS), physical activity, family history of diabetes; family history of myocardial infarction, family history of cancer, multivitamin use, aspirin use, baseline history of hypertension and hypercholesterolaemia, intake of whole grains, fruit, vegetables, or red and processed meat, total energy, BMI, SSB intake	
			< 1 serving/mo	4154	1		
			1–4 servings/mo	1334	1.00 (0.94–1.07)		
			2–6 servings/wk	1520	0.95 (0.90–1.01)		
			1 to < 2 servings/day	730	0.98 (0.91–1.06)		
			≥ 2 servings/day	580	1.05 (0.96–1.15)		
			Continuous (per serving/ day)	8318	1.01 (0.98–1.04)		
			Trend-test *P* value: 0.50				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mullee et al. (2019) Europe Enrolment, 1992–2000/ follow-up, through 2009–2013 (depending on study centre; mean, 16.4 yr) Cohort	451 743; EPIC cohort study participants from 10 European countries (Denmark, France, Greece, Germany, Italy, Netherlands, Norway, Spain, Sweden, and the United Kingdom); men and women excluding those with prevalent cancer, heart disease, stroke, or diabetes or with implausible dietary intake; for artificially sweetened soft drinks, participants from Italy, Spain, and Sweden were not included. Exposure assessment method: questionnaire; exposure to artificially sweetened soft drinks overall assessed once at baseline through country-specific validated tools (mainly FFQ) covering the usual diet over the past year; no specific assessment of aspartame or AS content of the artificially sweetened soft drinks	All cancers combined: mortality	Consumption of artificially sweetened soft drink (glass, 250 mL) (HR): < 1 glass/mo 1 to 4 glasses/mo > 1 to 6 glasses/ wk 1 to < 2 glasses/ day ≥ 2 glasses/day Trend-test *P* value: 0.23	 9359 1246 1251 72 303	 1 0.96 (0.90–1.02) 1.00 (0.94–1.06) 0.92 (0.73–1.16) 1.10 (0.97–1.23)	Age, centre, sex, BMI, physical activity index, educational status, alcohol consumption, smoking status, smoking intensity, smoking duration, ever use of contraceptive pill, menopausal status, ever use of menopausal hormone therapy, intakes of total energy, red and processed meat, fruits and vegetables, coffee, fruit and vegetable juice, sugar-sweetened soft drinks	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption (several types of beverage) in several western Europe countries at a period relevant for aspartame exposure (between 1991 and 2000). Key limitations were that no other sources of aspartame were considered (although these were more limited); uncertainty regarding the aspartame content in ASBs in every country; and that there was only one assessment at baseline.

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mullee et al. (2019) (cont.)		All cancers combined, mortality	Consumption of artificially sweetened soft drink (glass, 250 mL), men (HR):			Age, centre, BMI, physical activity index, educational status, alcohol consumption, smoking status, smoking intensity, smoking duration, intakes of total energy, red and processed meat, fruits and vegetables, coffee, fruit and vegetable juice, sugar-sweetened soft drinks	Other information: context of low ASB consumption in middle-aged adults. *Other strengths*: large population-based cohort spanning multiple countries with different behaviours; large number of cases; results adjusted for appropriate potential confounders, including BMI.
			< 1 glass/mo	NR	1		
			1 to 4 glasses/mo	NR	0.99 (0.89–1.09)		
			> 1 to 6 glasses/ wk	NR	1.10 (0.99–1.22)		
			1 to < 2 glasses/ day	NR	1.13 (0.76–1.66)		
			≥ 2 glasses/day	NR	1.14 (0.95–1.37)		
			Trend-test *P* value: 0.06				
		All cancers combined, mortality	Consumption of artificially sweetened soft drink (glass, 250 mL), women (HR):			Age, centre, BMI, physical activity index, educational status, alcohol consumption, smoking status, smoking intensity, smoking duration, ever use of contraceptive pill, menopausal status, ever use of menopausal hormone therapy, intakes of total energy, red and processed meat, fruits and vegetables, coffee, fruit and vegetable juice, sugar-sweetened soft drinks	
			< 1 glass/mo	NR	1		
			1 to 4 glasses/mo	NR	0.95 (0.87–1.02)		
			> 1 to 6 glasses/ wk	NR	0.95 (0.88–1.02)		
			1 to < 2 glasses/ day	NR	0.83 (0.62–1.11)		
			≥ 2 glasses/day	NR	1.06 (0.91–1.24)		
			Trend-test *P* value: 0.98				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Zhang et al. (2021) USA Enrolment, 1999–2014/ follow-up, through 2015 Cohort	31 402; NHANES participants, nationally representative sample of adult men and women aged ≥ 20 yr; participants excluded for current pregnancy or missing data Exposure assessment method: exposure to ASBs (sugar-free soft drinks and carbonated water) assessed at baseline with one or two 24 h dietary recalls	All cancers combined, mortality	Daily intake of ASBs (HR): None > 0 to < 1 serving/day 1 to < 2 servings/day ≥ 2 servings/day Continuous (per 1 serving/ day)	 727 68 64 24 883	 1 0.91 (0.64–1.29) 1.18 (0.84–1.66) 0.61 (0.35–1.04) 0.93 (0.83–1.06)	Age, sex, family income to poverty ratio, race, cigarette smoking, alcohol consumption, marital status, leisure time physical activity, BMI, prevalent high cholesterol, hypertension and diabetes, history of CVD and cancer, Healthy Eating Index-2015, total energy intake, SSB intake, complex sample design of NHANES	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption through dietary recalls, potentially capturing all types of ASB. Key limitations were the small number of dietary recalls (only one or two); no other sources were considered; only one assessment at baseline; and uncertainty regarding the aspartame content in ASBs in the USA after the mid-2000s (i.e. for the most part of the study assessment period, 1999–2014). *Other strengths*: prospective design; representative sample; consideration of nonlinear associations using cubic splines analysis. *Other limitations*: few cases among consumers of ASBs; likely non-differential exposure misclassification.

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) France Enrolment, 2009–2021/ follow-up until 22 January 2021 (median, 7.8 yr) Cohort	102 865; population-based cohort NutriNet-Santé (web-based); men and women aged ≥ 18 yr Exposure assessment method: records; participants are asked every 6 mo to complete a series of three validated web-based 24 h dietary records randomly assigned over a 2-wk period (2 weekdays, 1 weekend day); at least two 24 h dietary records during the first 2 yr of follow-up considered in analyses (mean ± SD, 5.6 ± 3.0)	All cancers combined, incidence	Aspartame intake (HR):			Age, sex, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, prevalent diabetes, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake	*Exposure assessment critique*: A key strength was that it was a prospective study using dietary records and quantitative assessment of aspartame based on food composition data updates for food supply changes over time. A key limitation was the baseline assessment in the main analysis but the sensitivity analysis was conducted using all 24 h dietary records available during follow-up. There was low aspartame use in the cohort (28%). *Other strengths*: large prospective cohort; large number of cases; sensitivity analyses excluded prevalent diabetes or used all available 24 h dietary records throughout follow-up.
			Non-consumers	2309	1		
			Lower consumers (men, < 14.45 mg/day; women, < 15.39 mg/day)	572	1.12 (1.02–1.23)		
			Higher consumers (men, ≥ 14.45 mg/day; women, ≥ 15.39 mg/day)	477	1.15 (1.03–1.28)		
			Trend-test *P* value: 0.002				
		All cancers combined, incidence	Aspartame intake, participants without diabetes (HR):			Age, sex, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake	
			Non-consumers	2238	1		
			Lower consumers (men, < 14.45 mg/day; women, < 15.39 mg/day)	542	1.13 (1.03–1.24)		
			Higher consumers (men, ≥ 14.45 mg/day; women, ≥ 15.39 mg/day)	439	1.16 (1.04–1.29)		
			Trend-test *P* value: 0.002				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Debras et al. (2022b) (cont.)		All cancers combined, incidence	Aspartame intake (time-dependent) (HR):			Age, sex, BMI, height, percentage weight gain during follow-up, physical activity, smoking status, number of smoked cigarettes in pack-years, educational level, number of 24 h dietary records, family history of cancer, prevalent diabetes, energy intake without alcohol, daily intakes of alcohol, sodium, saturated fatty acids, fibre, sugar, fruit and vegetables, whole-grain foods, and dairy products, other AS intake	*Other limitations*: self-selection may limit generalizability; potential for residual confounding and reverse causation.
			Non-consumers	2309	1		
			Lower consumers (men, < 14.45 mg/day; women, < 15.39 mg/day)	572	1.14 (1.05–1.25)		
			Higher consumers (men, ≥ 14.45 mg/day; women, ≥ 15.39 mg/day)	477	1.13 (1.02–1.25)		
			Trend-test *P* value: 0.003				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Fulgoni and Drewnowski (2022) USA Enrolment, 1988–1994/ follow-up, through 2019 Cohort	12 438; NHANES participants, nationally representative sample of adult men and women aged > 19 yrs; participants excluded for current pregnancy, history of myocardial infarction, chronic heart failure, stroke, cancer, or missing data; 978 cancer deaths overall (546 men, 432 women) Exposure assessment method: exposure to low-calorie sweeteners/ aspartame assessed through one or maximum two dietary recalls in several cycles of NHANES; aspartame specifically considered in NHANES 1988–1994 (beverages and added sweeteners); limited reporting of methodology	All cancers combined, mortality	Tertile of aspartame intake (HR):			Age, sex, race/ethnicity, education, current smoking status (yes/no), alcohol consumption, physical activity level, and BMI (continuous) as covariates; NHANES 1988–1994 exam weights	*Exposure assessment critique*: A key strength was the prospective assessment of aspartame exposure through 24 h recalls considering the most relevant source at the time of dietary assessment (1988–1994). Key limitations were the low number of dietary recalls (one recall for analysis with aspartame intake); and assessment at baseline only with up to 31 yr of follow-up and changes in the use of aspartame during that period. *Other strengths*: large representative cohort, large number of deaths. *Other limitations*: lacking detail on the mortality data used; likely bias from non-differential exposure misclassification.
			Non-consumers	NR	1		
			Tertile 1	NR	0.70 (0.49–0.99)		
			Tertile 2	NR	0.72 (0.49–1.06)		
			Tertile 3	NR	1.32 (0.94–1.85)		
			Trend-test *P* value: 0.9755				
		All cancers combined, mortality	Tertile of aspartame intake, men (HR):			Age, race/ethnicity, education, current smoking status (yes/no), alcohol consumption, physical activity level, and BMI (continuous) as covariates. NHANES 1988–1994 exam weights	
			Non-consumers	NR	1		
			Tertile 1	NR	0.61 (0.37–1.02)		
			Tertile 2	NR	0.55 (0.32–0.94)		
			Tertile 3	NR	1.50 (0.74–3.05)		
			Trend-test *P* value: 0.8547				
		All cancers combined, mortality	Tertile of aspartame intake, women (HR):				
			Non-consumers	NR	1		
			Tertile 1	NR	0.83 (0.52–1.34)		
			Tertile 2	NR	0.81 (0.52–1.26)		
			Tertile 3	NR	1.15 (0.81–1.65)		
			Trend-test *P* value: 0.9777				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Fulgoni and Drewnowski (2022) (cont.)		All cancers combined, mortality	Tertile of aspartame intake, age 19–50 yr (HR):			Age, sex, race/ethnicity, education, current smoking status (yes/no), alcohol consumption, physical activity level, and BMI (continuous) as covariates. NHANES 1988–1994 exam weights	
			Non-consumers	NR	1		
			Tertile 1	NR	0.38 (0.18–0.82)		
			Tertile 2	NR	0.57 (0.27–1.23)		
			Tertile 3	NR	1.54 (0.91–2.62)		
			Trend-test *P* value: 0.7825				
		All cancers combined, mortality	Tertile of aspartame intake, age 19–50 yr, men (HR):			Age, race/ethnicity, education, current smoking status (yes/no), alcohol consumption, physical activity level, and BMI (continuous) as covariates. NHANES 1988–1994 exam weights	
			Non-consumers	NR	1		
			Tertile 1	NR	0.44 (0.17–1.14)		
			Tertile 2	NR	1.19 (0.51–2.77)		
			Tertile 3	NR	1.46 (0.55–3.89)		
			Trend-test *P* value: 0.5494				
		All cancers combined, mortality	Tertile of aspartame intake, age 19–50 yr, women (HR):				
			Non-consumers	NR	1		
			Tertile 1	NR	0.35 (0.11–1.16)		
			Tertile 2	NR	0.46 (0.18–1.17)		
			Tertile 3	NR	1.28 (0.65–2.50)		
			Trend-test *P* value: 0.7151				
		All cancers combined, mortality	Tertile of aspartame intake, age 51+ yr (HR):			Age, sex, race/ethnicity, education, current smoking status (yes/no), alcohol consumption, physical activity level, and BMI (continuous) as covariates. NHANES 1988–1994 exam weights	
			Non-consumers	NR	1		
			Tertile 1	NR	0.86 (0.61–1.23)		
			Tertile 2	NR	0.65 (0.37–1.14)		
			Tertile 3	NR	0.99 (0.68–1.44)		
			Trend-test *P* value: 0.4396				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Fulgoni and Drewnowski (2022) (cont.)		All cancers combined, mortality	Tertile of aspartame intake, age 51+ yr, men (HR):			Age, race/ethnicity, education, current smoking status (yes/no), alcohol consumption, physical activity level, and BMI (continuous) as covariates. NHANES 1988–1994 exam weights	
			Non-consumers	NR	1		
			Tertile 1	NR	0.72 (0.38–1.36)		
			Tertile 2	NR	0.28 (0.13–0.56)		
			Tertile 3	NR	1.01 (0.56–1.81)		
			Trend-test *P* value: 0.0884				
		All cancers combined, mortality	Tertile of aspartame intake, age 51+ yr, women (HR):				
			Non-consumers	NR	1		
			Tertile 1	NR	1.06 (0.69–1.63)		
			Tertile 2	NR	0.95 (0.48–1.88)		
			Tertile 3	NR	1.24 (0.78–1.97)		
			Trend-test *P* value: 0.4677				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) USA Enrolment, 1982/follow-up, through 2016 (median, 27.7 yr) Cohort	934 777 (416 313 men, 518 464 women); CPS-II prospective cohort; adults aged ≥ 28 yr; excluded participants with personal history at baseline of diabetes or cancer other than nonmelanoma skin cancer, men aged > 90 yr or women aged > 95 yr at enrolment, and those reporting only prior but not current consumption of either SSBs or ASBs Exposure assessment method: questionnaire; exposure to ASBs assessed in 1982 through a question about the number of drinks/day of diet soda or ice teas (one pooled item) and potential changes over the past 10 yr; no specific assessment of aspartame content in ASBs	All cancers combined, mortality	ASB consumption (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption	*Exposure assessment critique*: A key strength was the prospective assessment of ASB consumption. Key limitations were that only one dietary assessment was carried out at baseline in 1982 that was before the use of aspartame in ASB, hence the relevance to aspartame exposure depends on the stability of ASB consumption over up to 34 yr of follow-up, but such information was not available; no other sources considered (although more limited); uncertainty of aspartame content in ASBs after the mid-2000s. Other information: exclusion of participants who reported only prior but not current consumption of either SSBs or ASBs at baseline.
			Never	NR	1		
			< 1 drink/day	NR	0.98 (0.96–0.99)		
			1 drink/day	NR	0.99 (0.97–1.01)		
			≥ 2 drinks/day	NR	0.99 (0.97–1.02)		
			Continuous (per drink/day)	135 093	1.00 (0.99–1.00)		
			Trend-test *P* value: 0.227				
		All cancers combined, mortality	ASB consumption, BMI-adjusted (HR):			Age, sex, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.96 (0.95–0.98)		
			1 drink/day	NR	0.98 (0.96–1.00)		
			≥ 2 drinks/day	NR	0.97 (0.95–0.99)		
			Continuous (per drink/day)	135 093	0.99 (0.99–1.00)		
			Trend-test *P* value: 0.001				
		All cancers combined, mortality	ASB consumption, men, BMI-adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	
			Never	NR	1		
			< 1 drink/day	NR	0.97 (0.94–1.00)		
			1 drink/day	NR	0.98 (0.94–1.01)		
			≥ 2 drinks/day	NR	0.98 (0.94–1.01)		
			Continuous (per drink/day)	70 834	1.00 (0.99–1.00)		
			Trend-test *P* value: 0.033				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
McCullough et al. (2022) (cont.)		All cancers combined, mortality	ASB consumption, women, BMI-adjusted (HR):			Age, race/ethnicity, smoking, marital status, education, red and processed meat consumption, fruit and vegetable consumption, alcohol consumption, SSB consumption, BMI	*Other strengths*: large cohort with long follow-up; ability to examine multiple cancer types, stratify by sex or BMI, and limit to never-smokers; comprehensive adjustment for confounders, including SSB consumption. *Other limitations*: likely bias from non-differential exposure misclassification.
			Never	NR	1		
			< 1 drink/day	NR	0.95 (0.93–0.97)		
			1 drink/day	NR	0.96 (0.93–0.99)		
			≥ 2 drinks/day	NR	0.95 (0.92–0.98)		
			Continuous (per drink/day)	64 259	0.98 (0.98–0.99)		
			Trend-test *P* value: < 0.0001				

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
You et al. (2022) USA Enrolment, 1993–2001/ follow-up through 2009 (median, 11.3 yr) Cohort	92 997; PLCO cancer screening trial participants, men and women aged 55–74 yr in 10 study centres; participants with history of cancer or diabetes were excluded Exposure assessment method: diet history questionnaire: FFQ with 156 items	All cancers combined, incidence	Type of soft drink consumption (HR):			Age, sex, race, study centre, arm, total energy intake, alcohol consumption, smoking status, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer	*Exposure assessment critique*: A key strength was the timing of exposure, which was consistent for aspartame being the major AS in beverage; ASBs were the major source of aspartame in this country and time frame. Key limitations were that there was no specific estimate of aspartame exposure, ASBs were used as a proxy; and only consumption vs non-consumption was considered (not dose); exposure data were reported at baseline only. *Other strengths*: prospective analysis. *Other limitations*: likely bias from non-differential exposure misclassification.
			No soft drink consumption	1089	1		
			Regular only	5202	1.03 (0.97–1.11)		
			Diet only	4557	1.06 (0.99–1.13)		
			Both	2078	0.99 (0.92–1.07)		
		All cancers combined, incidence	Type of soft drink consumption, men (HR):			Age, race, study centre, arm, total energy intake, alcohol consumption, smoking status, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer	
			No soft drink consumption	539	1		
			Regular only	3532	1.02 (0.93–1.12)		
			Diet only	2221	1.04 (0.94–1.14)		
			Both	1368	0.98 (0.89–1.09)		
		All cancers combined, incidence	Type of soft drink consumption, women (HR):			Age, race, study centre, arm, total energy intake, alcohol consumption, smoking status, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer, estrogen use	
			No soft drink consumption	[550]	1		
			Regular only	[1670]	1.03 (0.93–1.14)		
			Diet only	[2336]	1.07 (0.98–1.18)		
			Both	[710]	1.00 (0.89–1.12)		

Table 2.8 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
You et al. (2022) (cont.)		All cancers combined, incidence	Type of soft drink consumption, never-smokers (HR):			Age, sex, race, study centre, arm, total energy intake, alcohol consumption, BMI categories (baseline), physical activity, education, red meat intake, amounts of fruits and vegetables, coffee, family history of lung cancer	
			No soft drink consumption	446	1		
			Regular only	2183	1.04 (0.94–1.15)		
			Diet only	1858	1.09 (0.98–1.21)		
			Both	953	1.03 (0.92–1.16)		
		All cancers combined, incidence	Type of soft drink consumption, ever/current smokers (HR):				
			No soft drink consumption	643	1		
			Regular only	3017	1.04 (0.95–1.14)		
			Diet only	2699	1.02 (0.93–1.11)		
			Both	1124	0.95 (0.86–1.05)		

AICR, American Institute of Cancer Research; AS, artificial sweetener; ASB, artificially sweetened beverage; BMI, body mass index; CI, confidence interval; CPS-II, Cancer Prevention Study-II; CVD, cardiovascular disease; EPIC, European Prospective Investigation into Cancer and Nutrition; FDA, Food and Drug Administration; FFQ, food frequency questionnaire; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; mo, month(s); NHANES, National Health and Nutrition Examination Survey; NHS, Nurses' Health Study; NR, not reported; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer screening trial; RR, relative risk; SD, standard deviation; SSB, sugar-sweetened beverage; WCRF, World Cancer Research Fund; wk, week(s); yr, year(s).

was its estimation of aspartame intake from all dietary sources using brand-specific and time-matched composition data, which no other study provided; limitations included the relatively small proportion of aspartame users, meaning that the study could not evaluate finer classifications of amount consumed.]

In a prospective analysis of 80 647 women from the NHS cohort and 37 716 men from the HPFS cohort (Malik et al., 2019), 12 380 deaths from cancer occurred during a 34-year follow-up. ASB consumption was assessed 7–8 times during follow-up from 1980 in the NHS and 1986 in the HPFS, both studies ending follow-up in 2014. No association with all cancer mortality was observed (HR for ASB consumption of ≥ 2/day versus < 1/month, 1.04; 95% CI, 0.96–1.12). [An important strength of this analysis was that ASB consumption was assessed on multiple occasions, and the data were updated each time to account for changes in intakes. However, the results from this analysis were similar to those using the cumulative average of intake (HR for ASB consumption of ≥ 2/day versus < 1/month, 0.91; 95% CI, 0.84–1.00). As with other studies, the main limitations related to the lack of information on other sources of aspartame and the contribution of aspartame to artificial sweeteners used in beverages over time, although follow-up included the period when aspartame was the main artificial sweetener and soft drinks were the main source of aspartame in the USA.]

In the EPIC study, which included cohorts from seven countries (ASB data were not collected in all centres in Italy, Spain, or Sweden; therefore, those countries were excluded from this analysis) and more than 12 000 cancer deaths, the hazard ratio for ≥ 2 glasses/day of artificially sweetened soft drinks versus < 1 glass/month was 1.10 (95% CI, 0.97–1.23) (Mullee et al., 2019). [EPIC is a large prospective study with many cases, and the results were adjusted for appropriate potential confounders. The Working Group considered results adjusted for BMI to be the most informative. Although data were collected during a relevant time period for aspartame use in Europe, the collection of intake data at a single time point and the variation in products across countries and over time may lead to exposure misclassification and bias towards the null.]

Zhang et al. (2021) analysed NHANES data from 1999–2014 in relation to cancer mortality. Intake data were based on two 24-hour dietary recalls since 2003 (n = 22 348) or a single 24-hour dietary recall before 2003 (n = 9054). After a mean follow-up of 7.9 years, 3878 deaths, including 883 cancer deaths, were identified. A hazard ratio of 0.61 (95% CI, 0.35–1.04) was found for consumption of ≥ 2 servings/day of ASBs relative to no consumption. [The Working Group noted the wide confidence interval for this estimate. Notable strengths of the study included consideration of nonlinear associations using cubic splines analysis and use of a representative sample. The major weakness, as in most other studies, was the lack of specific data on aspartame intakes, and also that ASB intake may not be a good proxy for aspartame intake after 2003, the relatively small number of cancer deaths, and use of one or two dietary recalls that may not represent usual intake.]

Using 24-hour recall data from 12 438 participants aged > 19 years in the 1988–1994 NHANES survey, Fulgoni and Drewnowski (2022) evaluated aspartame intake in relation to all-cancer mortality. Results were reported overall and stratified by both sex and age (19–50 years, ≥ 51 years). For all ages combined, the hazard ratio was 1.32 (95% CI, 0.94–1.85; P for trend, 0.9755) for the top tertile of intake relative to non-consumers (Fulgoni and Drewnowski, 2022). [The Working Group acknowledged the wide confidence interval and the lack of a trend across increasing intakes. Strengths were the nationally representative sample and calculation of aspartame intake from diet beverages and tabletop sweeteners, which were the main sources of aspartame at the time; weaknesses included

that specific aspartame information was limited to the 1988–1994 cycle (NHANES III), so that only a smaller group with 978 deaths could be included in this analysis. Intake estimates were based on a single recall so could not be assumed to be representative of usual intake. No information was provided on mortality data, so it was not clear how cancer mortality was defined. Despite the potential strength of using specific aspartame intake in this analysis, the unclear outcome definition meant that the earlier analysis of NHANES data was considered to be more informative, although both analyses were consistent in not finding strong evidence linking aspartame or ASB intake with cancer mortality.]

During a median follow-up of 27.7 years, 135 093 CPS-II participants died from cancer (25.9% of all deaths) (McCullough et al., 2022). The cancer mortality hazard ratio for ≥ 2 drinks of ASBs relative to none was 0.99 (95% CI, 0.97–1.02) without adjustment for BMI and 0.97 (95% CI, 0.95–0.99) with BMI adjustment. The study used only a single assessment of ASB intake at baseline, which was before the approval and use of aspartame for soft drinks in the USA in 1983. [The Working Group considered that the results adjusted for BMI were the most informative. The strengths of the CPS-II study were its large size and prospective design. The Working Group noted that the relevance of this study with regard to aspartame exposure depended on the stability of ASB consumption over time since the introduction of aspartame into ASBs in 1983 in the USA. Although no information was available regarding potential variations in ASB intake over time during the (up to) 34 years of follow-up, some data from McCullough et al. (2014) in the CPS-II cohort suggested a stable ranking of participants with respect to ASB consumption between 1982 and 1999 for the subsample providing data at both time points. The Working Group still noted uncertainty regarding aspartame content in ASBs from the mid-2000s. Non-differential misclassification was therefore likely, probably leading to bias towards the null.] The only study reporting on the interaction between sex and ASB consumption (McCullough et al., 2022) did not find evidence of an effect of sex on the association (*P* for interaction, 0.145).

You et al. (2022) analysed prospective data from the PLCO Cancer Screening Trial, which followed more than 90 000 men and women for a median of 11.3 years. Hazard ratios slightly greater than 1 were found for use of diet soft drinks only, relative to no soft drink consumption, for several subgroups: overall HR, 1.06; 95% CI, 0.99–1.13; HR for women, 1.07; 95% CI, 0.98–1.18; HR for never-smokers, 1.09; 95% CI, 0.98–1.21. [A major limitation of this study was that it assessed soft drink intake in only four categories: none, regular only, diet only, and both regular and diet soft drinks, with no quantification or assessment of aspartame.]

Five meta-analyses were identified (Zhang et al., 2021; Yin et al., 2022; Pan et al., 2022, 2023; and Yan et al., 2022), none of which found evidence of an association between exposure and overall cancer mortality or incidence. [The Working Group determined that three of these studies did not contribute useful information, since too few studies were included. For example, only two studies were included in Zhang et al., with no overall risk estimate provided, and Pan et al. (cancer mortality in Pan et al., 2022; and cancer incidence in Pan et al., 2023) included four and two cohorts, respectively.] Yan et al. (2022) and Yin et al. (2022) identified more studies, including the study by Debras et al. (2022b) that specifically evaluated aspartame. [A limitation of the meta-analyses by Yin et al. (2022) and Yan et al. (2022) when estimating risk estimates for "overall cancer" pertained to their combining of individual reported cancer sites to produce "all cancer" estimates.]

2.8 Evidence synthesis for cancer in humans

This section provides a synthesis of studies of consumption of aspartame and ASBs and/or artificial sweeteners containing aspartame in relation to the risk of human cancer at various organ sites. A detailed definition of aspartame, as the agent of investigation in the present monograph, has been provided in Section 1.1. It is important to note that the body of evidence regarding the carcinogenicity of aspartame in humans was for the most part derived from several studies that assessed consumption of ASBs and only a few studies that more specifically focused on aspartame. In several cases, no clear distinction could be made between consumption of drinks and foods containing aspartame versus other artificial sweeteners, in which case the evidence was considered relevant only if the timing of the exposure assessment could be aligned with the time when aspartame was the prevailing artificial sweetener used in the respective country or region. Only the NutriNet-Santé prospective cohort provided information on exposure to aspartame across the entire spectrum of sources (beverages, dairy, tabletop sweeteners, and other sources, together with brand-specific information) with repeated 24-hour diet record assessments during the follow-up period. Two prospective cohort studies from the USA (NHS and HPFS) assessed aspartame intake from the two main sources (diet soft drinks and tabletop sweeteners, representing more than 90% of all aspartame-containing products on the market in the USA between the mid-1980s and mid-2000s) repeatedly (every 4 years) over two decades that specifically covered the time period when aspartame was approved for use and when it represented by far the most prevalent artificial sweetener in the USA (Rimm et al., 1992; Feskanich et al., 1993). The EPIC study measured diet with diet history questionnaires, including assessment of several hundreds of simple foods, mixed foods, and recipes and drinks that were adapted to the local diet. Diet questionnaires were complemented by 24-hour diet records collected in about 10% of the cohort (Riboli et al., 2002). In most of the participating centres, information on consumption of ASBs was collected (Navarrete-Muñoz et al., 2016; Stepien et al., 2016; Mullee et al., 2019; Heath et al., 2021; Zamora-Ros et al., 2022).

2.8.1 Studies evaluated

The association between aspartame or ASB consumption and different cancers was investigated in 12 prospective cohort studies and 13 retrospective case–control studies. Cancers that were studied more extensively were those of the digestive tract, including the colon and rectum (seven cohort and three case–control studies), liver (three studies including four cohorts), and pancreas (five cohort and four case–control studies); breast (six cohort and three case–control studies); urinary tract (four cohort and three case–control studies); prostate (five cohort and two case–control studies); and lymphatic and haematopoietic tissues (four cohort studies including five cohorts and one case–control study). Fewer studies were available for other cancer types such as brain (two cohort and two case–control studies), uterus (three cohort studies and one case–control study), thyroid (one cohort and one case–control study), larynx (one case–control study), ovary (two cohort studies, one case–control study), stomach (two cohort studies, three case–control studies), oesophagus (one cohort study, one case–control study), or lung (three cohort studies including four cohorts). With few exceptions (Australia, Argentina), these studies were conducted in the USA and Europe. In all these countries, the use of artificially sweetened products, containing aspartame as the main or one of the main artificial sweeteners, has been common over the past few decades; several detailed nutritional cohort

studies have been conducted, allowing for assessment of the association between aspartame use (primarily as its main dietary source, ASBs) and cancer. Although the studies were conducted in a limited geographical area, the results can probably be generalized to other populations. The studies were conducted in various regions of the USA and countries within Europe, with substantial diversity in dietary and lifestyle habits, as well as different cancer prevalence rates and average amounts of aspartame consumption.

In assessing the carcinogenicity of aspartame use, substantial weight was given to the results from the NutriNet-Santé study (Hercberg et al., 2010; Debras et al., 2022b), a prospective web-based cohort study of 102 865 individuals who had a median follow-up of 7.8 years (2009–2021). The NutriNet-Santé study collected detailed information on consumption of ASBs and other dietary sources of artificial sweeteners, by type, via multiple 24-hour dietary records collected every 6 months. Baseline diet was the average of all records over the first 2 years of follow-up, and all participants had at least two individual diet records over this period (92.3% had three or more records, Chazelas et al., 2019). In sensitivity analyses, a model with a time-dependent exposure variable used yearly average aspartame intake, thereby taking advantage of all available measurements conducted during the follow-up. The NutriNet-Santé study was the only one that applied a robust methodology for estimating individual food additives and was able to specifically single out exposure to aspartame, versus exposure to other sweeteners, by building a brand- and year-specific food composition table including specific data on the artificial sweetener content of drinks and foods. Although the NutriNet-Santé study results offered high-quality data, the sample sizes for incident cancer cases during the follow-up period tended to be large enough to examine individually only a few cancer sites (e.g. prostate and breast). Another characteristic of the NutriNet-Santé study was that, compared with other studies, a small proportion (28%) of cohort members were aspartame users, so the study could not look at finer stratification of the amount consumed. In addition, the potential for self-selection of women who were older, more health-conscious, and better educated may limit the generalizability of the results of the study, although the adjustment for many confounders minimized concerns about biases in risk estimates.

Several other cohort studies provided results on aspartame or ASB exposure and the risk of cancer. In particular, the NHS and HPFS (Schernhammer et al., 2005, 2012) used cumulatively updated diet assessments from the 1980s for up to two decades (based on repeated FFQs), potentially capturing very long-term exposure to aspartame from sodas and tabletop sweeteners in these populations. The studies controlled for BMI at baseline and updated diabetes diagnoses during follow-up. As noted above, other informative prospective cohort studies for the evaluation included the EPIC cohort and the pooled PLCO-NIH-AARP cohort.

In addition to the eleven cohort studies from the USA and Europe, one cohort study in Australia also provided results on several different cancer end-points: The MCCS used an FFQ to quantify the frequency of consumption of diet soft drinks at baseline in 1990–1994, which was after the approval of aspartame use in foods and drinks in Australia in 1986 (Hodge et al., 2018).

Of the total 13 case–control studies, only 11 were considered informative, although the degree to which each study was informative varied substantially. These studies were exclusively conducted in the USA and in Europe, except for a case–control study from Argentina (Andreatta et al., 2008). As generally is the case, the case–control studies were at greater risk of selection and information bias, and they tended to have relatively small numbers of cases. Some case–control studies were adjusted for multiple confounders, presented exposure–response anal-

yses, provided results stratified by BMI, and incorporated exposure only up to a certain period before diagnosis, avoiding reverse causation; however, other case–control studies were less informative because of a lack of information in one or more of the areas discussed above.

Case series, cross-sectional studies, and ecological studies were reviewed and considered by the Working Group but ultimately not included in this review because they were uninformative for the assessment of the association between aspartame consumption and cancer. In some cohort study publications, the outcome was cancer mortality, rather than incidence (NHS, HPFS: Malik et al., 2019; EPIC: Mullee et al., 2019, Heath et al., 2021; CPS-II: McCullough et al., 2022); however, other reports from the same cohorts presented data on cancer incidence for certain cancer types (NHS, HPFS: Schernhammer et al., 2005, 2012; EPIC: Stepien et al., 2016, Navarrete-Muñoz et al., 2016, Heath et al., 2021; with the latter reporting on both incidence and mortality; CPS-II: McCullough et al., 2014). The results of the reports on cancer incidence were qualitatively and quantitatively similar to those of the reports focused on cancer mortality. [The Working Group noted that, for cancer sites with medium to high lethality rates, mortality may be an acceptable proxy for incidence.]

2.8.2 Exposure assessment and misclassification of exposure

The quality of the exposure assessment was a limitation of all except one of the studies evaluated. Most of the existing studies of cancer in humans adopted different types of dietary assessment instruments, including FFQs, food consumption history questionnaires, and repeated 24-hour dietary recalls or records to estimate consumption of ASBs and use of tabletop sweetener packets. The EPIC study used more elaborate questionnaires on habitual diet across hundreds of country- and region-specific foods, separate assessment of usual portions and frequency of consumption, and consumption of foods and drinks during different seasons of the year. Other studies considered artificial sweeteners overall or ASB, and only five cohort studies (including six cohorts) (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014; Debras et al., 2022b; Fulgoni and Drewnowski, 2022) specifically considered aspartame as the exposure. Four of these studies (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014; Fulgoni and Drewnowski, 2022) were based in the USA and derived aspartame exposure from the consumption of ASBs and the use of aspartame tabletop sweetener packets, assessed through FFQs. The more recent cohort study, the French NutriNet-Santé study (Debras et al., 2022b), was the only one that derived aspartame exposure from all aspartame-containing foods and beverages assessed through repeated 24-hour dietary records, considering variations between products or brands and over time and with repeated exposure assessments. [The Working Group noted that the USA-based studies, even though they only considered the two main sources of aspartame (ASBs and tabletop packets), were likely to have captured more than 90% of all aspartame intake because of the time period during which they were conducted and the high prevalence of aspartame as the main or sole artificial sweetener used in the USA at that time (Rimm et al., 1992; Feskanich et al., 1993).]

A main limitation of many of the other cohort studies was that exposure was assessed only at a single time point, at baseline. Misclassification of exposure is inevitable when there is a single assessment and follow-up over longer time periods; however, because information on aspartame exposure was collected before the occurrence of disease in the prospective cohort studies, the resulting non-differential (with respect to cancer outcome) misclassification would tend to

bias results towards the null. Exposure assessments in the case–control studies were similarly incomplete (i.e. based on the assessment of consumption of ASBs or tabletop sweetener packets containing aspartame only) and additionally subject to recall bias, which is inherent in the design of retrospective case–control studies. In fact, recall bias (a potential limitation of all the included case–control studies) may bias estimates in either direction if cases were more likely than controls to recall artificial sweetener consumption (or vice versa). Exposure misclassification and the potential for selective exposure in relation to past medical history (e.g. selective consumption of ASBs among those newly diagnosed with diabetes) may occur to a various degree in both the cases and the controls; on the other hand, both may contribute to the potential for a bias towards the null.

Variation in aspartame consumption is driven by the geographical context or period and resulting differences in the type of artificial sweetener and amount of aspartame used in ASB, as well as an increase over time in the range of products (beyond ASBs and tabletop sweetener packets) containing aspartame, including the emergence of "diet" versions of yogurt, ice cream, or breakfast cereals. Mixed exposure to a variety of artificial sweeteners is also likely to have introduced the potential for exposure misclassification both in the exposed and reference groups, which would probably be non-differential with respect to cancer outcome, and to cause bias towards the null.

The NutriNet-Santé study conducted repeated assessments collecting detailed data on aspartame consumption, which included duration, frequency, and amount of use. Given the detailed and frequent repeat assessments of diet and the use of food composition databases to derive aspartame content for each single dietary item consumed, the data provided by NutriNet-Santé (Debras et al., 2022b) were the most detailed and therefore the quality of the exposure assessment was high. The Working Group considered differences between the results for this very detailed cohort study and those of the other available cohort studies and concluded that the accuracy of the NutriNet-Santé study exposure data may have provided a relatively more specific and precise exposure assessment, but that the three USA-based cohort studies (describing four cohorts) (Lim et al., 2006; Schernhammer et al., 2012; McCullough et al., 2014) were also of particular interest because they probably captured most of the aspartame consumed in these populations.

2.8.3 Confounding and selection bias

In its assessment of causality for consumption of aspartame or artificial sweeteners and risk of various cancers, the Working Group used directed acyclic graphs (DAGs) to determine the major confounding factors that should be adjusted for when estimating the effect of aspartame on cancer risk. The Working Group concluded that age, sex, BMI, socioeconomic status, diabetes, and sugar and/or sugar-sweetened beverages represented the minimal sufficient adjustment sets for estimating the effect of aspartame on certain cancers. Additionally, for specific cancer types (e.g. breast, liver), relevant cancer-specific confounders were also considered. The DAG drawn for liver cancer assessment is reported in Fig. S2.1 (see Annex 3, Supplementary material for Section 2, Cancer in Humans, also available from: https://publications.iarc.who.int/627). The consumption of sugar-sweetened beverages and sugar intake more generally was included because it may affect cancer risk either directly (e.g. Hur et al., 2021; Laguna et al., 2021; Yuan et al., 2022) or indirectly through its influence on BMI (Faruque et al., 2019). Aspartame use or the consumption of artificial sweeteners, although not entirely consistently, tended to be higher in individuals who have higher BMI (e.g. Bleich et al., 2014), diabetes (Mackenzie et al., 2006),

lower socioeconomic status, and a generally unhealthier lifestyle. All these attributes are also associated with increased risk of several cancers (e.g. Giovannucci et al., 2010). Most studies either adjusted for or matched on sex and age and adjusted for BMI. Likewise, many studies adjusted for lifestyle variables or stratified their results by BMI and diabetes. The results from the NutriNet-Santé study (Debras et al., 2022b), which were based on the most specific aspartame exposure assessment, were also thoroughly adjusted for the major confounders, including age, sex, lifestyle, and BMI. In sensitivity analyses, cases in participants with a history of diabetes at baseline were excluded.

Case–control studies varied in their adjustment for confounders; most adjusted for age, sex, and BMI; however, selection bias remained a concern in these studies. With the exception of one case–control study in Italy (Gallus et al., 2007; Bosetti et al., 2009), some of the population-based (e.g. Mayne et al., 2006, as reported in Gammon et al., 1997; Chan et al., 2009; Palomar-Cros et al., 2023, as reported in Castaño-Vinyals et al., 2015) and hospital-based (e.g. Davis et al., 2023) case–control studies may have been subject to selection bias because of low participation rates, if those who chose to participate varied from the source population in terms of sweetener use.

2.8.4 Reverse causation

It should be considered whether the association between aspartame consumption and cancer may be subject to reverse causation. In patients with a cancer diagnosis, early symptoms could potentially have led to changes in diet soda consumption habits, although the direction of any such changes remains speculative. Major reverse causation bias entirely explaining the observed associations is unlikely in prospective cohorts, since the participants did not have cancer at baseline. Some of the studies (e.g. Bao et al., 2008; Navarrete-Muñoz et al., 2016; Malik et al., 2019; Debras et al., 2022b) also applied lag-time analyses, excluding cases diagnosed during various periods of follow-up, and the results tended to remain unchanged.

2.8.5 Cancer of the liver

Three publications reporting on four cohorts (Stepien et al., 2016; Jones et al., 2022; McCullough et al., 2022) examined the association between ASB consumption and liver cancer risk. No other studies investigated associations with liver cancer. The Working Group carefully considered potential confounders at baseline of an association between aspartame and liver cancer (Fig. S2.1, Annex 3, Supplementary material for Section 2, Cancer in Humans, also available from: https://publications.iarc.who.int/627). The study in the EPIC cohort (Stepien et al., 2016) found that each increment of one 330 mL serving of artificially sweetened soft drinks per week was associated with an increased risk of hepatocellular cancer (HR, 1.06; 95% CI, 1.03–1.09; 151 cases). [The Working Group noted that this study was well adjusted for confounders for liver cancer, including BMI (height and weight measured at baseline by trained investigators), baseline diabetes, alcohol consumption, and smoking, and the investigators evaluated potential confounding by coffee and sugar-sweetened beverage intake. Although HBV or HCV infection is a strong risk factor for liver cancer, it is unlikely that there is an association between aspartame consumption and HBV or HCV infection, making it unlikely that HBV and HCV are confounders of the association between aspartame consumption and liver cancer. An association between aspartame and HBV or HCV via diabetes (Cacoub and Saadoun, 2021) is plausible but was addressed by adjustment for diabetes at baseline. Moreover, the prevalence of HBV or HCV infection in the EPIC cohort was very low (about 3%) (Trichopoulos et al., 2011).]

A pooled analysis of the NIH-AARP and PLCO cohorts (Jones et al., 2022) found a positive association with risk of liver cancer during the first 12-year follow-up interval among participants with diabetes at baseline (but not among those without diabetes). This interval corresponds to a time period when aspartame was the main artificial sweetener used in ASBs in the USA (see Section 1.6.1(c)). The hazard ratios for units of consumption per day were 1.13 (95% CI, 1.02–1.25) for ASBs overall; 1.13 (95% CI, 1.01–1.27) for artificially sweetened soda; and 1.17 (95% CI, 0.92–1.48) for artificially sweetened fruit punch. All findings were null for the second follow-up period beyond 12 years since baseline. These findings indicated that diabetes could be an effect modifier of the association, possibly due to greater ASB exposure; however, it was unclear whether biological mechanisms specific to individuals with diabetes could play a role in explaining these findings.

The study in the CPS-II cohort (McCullough et al., 2022) reported no association with liver cancer risk overall. There was a weak indication that, in analyses restricted to male never-smokers, the association between ≥ 2 drinks/day of artificially sweetened drinks (compared with 0 drinks/day) and liver cancer was positive (HR, 1.44; 95% CI, 0.99–2.08; *P* for trend, 0.040), although the hazard ratio was 1.21 after adjustment for BMI (*P* for trend, 0.335). Therefore, this study did not refute the findings of a positive association reported in the other studies. [The Working Group noted that a unique feature of this cohort was that its large size allowed restriction to never-smokers and stratification by sex. Additionally, participants with a history of diabetes at baseline were excluded in all analyses.]

A recent study by Debras et al. (2023) in the NutriNet-Santé cohort showed a positive association between aspartame consumption and diabetes incidence; therefore, it is possible that diabetes may be on a causal pathway between aspartame consumption and liver cancer risk. All these cohort studies controlled for baseline diabetes, by adjustment (Stepien et al., 2016), stratification (Jones et al., 2022), or exclusion (McCullough et al., 2022). These cohort studies did not control for incident diabetes during cohort follow-up, thereby allowing insulin resistance or diabetes to contribute to observed positive associations between aspartame and liver cancer. Other mechanisms may also play a role, since associations with ASB consumption were observed among diabetic patients.

The Working Group concluded that the three studies, which included four cohorts, examining the association between ASB consumption and cancer of the liver were informative. Other studies, including the highly informative NutriNet-Santé study, did not investigate liver cancer separately. Although some level of non-differential misclassification may exist in these studies, which would probably bias associations towards the null, the positive and statistically precise associations between aspartame use and liver cancer identified by these studies were considered to be important evidence. Although bias could not be ruled out with reasonable confidence, the findings demonstrated consistency, and the studies that were reviewed provided credible evidence for a positive association with liver cancer.

2.8.6 Cancer of the breast

The Working Group evaluated six cohort studies (NutriNet-Santé, NHS, NHS-II, CPS-II, EPIC, MCCS) and three case–control studies on consumption of aspartame, or ASBs or tabletop sweetener packets containing aspartame, and the risk of breast cancer.

The NutriNet-Santé study (Debras et al., 2022b) found a significantly increased risk of breast cancer overall (HR, 1.22; 95% CI, 1.01–1.48; *P* for trend, 0.036) for women with higher aspartame consumption, i.e. above the median for female aspartame users, compared with

non-users. The two NHS cohorts, NHS and NHS-II (Romanos-Nanclares et al., 2021), also had repeated diet exposure updates throughout follow-up. The studies found no increased risk of breast cancer associated with ASB consumption. Similarly, consumption of ASBs was not associated with increased breast cancer mortality (Malik et al., 2019) in the NHS cohort. The CPS-II cohort study (McCullough et al., 2022) adjusted in detail for confounding, including for BMI, and reported no association between consumption of ASBs and postmenopausal breast cancer risk and mortality. The EPIC cohort study (Mullee et al., 2019) reported no positive association between artificially sweetened soft drinks and breast cancer mortality, although a lower breast cancer mortality was observed among women consuming 1–4 glasses/month (HR, 0.79; 95% CI, 0.63–0.98) versus < 1 glass/month. Also, the study in the MCCS cohort (Hodge et al., 2018) found no association between consumption of artificially sweetened soft drinks and breast cancer risk.

Of the three case–control studies, one (Ewertz and Gill, 1990) was considered uninformative because the assessment of artificial sweetener consumption preceded approval for aspartame in the study country (Denmark). Another case–control study (Gallus et al., 2007) was conducted in Italy and reported an inverse association between breast cancer and consumption of artificial sweeteners other than saccharin. The large multicase–control study in Spain (MCC-Spain) (Palomar-Cros et al., 2023), investigated associations between use of artificial sweeteners and low- or no-calorie sweetened beverages and breast cancer risk (1510 cases). Overall, no increased risk of breast cancer was observed among women with high aspartame consumption (defined as third quartile or above). Among participants with diabetes, there was an inverse association when comparing high consumers with non-consumers (OR, 0.28; 95% CI, 0.08–0.83; *P* for trend, 0.03).

In sum, with the exception of one study in which aspartame was measured specifically and that suggested an elevated risk of breast cancer, the available studies on ASB consumption and breast cancer did not show positive associations. Although the high-quality NutriNet-Santé study (Debras et al., 2022b) found that intake of aspartame was associated with higher risk of breast cancer, the Working Group considered that, because of the potential for misclassification and inconsistent findings, the available studies were of insufficient informativeness about aspartame exposure and insufficient consistency to permit a conclusion to be drawn on the presence or absence of a causal association between aspartame consumption and breast cancer risk.

2.8.7 Cancers of lymphatic and haematopoietic tissues

The association between proxies of aspartame exposure and lymphatic and haematopoietic cancer risk was assessed by four cohort studies (NIH-AARP, NHS, HPFS, CPS-II). One case–control study (MCC-Spain) also contributed to this evaluation.

A study in the NHS and HPFS cohorts (Schernhammer et al., 2012) evaluated associations between aspartame-containing soda and incidence of haematopoietic cancers. With 22 years of follow-up and repeated exposure assessments covering the relevant period of aspartame use in ASBs in the USA, this study was particularly informative, comprising 1324 cases of NHL, 285 cases of multiple myeloma, and 339 cases of leukaemia. Compared with men who did not consume diet soda, men who consumed ≥ 1 daily servings of diet soda had an increased risk of NHL (RR, 1.31; 95% CI, 1.01–1.72; *P* for trend, 0.11) and multiple myeloma (RR, 2.02; 95% CI, 1.20–3.40; *P* for trend, 0.01, respectively). No association was observed for women (RR for NHL, 1.00; 95% CI, 0.78–1.26; *P* for trend, 0.999; and RR for multiple myeloma, 0.79; 95% CI, 0.45–1.36; *P*

for trend, 0.79). When data from the two cohorts were combined in a pooled analysis to maximize statistical precision, diet soda intake was associated with an increased risk of leukaemia (RR, 1.42; 95% CI, 1.00–2.02; *P* for trend, 0.05). Starting follow-up in 1994, when aspartame had become more widely used after the expiry of the patent in 1992, observed associations were confirmed among men, and an interaction with alcohol consumption was reported. Specifically, with follow-up from 1994, in the analysis among men, an association was observed between aspartame exposure (estimated from diet soda and aspartame packets consumed) and increased risk of NHL (RR for ≥ 143 mg/day versus none, 1.64; 95% CI, 1.17–2.29; *P* for trend, 0.002) and multiple myeloma (RR for ≥ 143 mg/day versus none, 3.36; 95% CI, 1.38–8.19; *P* for trend, 0.05); these associations were not observed in women. For leukaemia, the RR for ≥ 143 mg/day versus none was 1.56 (95% CI, 0.79–3.06; *P* for trend, 0.17) in men.

Two analyses from CPS-II reported on the association between ASB intake and lymphoid neoplasms. McCullough et al. (2022) examined ASB consumption and mortality from lymphoid neoplasms in the full CPS-II mortality cohort (at baseline in 1982, there were almost 935 000 participants), and McCullough et al. (2014) examined ASB consumption and estimated aspartame consumption in relation to NHL risk among 100 000 men and women in the CPS-II nutrition cohort (baseline, 1999), a subset of CPS-II participants followed for cancer incidence. Over 10 years of follow-up, no association was detected between consumption of artificially sweetened carbonated beverages or aspartame and NHL incident risk overall or by subtypes (multiple myeloma, diffuse large B-cell lymphoma, CLL/small lymphocytic lymphoma, and follicular and other B-cell lymphomas). Positive associations for estimated aspartame exposure from ASBs plus aspartame packets were observed for total NHL, diffuse large B-cell lymphoma, follicular, and other B-cell lymphomas in quintiles 2 and/or 3, but the associations were not linear across the range of intakes. For mortality in the parent CPS-II cohort (McCullough et al., 2022), no association was detected for NHL, multiple myeloma, or leukaemia in men, women, or sex-combined analyses. An interaction with BMI was detected for NHL: an inverse association for NHL was seen among people with a BMI of 18.5 to < 25 kg/m^2 (*P* for trend, 0.035; *P* for interaction, 0.029), whereas there was no association in the groups of people with a BMI of 25–30 kg/m^2 (*P* = 0.274) or BMI of > 30 kg/m^2 (*P* = 0.896).

The NIH-AARP study (Lim et al., 2006) reported no association between aspartame intake and all haematopoietic cancers (overall or in either men or women), Hodgkin lymphoma, multiple myeloma, NHL (or in two of its subtypes, small lymphocytic lymphoma and CLL; immunoblastic lymphoma and lymphoblastic lymphoma/leukaemia) and non-lymphoid leukaemia. The study was limited by a single baseline exposure assessment, relatively short follow-up (maximum, 5 years), and high age at study entry (mean, 62 years at baseline).

The MCC-Spain study (Palomar-Cros et al., 2023) found no association between high consumption of aspartame-containing products (low- or no-calorie soft drinks and tabletop sweeteners other than saccharin) and CLL risk overall.

The Working Group concluded that the body of evidence from four cohort studies, which did not include the NutriNet-Santé cohort, was small and did not consistently indicate a positive association between aspartame intake and cancers of lymphatic and haematopoietic tissues. Given the small number of studies and the diversity of their sizes, end-point definitions, and exposure definitions (each conveying some uncertainty), and despite the fact that some studies suggested an increased risk, no conclusion could be drawn on the presence or absence of a causal association

between aspartame consumption and risk of cancers of lymphatic and haematopoietic tissues.

2.8.8 Cancer of the pancreas

Four cohort studies, which included five cohorts, (Schernhammer et al., 2005; Bao et al., 2008; Navarrete-Muñoz et al., 2016; McCullough et al., 2022) and four case–control studies (Norell et al., 1986; Bosetti et al., 2009; Chan, et. al., 2009; Davis et al., 2023) examined the association between artificial sweetener exposure and pancreatic cancer risk. The results from the cohort studies were mixed: some findings were null (Schernhammer et al., 2005; Bao et al., 2008) but others were positive, including in one study overall (McCullough et al., 2022) and in another study among men but not women (Navarrete-Muñoz et al., 2016). In the CPS-II cohort, McCullough et al. (2022) reported an 11% increase in risk of pancreatic cancer in people consuming artificially sweetened drinks at a frequency of ≥ 2 drinks/day versus never (HR per ≥ 2 drinks/day, 1.11; 95% CI, 1.02–1.20) after adjusting for BMI in the multivariable-adjusted model; the risk among never-smokers was also elevated (HR per ≥ 2 drinks/day, 1.19; 95% CI, 1.05–1.34). Using data from seven participating countries in the EPIC cohort, the association between artificially sweetened soft drink consumption and adenocarcinoma of the exocrine pancreas was studied (Navarrete-Muñoz et al., 2016). Although no association between artificially sweetened soft drink consumption of > 92.2 g/day versus 0.1–2.0 g/day and pancreatic cancer risk was reported in models for men and women combined, a 25% increase in risk was observed in continuous models (per 336 g/day) among men but not women (*P* for interaction by sex, 0.004). In the NHS and HPFS cohort studies (Schernhammer et al., 2005), the investigators observed no association between frequency of diet soft drink consumption and pancreatic cancer risk. The study used repeated assessment of ASB, finely controlled for potential confounders, and stratified by BMI. The fact that overall consumption of ASBs was low in these cohorts was one of the limitations of this otherwise informative study. The NIH-AARP study (Bao et al., 2008) also found no association between diet soft drink consumption and pancreatic cancer risk. In sensitivity analyses, the first 2 years of follow-up were excluded to rule out an effect of subclinical pancreatic cancer on added sugar intake, again finding no association (*P* for trend, 0.19).

Among the four case–control studies, no association was observed between artificial sweetener consumption and pancreatic cancer in a small case–control study in Sweden (Norell et al., 1986) or between diet cola consumption and pancreatic cancer risk in a case–control study in the USA (Davis et al., 2023). Another US population-based case–control study (Chan et al., 2009) examined sugar-free carbonated beverage consumption and adenocarcinoma of the exocrine pancreas, finding a higher risk of pancreatic cancer with some indication of potential effect modification by diabetes: risk was elevated among participants without diabetes (OR for ≥ 1 serving/day versus 0, 1.6; 95% CI, 1.1–2.3), but not among those with diabetes (OR, 0.9; 95% CI, 0.3–2.6). No associations were found between consumption of low-calorie sweeteners other than saccharin and pancreatic cancer in the case–control study in Italy (Bosetti et al., 2009).

In sum, the available studies on the association between artificial sweetener exposure and risk of pancreatic cancer lacked sufficient consistency, quality, and precision to permit a conclusion to be drawn on the absence or presence of a causal association.

2.8.9 Cancer of the stomach

Two cohort studies (Hodge et al., 2018; McCullough et al., 2022) and three case–control study (Mayne et al., 2006; Bosetti et al., 2009; Palomar-Cros et al., 2023) examined the

association between artificial sweetener exposure and stomach cancer risk. The Australian MCCS (Hodge et al., 2018) and the study in the CPS-II cohort (McCullough et al., 2022) found no association between ASB consumption and cancers of the gastric cardia or stomach, respectively. Among the three case–control studies, the results were inconsistent. In the multicase–control study in Spain (MCC-Spain) (Palomar-Cros et al., 2023), the overall results were null for stomach cancer, but a positive association was observed for aspartame-containing products (no- or low-calorie soft drinks and tabletop sweeteners other than saccharin) for high versus no consumption among participants with diabetes (OR, 2.04; 95% CI, 0.70–5.40; *P* for trend, 0.05). The small body of available studies lacked sufficient informativeness, precision, and consistency to permit a conclusion to be drawn on the presence or absence of a causal association between artificial sweetener exposure and stomach cancer risk.

2.8.10 Cancer of the oesophagus

One cohort study (McCullough et al., 2022) and two case–control studies (Mayne et al., 2006; Gallus et al., 2007) examined the association between artificial sweetener exposure and oesophageal cancer risk. The study in the CPS-II cohort (McCullough et al., 2022) reported no association between ASB consumption and oesophageal cancer. No consistent associations were observed between diet carbonated beverage consumption and oesophageal adenocarcinoma or oesophageal squamous cell carcinoma in the case–control study in the USA (Mayne et al., 2006) or between diet carbonated beverage consumption and oesophageal cancer in the case–control study in Italy (Gallus et al., 2007). In sum, the small body of available studies were of insufficient quality, lacked consistency, and did not provide support for a positive association between aspartame consumption and oesophageal cancer.

2.8.11 Cancers of the colon and rectum

Studies in seven cohorts (NutriNet-Santé, CPS-II, EPIC, NHS, NHS-II, HPFS, and MCCS) and two case–control studies were considered informative for the assessment of the association between aspartame intake, either directly or through consumption of ASB, and colon and/or rectal cancer incidence or mortality.

The NutriNet-Santé study (Chazelas et al., 2019) found no association between consumption of ASBs and the risk of colorectal cancer (sub-distribution hazard ratio for top quartile versus bottom, sHR, 0.80; 95% CI, 0.44 to 1.46). These results were adjusted in great detail for major confounders. None of the other five cohort studies reported a positive association between consumption of artificially sweetened soft drinks and colorectal cancer risk (Hodge et al., 2018; Malik et al., 2019; Mullee et al., 2019; Hur et al., 2021; McCullough et al., 2022). Four of the five cohort studies examined mortality (Malik et al., 2019; Mullee et al., 2019; Hur et al., 2021; McCullough et al., 2022). Two of these cohort studies were considered less informative because of the collection of intake data at a single time point, together with variation in products across countries over time (Mullee et al., 2019), or with collection of data 1 year before the approval of aspartame use in ASBs (McCullough et al., 2022), which would lead to non-differential exposure misclassification.

There were also two informative case–control studies from Italy (Gallus et al., 2007) and Spain (Palomar-Cros et al., 2023). The Italian case–control study found no association between artificial sweetener consumption and cancer of the colon or rectum. The MCC-Spain study considered aspartame-containing products (low- or no-calorie soft drinks and tabletop sweeteners other than saccharin) and found no association between high aspartame consumption and colorectal cancer risk either overall or among participants with diabetes.

The Working Group concluded that the available studies that were considered informative were consistent with there being no positive association between aspartame consumption and cancer of the colon or rectum. Although bias could not be ruled out (considering the generally weak exposure assessments), given the consistency of null findings, the studies that were reviewed did not provide support for a positive association with cancers of the colon or rectum.

2.8.12 Cancer of the prostate

The Working Group evaluated five cohort studies (HPFS, CPS-II, NutriNet-Santé, MCCS, EPIC) and two case–control studies on aspartame consumption, or consumption of ASBs or tabletop sweetener packets containing aspartame, and the risk of prostate cancer.

The NutriNet-Santé study (Debras et al., 2022b), although of limited power, found no association between higher aspartame consumption (i.e. above the median among male aspartame users) and prostate cancer (HR versus non-users, multivariable-adjusted, 1.28; 95% CI, 0.91–1.79; *P* for trend, 0.28). The prospective study in the HPFS cohort in the USA (Malik et al., 2019) found no association between consumption of ASBs and prostate cancer mortality (HR for ≥ 2 servings/day versus < 1 serving/month, 1.01; 95% CI, 0.67–1.52). The study in the large CPS-II cohort (McCullough et al., 2022) found no increased risk of prostate cancer mortality with ASB consumption overall, and a suggestive interaction with BMI (*P* for interaction, 0.013); there was a positive association only among participants categorized as obese. The study in the EPIC cohort (Mullee et al., 2019) reported null findings for prostate cancer mortality (HR for artificially sweetened soft drinks consumption of ≥ 1 glass/day versus < 1 glass/month, 1.05; 95% CI, 0.64–1.75; *P* for trend, 0.53), although higher mortality from prostate cancer was observed among those consuming > 1–6 glasses per week (HR, 1.36; 95% CI, 1.05–1.78). The MCCS cohort found no association between artificially sweetened soft drinks and risk of aggressive prostate cancer (HR for ≥ 1 time per day versus < 1 time per month, 0.81; 95% CI, 0.49–1.33) (Hodge et al., 2018).

The case–control study in Italy (Gallus et al., 2007), which included 1294 prostate cancer cases and 1451 controls, reported that the overall association with consumption of sachets of artificial sweeteners other than saccharin was null. The MCC-Spain study (Palomar-Cros et al., 2023) investigated associations between consumption of artificial sweeteners and risk of prostate cancer (972 cases). Overall, no increased risk of prostate cancer was observed among those with high aspartame consumption (defined as third quartile or above) versus non-consumers, although the odds ratio among participants with diabetes was 1.91 (95% CI, 0.87–4.20; *P* for trend, 0.3).

In sum, of all the available studies, none reported an overall positive association between aspartame consumption and prostate cancer incidence or mortality, and two studies reported a positive association with mortality or incidence of prostate cancer in subgroups (among obese men and among those with diabetes). The available studies were of insufficient quality and consistency to permit a conclusion to be drawn on the presence or absence of a causal association.

2.8.13 Cancers of the urinary tract

Of the informative studies investigating the role of consumption of artificial sweeteners, particularly aspartame, and cancers of the urinary tract (kidney, bladder, and lower urinary tract combined), four were cohort studies (CPS-II, WHI-OS, MCCS, EPIC) (Hodge et al., 2018; Heath et al., 2021; McCullough et al., 2022; Ringel et al., 2023) and three were case–control studies (Nomura et al., 1991; Gallus et al., 2007; Andreatta et al., 2008).

The study in the CPS-II cohort (McCullough et al., 2022) investigated the association between ASB consumption and kidney or bladder cancer mortality, finding no association. The USA-based WHI-OS (Ringel et al., 2023) found no increased risk of urinary bladder cancer or kidney cancer among participants consuming ≥ 1 serving/day compared with those never consuming or consuming < 1 serving/week. In the MCCS cohort, there was no significant association observed between artificially sweetened soft drink consumption and kidney cancer (Hodge et al., 2018). In the EPIC cohort, Heath et al. (2021) found that the risk of either being diagnosed with or dying from renal cell carcinoma was not associated with artificially sweetened soft drink intake, assessed at baseline.

The case–control study in Italy by Gallus et al. (2007) found no association between exposure to artificial sweeteners other than saccharin and renal cell carcinoma. Two additional case–control studies examined the association between several proxies of aspartame use and (lower) urinary tract tumours. In the first, a case–control study in Argentina (Andreatta et al., 2008), long-term (≥ 10 years) consumption of artificial sweeteners, exclusively when used as an additive in infusions (tea, coffee, mate), without consideration of consumption from other sources such as soft drinks or dietetic foods, was associated with a significantly increased risk of urinary tract tumours, compared with non-use (OR, 2.18; 95% CI, 1.22–3.89). [The Working Group noted that there were very small numbers of cases and controls exposed and probably exposure misclassification.] A case–control study in Hawaii, USA (Nomura et al., 1991) evaluated consumption of artificial sweeteners on the basis of the use of saccharin and ingestion of diet beverages; no association was observed between lower urinary tract cancer and either ever consumption or cumulative consumption of diet beverages.

In sum, given the weak exposure definitions and potential for misclassification, bias could not be ruled out. The available studies were of insufficient quality and statistical precision to permit a conclusion to be drawn on the presence or absence of a causal association between aspartame consumption and risk of cancers of the urinary tract.

2.8.14 *Cancer of the brain*

In total, two cohort studies reported on proxies of aspartame intake and ASB consumption and brain cancer (NIH-AARP, CPS-II) (Lim et al., 2006; McCullough et al., 2022). In addition, two case–control studies reported on aspartame and artificial sweeteners and cancers of the brain (Gurney et al., 1997; Cabaniols et al., 2011). The CPS-II cohort study (McCullough et al., 2022) found no association between ASB intake and the risk of dying from cancer of the brain. The study in the NIH-AARP cohort (Lim et al., 2006) found that aspartame was not positively associated with glioma overall or with glioblastoma (the largest subtype of brain tumours) when non-users of aspartame was set as the reference category.

One USA-based case–control study (Gurney et al., 1997) investigated the risk of brain tumours in childhood in relation to the consumption of aspartame during infancy and childhood. Results indicated no association between aspartame consumption and brain tumour risk in childhood. A case–control study in France (Cabaniols et al., 2011) reported no association between regular (≥ 1 intake/week) aspartame consumption (no further definition of the aspartame assessment was provided) and brain cancer.

The Working Group concluded that all the studies found no association between aspartame intake and cancers of the brain. Given the insufficient informativeness of these studies and the lack of consistency, the evidence did not permit a conclusion to be drawn on the presence or absence of a causal association between

aspartame consumption and risk of cancers of the brain.

2.8.15 Other cancers

One cohort study also examined the association between artificial sweetener exposure and cancers of the small intestine, gall bladder, larynx, lip, oral cavity, and pharynx. In the CPS-II cohort, McCullough et al. (2022) reported an association between ASB consumption and small intestine cancer mortality that appeared to be elevated (HR per drink/day, 1.11; 95% CI, 1.00–1.22; *P* for trend, 0.244; after adjustment for BMI). Furthermore, among men, the association with oral, pharyngeal, and laryngeal cancer mortality combined per drink/day was 1.07 (95% CI, 1.00–1.14; *P* for trend, 0.034; after adjustment for BMI), but several limitations affected the interpretability of this finding.

Additional cohort studies reported on cancers of the thyroid (Zamora-Ros et al., 2022), lung (Malik et al., 2019; McCullough et al., 2022; You et al., 2022), and ovary or endometrium (Inoue-Choi et al., 2013; Hodge et al., 2018; McCullough et al., 2022). Four case–control studies reported on cancers of the uterus (Bosetti et al., 2009), thyroid (Singh et al., 2020), and larynx and ovary (Gallus et al., 2007). There were no findings of increased risk for ovary, endometrial, laryngeal, or lung cancer, and the evidence was too sparse and of insufficient informativeness to permit conclusions to be drawn on the presence or absence of causal associations between aspartame consumption and risk of cancer at any of these sites.

2.8.16 Obesity-related cancers and other groupings

Overall, three cohort studies reported on aspartame or ASB consumption and the risk of developing or dying from obesity-related cancers. A separate analysis from one of these cohorts examined the association between artificially sweetened soft drinks and non-obesity-related cancers.

The NutriNet-Santé study (Debras et al., 2022b) found that, compared with non-consumers, the hazard ratio (adjusted for BMI) of developing one of the obesity-related cancers considered in their analyses was 1.08 (95% CI, 0.96–1.22) for lower-level aspartame consumers and 1.15 (95% CI, 1.01–1.32) for higher-level consumers (i.e. above the sex-specific median of exposure) (*P* for trend, 0.026). [The Working Group noted that "absence of excess body fatness" has been found to be cancer-preventive by the *IARC Handbooks* programme, with *sufficient* or *limited* evidence for several cancer sites (IARC, 2018c).]

In the CPS-II cohort, McCullough et al. (2022) evaluated the association between ASB consumption (soft drinks and iced tea) and obesity-related cancers. In multivariable-adjusted statistical analyses (additionally adjusted for BMI), the hazard ratio for consumption of ≥ 2 drinks/day versus none was 1.02 (95% CI, 0.96–1.08; *P* for trend, 0.599) for men and 0.98 (95% CI, 0.94–1.02; *P* for trend, 0.213) for women.

The MCCS cohort (Hodge et al., 2018) included cancers of the liver, prostate (aggressive), ovary, gallbladder, kidney, colorectum, oesophagus (adenocarcinoma), breast (postmenopausal), pancreas, endometrium, and gastric cardia as being obesity-related. The study found no association between ASBs consumption and obesity-related cancers. In the MCCS cohort (Bassett at al., 2020), intake of > 1 serving/day of artificially sweetened soft drinks versus none or < 1/month was also associated with an increased risk of non-obesity-related cancers: the hazard ratio was 1.23 (95% CI, 1.02–1.48; *P* for trend, 0.006), or 1.10 (95% CI, 1.03–1.17) for each extra serving per day.

The three cohort studies reporting on obesity-related cancers each included a slightly different selection of cancer sites. In sum, given

the small body of evidence based on three cohort studies with mixed findings as well as diversity in size, end-point definitions, and exposure definitions (each having substantial uncertainty), no conclusion could be drawn on the presence or absence of a causal association between aspartame consumption and risk of obesity-related cancers as a group.

2.8.17 Cancer of all sites combined

Studies in seven cohorts looked at overall cancer incidence (NutriNet-Santé, PLCO), or mortality (EPIC, NHS, HPFS, NHANES, CPS-II) in relation to different estimates of aspartame consumption and were considered to be informative.

In the NutriNet-Santé study (Debras et al., 2022b), individuals classified as having a lower or higher intake of aspartame (from an average of 5.6 dietary records per participant) had an elevated cancer risk compared with non-consumers (HR for higher-consumers, 1.15; 95% CI, 1.03–1.28; *P* for trend, 0.002). In the NHS and HPFS cohorts (Malik et al., 2019), no association was observed between ASB consumption and cancer mortality. In the CPS-II cohort, McCullough et al. (2022) reported a hazard ratio for cancer deaths of 0.99 (95% CI, 0.97–1.02) for ASB consumption of ≥ 2 drinks/day relative to none without adjustment for BMI, and 0.97 (95% CI, 0.95–0.99) after adjustment for BMI. In the PLCO cohort, You et al. (2022) found no evidence that consumption of diet soft drinks only, relative to no soft drink consumption, was associated with all cancer risk. Similarly, the EPIC study (Mullee et al., 2019) found no association between artificially sweetened soft drink consumption and cancer death. In NHANES, two independent studies (Zhang et al., 2021; Fulgoni and Drewnowski, 2022) found that consumption of ≥ 2 servings/day of ASBs relative to no consumption (Zhang et al., 2021) or increasing tertile of aspartame intake (Fulgoni and Drewnowski, 2022) were not associated with overall cancer mortality.

The available evidence, based on seven cohort studies with diverse size, exposure definitions (most with substantial uncertainty) and findings, did not permit a conclusion to be drawn on the presence or absence of a causal association between aspartame consumption and risk of all cancers combined.

2.8.18 Results stratified by sex and other attributes of the study participants

Where data were provided, mostly null associations between aspartame and ASB consumption and cancer risk were seen for both men and women. Direct comparisons between two cohorts – NHS (women only) and HPFS (men only) (Schernhammer et al., 2012) – suggested differences by sex, with higher risks of NHL and multiple myeloma associated with artificial sweetener consumption in men.

A study reporting on the interaction between sex and ASB consumption was conducted in the context of all cancer mortality (McCullough et al., 2022) and did not find evidence of an effect of sex on the association (*P* for interaction, 0.145). In the EPIC cohort, the association between ASB consumption and adenocarcinoma of the exocrine pancreas was stratified by sex (Navarrete-Muñoz et al., 2016); a 25% increase in risk was observed in continuous models (per 336 g/day) among men, but not women (*P* for interaction by sex, 0.004). Overall, there was no pattern suggesting consistent effect modification by sex.

Several studies examined effect modification by BMI. For example, in the CPS-II cohort McCullough et al. (2022) found no overall increase in risk of prostate cancer death, and a suggestive interaction with BMI (*P* for interaction, 0.013) with a positive association only among obese participants. An interaction with BMI was also detected for NHL in the same cohort,

showing an inverse association for NHL among participants with a BMI of 18.5 to < 25 kg/m^2, and no association among participants with a BMI of 25–30 kg/m^2 or > 30 kg/m^2. On the other hand, the NutriNet-Santé study (Debras et al., 2022b) found no indication of BMI interaction, regardless of cancer end-point. Overall, no consistent patterns emerged regarding effect modification by BMI.

A combined analysis of the NIH-AARP and PLCO cohorts (Jones et al., 2022) found positive associations between risk of liver cancer and several exposure types among participants with diabetes (but not among those without diabetes). In the MCC-Spain study (Palomar-Cros et al., 2023), although the results were negative overall, there was a positive association between aspartame-containing products and cancers of the stomach (OR, 2.04; 95% CI, 0.70–5.40; *P* for trend, 0.05) and prostate (OR, 1.91; 95% CI, 0.87–4.20; *P* for trend, 0.3) for high consumption versus no consumption, among participants with diabetes. In contrast, in the same study, there was an inverse association between aspartame consumption and breast cancer (OR, 0.28; 95% CI, 0.08–0.83; *P* for trend, 0.03) for aspartame consumers compared with non-consumers, among participants with diabetes. For CLL, the risk of which was null overall and among participants with diabetes, a positive association (OR, 2.15; 95% CI, 0.93–4.51; *P* for trend, 0.4) was found between high consumption of aspartame and CLL among participants without diabetes. A case–control study in the USA (Chan et al., 2009) examined ASB consumption and adenocarcinoma of the exocrine pancreas, reporting some indication of potential effect modification by diabetes: risk was elevated among those without diabetes but not among those with diabetes.

In sum, although there was some suggestion of effect modification by BMI and diabetes, the direction of this modification remained unclear given the inconsistency in the results of the available studies, which did not allow any conclusions to be drawn.

3. Cancer in Experimental Animals

3.1 Mouse

See Table 3.1.

3.1.1 Oral administration (drinking-water)

In a study conducted in a genetically modified C57BL/6 Ela1-Tag mouse model (which expresses the simian virus SV40 large T antigen under the control of the elastase 1 acinar cell promoter, giving spontaneous formation of pancreatic cancers of acinar origin), 25 male mice (previously treated with aspartame in utero) were treated with drinking-water containing aspartame (purity not reported) at a concentration of 0% (13 mice) or 0.035% (12 mice) from birth until age 21 weeks. Survival rates were unaffected in all treated groups compared with controls. No data on body weight or water consumption were available (Dooley et al., 2017).

There were no effects on the cumulative incidence of pancreatic acinar carcinoma, the age of tumour onset, tumour growth rate, or tumour-induced mortality. [The Working Group noted that this study was limited by the use of a single low dose and a single sex, the lack of information on aspartame purity, and the limited number of organs examined without detailed histopathological information being reported. Therefore, the Working Group judged this study to be inadequate for the evaluation of the carcinogenicity of aspartame in experimental animals.]

3.1.2 Oral administration (feed)

In a study of carcinogenicity conducted in 1974 (EFSA_E75, 2011), 182 male and 178 female HAM-ICR Swiss mice (age, 28 days) were

Table 3.1 Studies of carcinogenicity with aspartame in mice

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, HAM-ICR Swiss (M) 28 days 104 wk EFSA E75 (2011)	Oral administration (feed) Purity, NR (conversion product, diketopiperazine, SC-19192, 0.8–1.2%) Feed 0, 1, 2, 4 g/kg bw per day Dose adjusted by body-weight change 72, 36, 37, 37 17, 9, 10, 6	No significant increase in tumour incidence in treated animals		*Principal strengths*: long-term study (> 2 yr); multiple-dose study; use of males and females. *Principal limitations*: study from pre-GLP era (1974); uncertainty regarding purity; < 50 animals per treated group; lack of measurement of stability and homogeneity of aspartame in the diet. *Other comments*: original document was the report submitted to Searle Laboratories in 1974.
Full carcinogenicity Mouse, HAM-ICR Swiss (F) 28 days 104 wk EFSA E75 (2011)	Oral administration (feed) Purity, NR (conversion product, diketopiperazine, SC-19192, 0.8–1.2%) Feed 0, 1, 2, 4 g/kg bw per day Dose adjusted by body-weight change 72, 36, 35, 35 26, 11, 11, 10	No significant increase in tumour incidence in treated animals		*Principal strengths*: long-term study (> 2 yr); multiple-dose study; use of males and females. *Principal limitations*: number of animals in treated group was 35–36; study from pre-GLP era (1974); uncertainty regarding purity. *Other comments*: original document was the report submitted to Searle Laboratories in 1974.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse (transgenic), FVB/N-TgN(v-Ha-*ras*)Led (Tg.AC) Hemizygous (M) 6 wk 40 wk NTP (2005)	Oral administration (feed) Purity, > 98% Feed 0, 3125, 6250, 12 500, 25 000, 50 000 ppm (approx. 0, 490, 980, 1960, 3960, and 7660 mg/kg bw per day) 15, 15, 15, 15, 15, 15 9, 12, 8, 12, 11, 10	*Lung* Bronchioloalveolar adenoma 0/14, 0/15, 2/14 (14%), 1/15 (7%), 2/14 (14%), 2/14 (14%) *Salivary gland* Duct, carcinoma 0/15, 0/15, 2/15 (13%), 0/15, 1/15 (7%), 1/15 (7%)	 NS NS	*Principal strengths*: GLP study; multiple-dose study; use of males and females. *Principal limitations*: low sensitivity of this transgenic mouse model under the reported study design. *Other comments*: there were no statistically significant differences in feed consumption, body weight, and survival.
Full carcinogenicity Mouse (transgenic), FVB/N-TgN(v-Ha-ras)Led (Tg.AC) homozygous (F) 6 wk 40 wk NTP (2005)	Oral administration (feed) Purity, > 98% Feed 0, 3125, 6250, 12 500, 25 000, 50 000 ppm (approx. 0, 550, 1100, 2260, 4420, and 8180 mg/kg bw per day) 15, 15, 15, 15, 15, 15 11, 10, 9, 9, 11, 8	*Salivary gland* Duct, carcinoma 0/15, 0/15, 0/15, 1/15 (7%), 0/15, 1/15 (7%)	 NS	*Principal strengths*: GLP study; multiple-dose study; use of males and females. *Principal limitations*: low sensitivity of this transgenic mouse model under the reported study design. *Other comments*: body weight in group at 50 000 ppm was greater than in control group; there were no statistically significant differences in feed consumption and survival.
Full carcinogenicity Mouse (transgenic), B6.129-*Trp53*^{tm1Brd} (N5) haploinsufficient (M) 7 wk 40 wk NTP (2005)	Oral administration (feed) Purity, > 98% Feed 0, 3125, 6250, 12 500, 25 000, 50 000 ppm (approx. 0, 490, 970, 1860, 3800, and 7280 mg/kg bw per day) 15, 15, 15, 15, 15, 15 14, 15, 13, 15, 14, 14	No significant increase in tumour incidence in treated animals		*Principal strengths*: GLP study; multiple-dose study; use of males and females. *Principal limitations*: low sensitivity of this transgenic mouse model under the reported study design. *Other comments*: mean body weights in groups at 6250, 12 500, 25 000, and 50 000 ppm were less than those in the control group for several weeks near the end of the study; there were no statistically significant differences in feed consumption and survival.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse (transgenic), B6.129-*Trp53*^{tm1Brd} (N5) haploinsufficient (F) 7 wk 40 wk NTP (2005)	Oral administration (feed) Purity, > 98% Feed 0, 3125, 6250, 12 500, 25 000, 50 000 ppm (approx. 0, 630, 1210, 2490, 5020, and 9620 mg/kg bw per day) 15, 15, 15, 15, 15, 15 14, 14, 14, 15, 15, 15	No significant increase in tumour incidence in treated animals		*Principal strengths*: GLP study; multiple-dose study; use of males and females. *Principal limitations*: low sensitivity of this transgenic mouse model under the reported study design. *Other comments*: there were no statistically significant differences in feed consumption, body weight, and survival.
Full carcinogenicity Mouse (transgenic), B6.129-*Cdkn2a*^{tm1Rdp} (N2) deficient (M) 7–9 wk 40 wk NTP (2005)	Oral administration (feed) Purity, > 98% Feed 0, 3125, 6250, 12 500, 25 000, 50 000 ppm (approx. 0, 490, 960, 1900, 3700, and 7400 mg/kg bw per day) 15, 15, 15, 15, 15, 15 14, 14, 15, 14, 14, 15	No significant increase in tumour incidence in treated animals		*Principal strengths*: GLP study; multiple-dose study; use of males and females. *Principal limitations*: low sensitivity of this transgenic mouse model under the current study design. *Other comments*: mean body weights in the group at 3125 ppm after week 29 and at 6250 ppm after week 16 were less than those in the control group; there were no statistically significant differences in feed consumption and survival.
Full carcinogenicity Mouse (transgenic), B6.129-*Cdkn2a*^{tm1Rdp} (N2) deficient (F) 7–9 wk 40 wk NTP (2005)	Oral administration (feed) Purity, > 98% Feed 0, 3125, 6250, 12 500, 25 000, 50 000 ppm (approx. 0, 610, 1200, 2300, 4850, and 9560 mg/kg bw per day) 15, 15, 15, 15, 15, 15 13, 15, 13, 15, 15, 14	*All organs* Haemangiosarcoma 0/15, 0/15, 2/15 (13%), 0/15, 0/15, 1/15 (7%)	NS	*Principal strengths*: GLP study; multiple-dose study; use of males and females. *Principal limitations*: low sensitivity of this transgenic mouse model under the reported study design. *Other comments*: there were no statistically significant differences in feed consumption, body weight, and survival.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, Swiss (M) Day 12 of gestation 130 wk Soffritti et al. (2010)	Oral administration (feed) Purity, 98.7% (impurities, diketopiperazine, 0.2%, and L-phenylalanine, 0.1%) Feed 0, 2000, 8000, 16 000, 32 000 ppm (approx. 0, 247, 987, 1919, and 3909 mg/kg bw per day) 117, 103, 62, 64, 83 NR, NR, NR, NR, NR	*Liver* Hepatocellular adenoma 9/117 (7.7%), 10/103 (9.7%), 4/62 (6.5%), 6/64 (9.4%), 2/83 (2.4%)	NS	*Principal strengths*: long-term study (> 2 yr); multiple-dose study; use of males and females; adequate number of animals per group; adequate duration of exposure and observation. *Principal limitations*: uncertainties regarding litter effects; conditions of animal husbandry not clear; see text for details. *Other comments*: no statistically significant differences in feed consumption, body weight, and survival; all organs and tissues were preserved in 70% alcohol, apart from bone tissues, which were preserved in 10% formalin. Historical controls (1047 male Swiss mice): hepatocellular carcinoma 3.2%, (range, 0–26.3%); bronchioloalveolar carcinoma 1.45%, (range, 0–14.3%). A statistical reanalysis by Gnudi et al. (2023) showed a significant positive trend for leukaemia (all types), with the incidence being significantly increased at all doses; a significant increase in the incidence of lymphoblastic leukaemia at all doses, of monocytic leukaemia, and of the total myeloid tumours at 16 000 ppm, as well as a significant increase in the incidence of lymphoblastic lymphoma or lymphoblastic leukaemia (combined) at 8000 and 32 000 ppm were also reported (see text for more details).
		Hepatocellular carcinoma 6/117 (5.1%), 12/103 (11.7%), 9/62 (14.5%)*, 10/64 (15.6%)**, 15/83 (18.1%)***	$P < 0.01$, trend test [test not specified] *[$P = 0.0331$, Fisher exact test]; **$P < 0.05$, Cox proportional hazard model [$P = 0.0196$, Fisher exact test]; ***$P < 0.01$, Cox proportional hazard model [$P = 0.0035$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 15/117 (12.8%), 22/103 (21.4%), 13/62 (21.0%), 16/64 (25.0%)*, 17/83 (20.5%)	*$P < 0.05$, logistic analysis [$P = 0.0321$, Fisher exact test]	
		Lung Bronchioloalveolar adenoma 8/117 (6.8%), 9/103 (8.7%), 7/62 (11.3%), 7/64 (10.9%), 6/82 (7.2%)	NS	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, Swiss (M) Day 12 of gestation 130 wk Soffritti et al. (2010) (cont.)		Bronchioloalveolar carcinoma 7/117 (6.0%), 6/103 (5.8%), 7/62 (11.3%), 8/64 (12.5%), 11/83 (13.3%)*	$P < 0.05$, trend test [test not specified] *$P < 0.01$, Cox proportional hazard model [NS, Fisher exact test]	
		Bronchioloalveolar adenoma or carcinoma (combined) 15/117 (12.9%), 15/103 (14.6%), 14/62 (22.6%), 15/64 (23.4%), 17/83 (20.5%)*	$P < 0.05$, logistic analysis trend test *$P < 0.05$, Cox proportional hazard model [NS, Fisher exact test]	
Full carcinogenicity Mouse, Swiss (F) Day 12 of gestation 130 wk Soffritti et al. (2010)	Oral administration (feed) Purity, 98.7% (impurities, diketopiperazine, 0.2%, and L-phenylalanine, 0.1%) Feed 0, 2000, 8000, 16 000, 32 000 ppm (approx. 0, 247, 987, 1919, and 3909 mg/kg bw per day) 102, 122, 73, 64, 62 NR, NR, NR, NR, NR	*Liver* Hepatocellular adenoma 1/102 (1.0%), 6/122 (4.9%), 2/73 (2.7%), 0/64, 0/62	NS	*Principal strengths*: long-term study (> 2 yr); multiple-dose study; use of males and females. *Principal limitations*: study not conducted under GLP. *Other comments*: no statistically significant differences in feed consumption, body weight, and survival; all organs and tissues were preserved in 70% alcohol, apart from bone tissues, which were preserved in 10% formalin. In the historical controls (999 female Swiss mice), the overall incidence of hepatocellular carcinoma was 0.2% (range, 0–2.1%) A statistical reanalysis by Gnudi et al. (2023) showed a significant increase in the incidence of leukaemia (all types) at the lowest dose (see text for more details).
		Hepatocellular carcinoma 0/102, 2/122 (1.6%), 0/73, 2/64 (3.1%), 0/62	NS	
		Hepatocellular adenoma or carcinoma (combined) 1/102 (1%), 8/122 (6.5%), 2/73 (2.7%), 2/64 (3.1%), 0/62	NS	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, Swiss (F) Day 12 of gestation 130 wk Soffritti et al. (2010) (cont.)		*Lung* Bronchioloalveolar adenoma 4/102 (3.9%), 9/122 (7.4%), 3/73 (4.1%), 2/64 (3.1%), 3/62 (4.8%) Bronchioloalveolar carcinoma 7/102 (6.9%), 10/122 (8.2%), 6/73 (8.2%), 7/64 (10.9%), 2/62 (3.2%) Bronchioloalveolar adenoma or carcinoma (combined) 11/102 (10.8%), 19/122 (15.6%), 9/73 (19.2%), 9/64 (23.4%), 5/62 (8.06%)	NS NS NS	

approx., approximately; bw, body weight; F, female; GLP, Good Laboratory Practice; M, male; NR, not reported; NS, not significant; ppm, parts per million; wk, week(s); yr, year(s).

treated with feed containing aspartame (purity not reported; impurities, concentrations of the conversion product, diketopiperazine (SC-19192), ranged from 0.8% to 1.2%) at a dose of 0 (control), 1, 2, or 4 g/kg body weight (bw) per day, adjusted according to body-weight change, for up to 104 weeks. The feed and drinking-water were available ad libitum. Survival rates were unaffected in all treated groups of males and females compared with controls. At study termination, survival was 17/72, 9/36, 10/37, and 6/37 in males, and 26/72, 11/36, 11/35, and 10/35 in females, for the groups at 0 (control), 1, 2, or 4 g/kg bw per day, respectively. Body-weight gains for males at the three treatment doses during the first year were statistically lower than those for the male controls, with actual group mean differences not exceeding 3% at week 52; mean terminal body weights in the second year were similar to those of male controls. Body weights for the females were generally similar to those of the female controls throughout the study. Total food consumption during the first year was significantly lower for males in the groups at 1 g/kg bw and 4 g/kg bw than for the controls, slightly but not significantly lower for males in the group at 2 g/kg bw per day and females in the groups at 2 and 4 g/kg bw per day than for the respective controls, and similar to that for controls for females in the group at 1 g/kg bw per day. During the second year, mean group food consumption for males and females tended to be slightly below that for the respective controls. At each treatment dose for each sex, mean daily consumption of aspartame for the entire 104-week test period was within ± 4% of the planned dosage levels (EFSA_E75, 2011).

Histopathological examination was performed on all gross lesions from all animals at each treatment dose, and 20–27 grossly unremarkable organs from all mice in the control group and in the group at the highest dose, and from about two thirds and one third of the mice in the groups at 2 and 1 g/kg bw per day, respectively. Dietary administration of aspartame did not cause a significant increase in the incidence of any type of malignant neoplasm in either sex.

Regarding non-neoplastic lesions, no consistent alterations were detected in the tissues evaluated that could be attributed to the administration of aspartame. [The Working Group noted that this long-term study used multiple dose groups and both sexes, and that the duration covered most of the mouse lifespan. However, it was limited by selective histopathology, the lack of measurement of the stability and homogeneity of aspartame in the diet, the lack of information on aspartame purity, and the use of fewer than 50 animals in each dosed group (35–37 mice per group).]

In a study that complied with Good Laboratory Practice (GLP) and used a genetically modified FVB/N-TgN(v-Ha-*ras*)Led (Tg.AC) hemizygous mouse model [this model introduced the v-Ha-*ras* coding sequence with point mutations in codons 12 and 59 and an SV40 polyadenylation sequence under the control of the promoter of the mouse embryonic *zeta*-globin gene, giving a reporter phenotype (skin papilloma) in response to genotoxic or nongenotoxic carcinogens], groups of 15 male and 15 female transgenic mice (age, 6 weeks) were treated with feed containing aspartame (purity, > 98%) at a concentration of 0 (control), 3125, 6250, 12 500, 25 000, or 50 000 ppm (approximately equivalent to doses of 0, 490, 980, 1960, 3960, and 7660 mg/kg bw per day in males and 0, 550, 1100, 2260, 4420, and 8180 mg/kg bw per day in females) for up to 40 weeks. Basal diet and drinking-water were available ad libitum. Survival rates were unaffected in all treated groups of males and females compared with controls. Body weights in the exposed groups were similar to those in the control group for males and greater than those in the control group for females at 50 000 ppm (statistical data were not shown). Food consumption was unaffected in all treated groups of males and females compared with controls (NTP, 2005).

Histopathological examination showed no evidence of an increased incidence of neoplastic or non-neoplastic lesions in males or females in all treated groups. [The Working Group noted that this was a well-conducted study that followed the National Toxicology Program (NTP) guidelines for transgenic mouse studies and that complied with GLP and used multiple doses and both sexes. The Working Group concurred with the study authors' conclusion that this transgenic mouse model using the study design reported may not have sufficient sensitivity to detect a carcinogenic effect of aspartame.]

In a study that complied with GLP and that used a genetically modified B6.129-*Trp53*^{tm1Brd} (N5) haploinsufficient mouse model [this model introduced a null mutation into one *p53* allele by insertion of a neo cassette, thus deleting a 450-base pair gene fragment, and giving a phenotype that is susceptible to mostly genotoxic carcinogens], groups of 15 male and 15 female transgenic mice (age, 7 weeks) were treated with feed containing aspartame (purity, > 98%) at a concentration of 0 (control), 3125, 6250, 12 500, 25 000, or 50 000 ppm (approximately equivalent to doses of 0, 490, 970, 1860, 3800, and 7280 mg/kg bw per day in males and 0, 630, 1210, 2490, 5020, and 9620 mg/kg bw per day in females) for up to 40 weeks. Basal diet and drinking-water were available ad libitum. Survival rates were unaffected in all treated groups of males and females compared with controls. Body weights in all exposed groups of males and females were similar to those in the control groups. Food consumption was unaffected in all treated groups of males and females compared with controls (NTP, 2005).

Histopathological examination showed no evidence of increased neoplastic or non-neoplastic lesions in males or females in all treated groups. [The Working Group noted that this was a well-conducted study that followed the NTP guidelines for transgenic mouse studies, complied with GLP, and used multiple doses and both sexes. The Working Group concurred with the study authors' conclusion that this transgenic mouse model using the study design reported may not have sufficient sensitivity to detect a carcinogenic effect of aspartame.]

In a study that complied with GLP and used a genetically modified B6.129-*Cdkn2a*^{tm1Rdp} (N2) deficient mouse model [this model had a targeted deletion in the *Cdkn2a* locus (*Cdkn2a* deletion), causing disruption in p16^{Ink4a} and p19^{Arf} protein function and giving a phenotype of increased cell proliferation], groups of 15 male and 15 female transgenic mice (age, 7–9 weeks) were treated with feed containing aspartame (purity, > 98%) at a concentration of 0 (control), 3125, 6250, 125 000, 25 000, or 50 000 ppm (approximately equivalent to doses of 0, 490, 960, 1900, 3700, and 7400 mg/kg bw per day in males and 0, 610, 1200, 2300, 4850, and 9560 mg/kg bw per day in females) for up to 40 weeks. Basal diet and drinking-water were available ad libitum. Survival rates were unaffected in all treated groups of males and females compared with controls. Body weights in the exposed groups were less than those in the control group for males at 3125 ppm (after 29 weeks) and at 6250 ppm (after 16 weeks), and similar to those in the control group for all treated groups of females. Food consumption was unaffected in all treated groups of males and females compared with controls (NTP, 2005).

Histopathological examination showed no evidence of an increased incidence of neoplastic lesions in males or females. Regarding non-neoplastic lesions, minimal to mild cytoplastic vacuolization of periportal hepatocytes was found in control and exposed male mice, with an increase in incidence in the group at 6250 ppm compared with controls. [The Working Group noted that this was a well-conducted study that followed the NTP guidelines for transgenic mouse studies and that complied with GLP and used multiple doses and both sexes. The Working Group concurred with the author's statement that this transgenic mouse model using the study

design reported may not have sufficient sensitivity to detect a carcinogenic effect of aspartame.]

3.1.3 *Transplacental and perinatal administration followed by oral administration (feed)*

In a study of chronic toxicity and carcinogenicity, 429 male and 423 female Swiss mice were obtained by mating groups containing an equal number of males and females (three groups of 40 and two groups of 60 mice, with a total of 240 overall, no further details provided). Dams were treated with feed containing aspartame (purity, 98.7%; impurities, 0.2% diketopiperazine and 0.1% L-phenylalanine) at a concentration of 0 (control), 2000, 8000, 16 000, or 32 000 ppm (approximately equivalent to doses of 0, 247, 987, 1919, and 3909 mg/kg bw per day) starting from day 12 of gestation. The pups were weaned at age 4–5 weeks and were fed the same diet until week 130. Feed and drinking-water were available ad libitum. The initial numbers of mice assigned to the groups at 0, 2000, 8000, 16 000, and 32 000 ppm groups were 117, 103, 62, 64, and 83 for males and 102, 122, 73, 64, and 62 for females, respectively. Surviving animals (67 in total, equally distributed in groups and sex) were killed at age 130 weeks. The survival rate per group at study termination was not reported; however, the authors reported that there were no statistically significant differences in survival or in the breeding of the offspring for all groups of males and females compared with controls. No differences were observed in body weight in the treated groups compared with controls. Histopathology was performed on all organs and tissues (all were preserved in 70% ethanol, except for bone, which was preserved in 10% formalin). All slides were evaluated by a junior pathologist (with at least 4 years of experience), and all tumours and lesions of oncological interest were reviewed by two senior pathologists (with more than 20 years of experience) (Soffritti et al., 2010).

In male mice, there was a significant positive trend ($P < 0.01$, trend test [method not reported]) in the incidence of hepatocellular carcinoma – 6/117 (5.1%), 12/103 (11.7%), 9/62 (14.5%), 10/64 (15.6%), and 15/83 (18.1%) for the groups at 0 (control), 2000, 8000, 16 000 and 32 000 ppm, respectively – with the incidence being significantly increased at 16 000 and 32 000 ppm ($P < 0.05$ and $P < 0.01$, respectively, Cox proportional hazard model) and at 8000, 16 000, and 32 000 ppm [$P = 0.0331$, $P = 0.0196$, and $P = 0.0035$, respectively, Fisher exact test]. The incidence of hepatocellular carcinoma in all groups was within the upper bound of the range for historical controls (1047 male Swiss mice, overall incidence of 3.2%; range, 0–26.3%) from this laboratory. The incidence of hepatocellular adenoma or carcinoma (combined) – 15/117 (12.8%), 22/103 (21.4%), 13/62 (21.0%), 16/64 (25.0%), and 17/83 (20.5%) for the groups at 0 (control), 2000, 8000, 16 000 and 32 000 ppm, respectively – was significantly increased at 16 000 ppm ($P < 0.05$, logistic analysis; [$P = 0.0321$, Fisher exact test]). The incidence of hepatocellular adenoma, described as "typically loss of the normal lobular architecture, cells occurred in irregular plates, 1–3 cell layers thick, disposed perpendicular or obliquely on the surrounding parenchyma", did not show dose-related changes or a significant increase in all exposed groups. There was a significant positive trend ($P < 0.05$, trend test [method not reported]) in the incidence of bronchioloalveolar carcinoma – 7/117 (6.0%), 6/103 (5.8%), 7/62 (11.3%), 8/64 (12.5%), and 11/83 (13.3%) for the groups at 0 (control), 2000, 8000, 16 000 and 32 000 ppm, respectively – with the incidence being significantly increased at 32 000 ppm ($P < 0.01$, Cox proportional hazard model). The incidence of bronchioloalveolar carcinoma in all groups was within the upper bound of the range for historical controls (1047 male Swiss mice, overall incidence, 1.45%; range, 0–14.3%) from this laboratory. There was a significant positive trend ($P < 0.05$, logistic analysis) in the incidence

of bronchioloalveolar adenoma or carcinoma (combined) – 15/117 (12.9%), 15/103 (14.6%), 14/62 (22.6%), 15/64 (23.4%), and 17/83 (20.5%) for the groups at 0 (control), 2000, 8000, 16 000 and 32 000 ppm treated groups, respectively – with incidence being significantly increased at 32 000 ppm ($P < 0.05$, Cox proportional hazard model). The incidence in historical controls was not reported for adenoma or carcinoma (combined). The incidence of bronchioloalveolar adenoma did not show dose-related changes or a significant increase in any of the groups of males. In female mice, dietary exposure to aspartame did not cause a significant increase in the incidence of any tumours.

Regarding non-neoplastic lesions, no information was available to the Working Group.

[The Working Group noted that this long-term study used multiple dose groups, adequate numbers of animals of both sexes, and an adequate duration, and it provided purity and stability data for aspartame. However, the Working Group considered that there were uncertainties regarding litter effects (e.g. there was no information regarding the number of litters per treatment group, the number of pups per litter per treatment group, whether the treatments were loaded in a balanced fashion, and the method for animal selection), potentially resulting in false-positive results. The conditions of animal husbandry were unclear. In addition, the incidence of hepatocellular carcinoma and bronchioloalveolar carcinoma in all groups was within the upper bound of the range for historical controls for male Swiss mice from this institute. The incidence in historical controls was not reported for bronchioloalveolar adenoma or carcinoma (combined). There were concerns about the reliability of histological diagnoses of solid tumours in autolytic tissues. A minority of the Working Group considered, that although concerns were expressed about potential litter effects in prenatal studies, the large sample size per group (n = 70–150), the significant dose–response trend, and pairwise testing of multiple tumour types suggested the absence of a potential bias caused by litter effects. Although the incidence of hepatocellular carcinoma and bronchioloalveolar carcinoma in all groups was within the upper bound of the range for historical controls for male Swiss mice from this institute, the Preamble to the *IARC Monographs* (see the front matter of the present volume; IARC, 2019) notes that concurrent controls are generally preferred over historical controls, except in the case of rare tumours. While concerns were expressed about the reliability of histological diagnoses in autolytic tissues, a minority of the Working Group considered that these bore no significance because the moribund animals were monitored two to three times daily to avoid such concerns.]

[To help with the interpretation of the study results, the Working Group deemed it important that the data be reanalysed and thus encouraged the Ramazzini Institute to provide a more detailed statistical analysis of haemolymphoreticular tumour diagnoses not previously reported in the Soffritti et al. (2010) study in mice treated with aspartame.] The statistical reanalysis of data for males by Gnudi et al. (2023) showed a significant positive trend for leukaemia (all types) ($P = 0.0492$, Cochran–Armitage trend test), with incidence being significantly increased) at all doses ($P = 0.018$, $P = 0.001$, $P = 0.001$, and $P = 0.007$, Fisher exact test; for the groups at 2000, 8000, 16 000 and 32 000 ppm treated groups, respectively). A significant increase in the incidence of lymphoblastic leukaemia (P = 0.012, P = 0.001, and P = 0.021, Fisher exact test; for the groups at 2000, 8000, and 32 000 ppm, respectively), of monocytic leukaemia (P = 0.043, Fisher exact test; for the group at 16 000 ppm), and for total myeloid tumours ($P = 0.024$, Fisher exact test; for the group at 16 000 ppm) was also reported. A significant increase in lymphoblastic lymphoma or lymphoblastic leukaemia (combined) was

observed at 8000 and 32 000 ppm [$P = 0.0039$ and 0.0330, respectively, Fisher exact test].

In females, Gnudi et al. (2023) showed a significant increase in the incidence of leukaemia (all types) ($P = 0.007$, Fisher exact test; for the group at 2000 ppm).

[The Working Group noted that the incidence of lymphoma or leukaemia (combined) in the treated groups did not exceed the incidence in historical controls, and no indication of the historical control incidence for individual haematolymphoid tumours was available, especially for rare tumours with low incidence. A minority of the Working Group noted that the Preamble to the *IARC Monographs* (see the front matter of the present volume) states that concurrent controls are generally preferred over historical controls, except in the case of rare tumours. This minority also emphasized that the diagnostic criteria were adequate for myeloid and lymphoid tumours and leukaemia, with the exception of immunoblastic lymphoma of the lung, for which the differentiation between reactive hyperplasia and neoplasia has been recognized as challenging by the NTP, Ramazzini Institute, and pathologists from other institutes (Malarkey and Bucher, 2011; Tibaldi et al., 2020).]

3.1.4 Intravesical pellet implantation

In a 26-week study, a total of 400 female Swiss albino mice (age, 60–90 days) were implanted intravesically (urinary bladder) with a pellet containing one part aspartame (purity not reported) at a dose of 0 or 4.0–4.4 mg per mouse and four parts cholesterol, for up to 26 weeks. The control group of 200 mice (group A, $n = 100$; group B, $n = 100$) was exposed only to pellets of purified cholesterol. Survival and body weight did not differ statistically between the control and treated groups (EFSA_E58, 2011; also reported by Bryan, 1984).

Urinary bladder neoplasia was observed at 175 days post-implantation. There were no statistical differences in tumour stage or incidence of neoplasia in the urinary bladder between the control and treated groups. The incidence of other tumours observed in each group was low, and histopathological data were not available. [The Working Group noted that this study was limited by the use of a single dose, the short duration (26 weeks), the lack of information on purity, and the use of an uncommon route of exposure in a single sex. Therefore, the Working Group judged this study to be inadequate for the evaluation of the carcinogenicity of aspartame in experimental animals.]

In a concurrent study, a total of 200 (group A, $n = 100$; group B, $n = 100$) female Swiss albino mice (age, 60–90 days) were implanted intravesically (urinary bladder) with a pellet containing one part aspartame (purity not reported) at a dose of 0 or 4.0–4.4 mg per mouse and four parts cholesterol, for up to 56 weeks. The control group of 200 mice (group A, $n = 100$; group B, $n = 100$) was exposed only to pellets of purified cholesterol. Survival and body weight did not differ statistically between the control and treated groups. All mice underwent complete necropsy with histopathological evaluation (EFSA_E72, 2011; also reported by Bryan, 1984).

There were no statistical differences in tumour stage or the incidence of neoplasia in the urinary bladder between the control and treated groups. There was no difference in the incidence of other tumours observed in each group (Prejean et al., 1973). No data on non-neoplastic lesions were provided. [The Working Group noted that this study was limited by the use of a single dose, the lack of information on purity, and the use of an uncommon route of exposure in a single sex. Therefore, the Working Group judged this study to be inadequate for the evaluation of the carcinogenicity of aspartame in experimental animals.]

3.2 Rat

See Table 3.2.

3.2.1 Oral administration (feed)

In studies published by Soffritti et al. (2005, 2006), also reported by Belpoggi et al. (2006), groups of 100 or 150 male and female Sprague-Dawley rats (age, 8 weeks) were treated with feed containing aspartame (purity, > 98%; impurities, concentrations of the decomposition products diketopiperazine and L-phenylalanine were 1.5% and < 0.5%, respectively) at a concentration of 0, 80, 400, 2000, 10 000, 50 000, or 100 000 ppm (approximately equivalent to daily intakes of 0, 4, 20, 100, 500, 2500, and 5000 mg/kg bw). The Sprague-Dawley rats were obtained from the breeding colony maintained at the Ramazzini Institute, Italy. At age 4–5 weeks, the rats were randomized to include no more than one male and one female from each litter in the same group, to control for litter effects. The exposure lasted until the natural death of the rats, and all rats found moribund or dead were promptly necropsied for gross and microscopic evaluation of the extensive list of tissues identified in the study protocol. The in-life phase of the study lasted 159 weeks (Soffritti et al., 2005, 2006; also reported by Belpoggi et al., 2006). There were no significant differences in water consumption, slight dose-dependent decreases in feed consumption, and no differences in body-weight gain or survival across groups. The rats at 100 000 ppm developed yellowing of the hair coat, which was reported to be also observed in a study from the Ramazzini Institute in rats treated with formaldehyde (Soffritti et al., 2002).

In male rats, an overall increase in the incidence of animals bearing malignant tumours was observed, with a significant positive trend ($P \leq 0.05$, Cochran–Armitage trend test). There was a significant positive trend ($P \leq 0.05$, Cochran–Armitage trend test; $P \leq 0.05$, poly-3 trend test) in the incidence of lymphoma and leukaemia (combined) (multiple organs) – 31/150 (20.7%), 23/150 (15.3%), 25/150 (16.7%), 33/150 (22%), 15/100 (15%), 20/100 (20%), and 29/100 (29%), at 0 (control), 80, 400, 2000, 10 000, 50 000, and 100 000 ppm, respectively – with the incidence in all treated groups being within the upper bound of the range observed in historical controls (2265 males, 20.6%; range, 8.0–30.9%) from this laboratory (Soffritti et al., 2005, 2006). There was a significant positive trend ($P \leq 0.05$, Cochran–Armitage trend test; $P \leq 0.05$, poly-3 trend test) in the incidence of malignant schwannoma of the peripheral nerve, most frequently observed in cranial nerves and other sites (mainly spinal nerve roots) – 1/150 (0.7%), 1/150 (0.7%), 3/150 (2%), 2/150 (1.3%), 2/100 (2%), 3/100 (3%), and 4/100 (4%) at 0 (control), 80, 400, 2000, 10 000, 50 000, and 100 000 ppm, respectively – with the incidence at 50 000 and 100 000 ppm exceeding the upper bound of the range observed in historical controls (2265 males, 0.5%; range, 0–2.0%) from this laboratory (Soffritti et al., 2005, 2006). All the schwannoma diagnoses were supported by positive immunohistochemical staining for S100 protein. [The Working Group noted that malignant schwannoma is a rare tumour in this animal model.] Metastases of malignant schwannoma were observed in three rats at 100 000 ppm. The incidence of dysplastic papilloma and carcinoma (combined) of the renal pelvis and ureter exceeded that observed in historical controls (2265 males, 0%) from this laboratory (Soffritti et al., 2005, 2006). [The Working Group considered it satisfactory to combine the "dysplastic papillomas" with the carcinomas, while excluding "dysplastic hyperplasia".] In addition, two transitional cell carcinomas of the urinary bladder were reported in male rats at 10 000 ppm (Soffritti et al., 2006). [The Working Group noted that transitional cell carcinoma is a rare tumour among historical controls in this colony of Sprague-Dawley rats.]

Table 3.2 Studies of carcinogenicity with aspartame in rats

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, Sprague-Dawley (M) 8 wk Lifetime Soffritti et al. (2006)	Oral administration (feed) Purity, > 98% (impurities, diketopiperazine, < 1.5%, and L-phenylalanine, < 0.5%) Feed 0, 80, 400, 2000, 10 000, 50 000, 100 000 ppm, daily 150, 150, 150, 150, 100, 100, 100 150, 150, 150, 150, 100, 100, 100	*Systemic (multiple organs)* Lymphoma or leukaemia (combined) 31/150 (20.7%), 23/150 (15.3%), 25/150 (16.7%), 33/150 (22%), 15/100 (15%), 20/100 (20%), 29/100 (29%) *Peripheral nerve (cranial and others)* Malignant schwannoma 1/150 (0.7%), 1/150 (0.7%), 3/150 (2%), 2/150 (1.3%), 2/100 (2%), 3/100 (3%), 4/100 (4%) *Renal pelvis and ureter* Dysplastic papilloma 0/150, 0/149, 1/149 (0.7%), 0/150, 0/100, 0/100, 0/100 Carcinoma 0/150, 0/149, 0/149, 1/150 (0.7%), 1/100 (1%), 1/100 (1%), 1/100 (1%) Dysplastic papilloma and carcinoma (combined) 0/150, 0/149, 1/149 (0.7%), 1/150 (0.7%), 1/100 (1%), 1/100 (1%), 1/100 (1%)	$P \leq 0.05$, Cochran–Armitage trend test; $P \leq 0.05$ poly-3 trend test $P \leq 0.05$, Cochran–Armitage trend test; $P \leq 0.05$, poly-3 trend test NS NS NS	*Principal strengths*: multiple-dose study; males and females used; large group size (100–150/group); lifetime exposure (> 2 yr). *Principal limitations*: details of pathology descriptions not available. *Other comments*: combinations of some lesions in this study are not commonly used, e.g. (i) histiocytic sarcoma was combined with lymphoma/leukaemia; (ii) hyperplasia was combined with tumours (dysplastic hyperplasia lesions of renal pelvis and ureter were combined with dysplastic papilloma and carcinoma). See text for details on the histological reanalysis by Tibaldi et al. (2020), and the statistical reanalysis by Gnudi et al. (2023). Historical controls: lymphoma and leukaemia (combined), 2265 males, 20.6% (range, 8.0–30.9%); malignant schwannoma, 2265 males, 0.5% (range, 0–2.0%); olfactory neuroblastoma, 2265 males, 0.1% (range, 0–1.8%); renal pelvis and ureter transitional cell carcinomas (combined), 2265 males, 0%.

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, Sprague-Dawley (F) 8 wk Lifetime Soffritti et al. (2006)	Oral administration (feed) Purity, > 98% (impurities, diketopiperazine, < 1.5%, and L-phenylalanine, < 0.5%) Feed 0, 80, 400, 2000, 10 000, 50 000, 100 000 ppm, daily 150, 150, 150, 150, 100, 100, 100 150, 150, 150, 150, 100, 100, 100	*Systemic (multiple organs)* Lymphoblastic lymphoma 2/150 (1.3%), 3/150 (2%), 7/150 (4.7%), 5/150 (3.3%), 2/100 (2%), 2/100 (2%), 1/100 (1%) Lymphoblastic leukaemia 0/150, 0/150, 0/150, 1/150 (0.7%), 0/100, 0/100, 0/100 Lymphocytic lymphoma 2/150 (1.3%), 5/150 (3.3%), 2/150 (1.3%), 1/150 (0.7%), 2/100 (2%), 0/100, 2/100 (2%) Lymphoimmunoblastic lymphoma 5/150 (3.3%), 6/150 (4%), 8/150 (5.3%), 8/150 (5.3%), 3/100 (3%), 10/100 (10%), 11/100 (11%) Histiocytic sarcoma 4/150 (2.7%), 6/150 (4%), 9/150 (6%), 8/150 (5.3), 10/100 (10%), 8/100 (8%), 7/100 (7%)	 NS [One animal at 400 ppm dose group had lymphoblastic lymphoma and histiocytic sarcoma] NS NS NS NS	*Principal strengths*: multiple-dose study; males and females used; large group size (100–150/group); lifetime exposure (> 2 yr). *Principal limitations*: details of pathology descriptions were not available. *Other comments*: combinations of some lesions in this study are not commonly used, e.g. (i) histiocytic sarcoma was combined with lymphoma/leukaemia; (ii) hyperplasia was combined with tumours (dysplastic hyperplasia lesions of renal pelvis and ureter were combined with dysplastic papilloma and carcinoma). See the text for details on the histological reanalysis by Tibaldi et al. (2020), and the statistical reanalysis by Gnudi et al. (2023). Historical controls: lymphoma and leukaemia (combined), 2274 females, 13.3% (range, 4.0–25.0%); malignant schwannoma, 2274 females, 0.1% (range, 0–2.0%); renal pelvis and ureter transitional cell carcinomas, 2274 females, 0.04% (range, 0–1.0%); olfactory neuroblastoma, 2274 females, 0.1% (range, 0–1.8%).

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, Sprague-Dawley (F) 8 wk Lifetime Soffritti et al. (2006) (cont.)		Monocytic leukaemia 0/150, 2/150 (1.3%), 5/150 (3.3%), 4/150 (2.7%), 2/100 (2%), 4/100 (4%), 2/100 (2%)	NS	
		Myeloid leukaemia 0/150, 0/150, 0/150, 1/150 (0.7%), 0/100, 1/100 (1%), 2/100 (2%)	NS	
		Lymphoma or leukaemia (combined) 13/150 (8.7%), 22/150 (14.7%), 30/150 (20%)**, 28/150 (18.7%)*, 19/100 (19%)*, 25/100 (25%)**, 25/100 (25%)**	$P \leq 0.01$, Cochran–Armitage trend test; $P \leq 0.05$, poly-3 trend test *$P \leq 0.05$, poly-3 test; [$P \leq 0.01$, Fisher exact test] **$P \leq 0.01$, poly-3 test; [$P \leq 0.01$, Fisher exact test]	
		Peripheral nerve (cranial and others) Malignant schwannoma 0/150, 2/150 (1.3%), 0/150, 3/150 (2%), 1/100 (1%), 1/100 (1%), 2/100 (2%)	NS	

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, Sprague-Dawley (F) 8 wk Lifetime Soffritti et al. (2006) (cont.)		*Renal pelvis and ureter* Dysplastic papilloma 0/150, 1/150 (0.7%), 1/150 (0.7%), 1/150 (0.7%), 1/100 (1%), 1/100 (1%), 3/100 (3%)	$P \leq 0.05$, Cochran–Armitage trend test	
		Carcinoma 0/150, 1/150 (0.7%), 3/150 (2%), 3/150 (2%), 3/100 (3%), 3/100 (3%), 4/100 (4%)*	*$P \leq 0.05$, poly-3 test; [$P = 0.0247$, Fisher exact test] [One rat at 2000 and at 10 000 ppm had tumours in both kidneys]	
		Dysplastic papilloma or carcinoma (combined) 0/150 (0%), 2/150 (1.3%), 4/150 (2.7%), 4/150 (2.7%), 4/100 (4%), 4/100 (4%), 7/100 (7%)*	[$P = 0.041$, Cochran–Armitage trend test] [*$P = 0.0014$, Fisher exact test] [One rat at 2000 and 10 000 ppm had tumours in both kidneys]	
		Mammary glands Carcinoma 8/150 (5.3%), 15/150 (10%), 16/150 (10.7%), 12/150 (8%), 7/100 (7%), 18/100 (18%)*, 7/100 (7%)	[*$P = 0.0015$, Fisher exact test]	

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, SLC Wistar (M) 6 wk 104 wk Ishii (1981)	Oral administration (feed) Purity, NR Feed 0, 1, 2, 4 g/kg bw per day 60, 60, 60, 60 26, 16, 28, 25	*Brain* Glioma (astrocytoma, atypical astrocytoma, oligodendroglioma, combined) 0/60, 1/60 (1.7%), 0/60, 1/60 (1.7%)	NS	*Principal strengths*: males and females used; multiple-dose study; adequate number of animals used; adequate duration of exposure and observation. *Principal limitations*: purity, NR; lack of detailed histopathology. Other comments: histological reanalysis by Shibui et al. (2019) and EFSA_UA04 (2011)
Full carcinogenicity Rat, SLC Wistar (F) 6 wk 104 wk Ishii (1981)	Oral administration (feed) Purity, NR Feed 0, 1, 2, 4 g/kg bw per day 60, 60, 60, 60 49, 40, 51, 43	*Brain* Glioma (astrocytoma, atypical astrocytoma, oligodendroglioma, combined) 1/60 (1.7%), 0/60, 1/60 (1.7%), 0/60	NS	*Principal strengths*: males and females used; multiple-dose study; adequate number of animals used; adequate duration of exposure and observation. *Principal limitations*: purity, NR; lack of detailed histopathology. Other comments: histological reanalysis by Shibui et al. (2019) and EFSA_UA04 (2011).
Full carcinogenicity Rat, SLC Wistar (M) 6 wk 52 wk Ishii (1981)	Oral administration (feed) Purity, NR Feed 0, 1, 2, 4 g/kg bw per day 16, 16, 16, 16 16, 16, 16, 16	No significant increase in tumour incidence in treated animals		*Principal strengths*: males and females used; multiple-dose study. *Principal limitations*: purity, NR.
Full carcinogenicity Rat, SLC Wistar (F) 6 wk 52 wk Ishii (1981)	Oral administration (feed) Purity, NR Feed 0, 1, 2, 4 g/kg bw per day 16, 16, 16, 16 16, 16, 16, 16	No significant increase in tumour incidence in treated animals		*Principal strengths*: males and females used; multiple-dose study. *Principal limitations*: purity, NR.

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, SLC Wistar (M) 6 wk 26 wk Ishii (1981)	Oral administration (feed) Purity, NR Feed 0, 1, 2, 4 g/kg bw per day 16, 16, 16, 16 16, 16, 16, 16	No significant increase in tumour incidence in treated animals		*Principal strengths*: males and females used; multiple-dose study. *Principal limitations*: purity, NR
Full carcinogenicity Rat, SLC Wistar (F) 6 wk 26 wk Ishii (1981)	Oral administration (feed) Purity, NR Feed 0, 1, 2, 4 g/kg bw per day 16, 16, 16, 16 16, 16, 16, 16	No significant increase in tumour incidence in treated animals		*Principal strengths*: males and females used; multiple-dose study. *Principal limitations*: purity, NR.
Full carcinogenicity Rat, SLC Wistar (M) 6 wk 26 wk Shibui et al. (2019)	Oral administration (feed) Purity, NR Feed 0, 1, 2, 4 g/kg bw per day 60, 60, 60, 60 60, 60, 60, 60	*Lung* Bronchioloalveolar adenoma 1/60 (1.7%), 3/60 (5%), 0/60, 2/60 (3.3%) Bronchioloalveolar carcinoma 0/60, 0/60, 2/60 (3.3%), 0/60 Bronchioloalveolar adenoma or carcinoma (combined) 1/60 (1.7%), 3/60 (5%), 2/60 (3.3%), 2/60 (3.3%)	NS NS NS	*Principal strengths*: males and females used; multiple-dose study. *Principal limitations*: purity, NR. *Other comments*: histological reanalysis of Ishii et al. (1981) by Shibui et al. (2019) also reported by EFSA_UA04 (2011).

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, SLC Wistar (M) 6 wk 26 wk Shibui et al. (2019) (cont.)		*Pituitary gland (pars distalis)* Adenoma 5/48 (10.4%), 8/48 (16.7%), 12/53 (22.6%), 5/53 (9.4%) Carcinoma 0/48, 1/48 (2%), 0/53, 0/53 *Adrenal gland* Benign pheochromocytoma 4/59 (6.8%), 9/59 (15.3%), 4/60 (6.7%), 10/60 (16.7%)	 NS NS NS	
Full carcinogenicity Rat, SLC Wistar (F) 6 wk 26 wk Shibui et al. (2019)	Oral administration (feed) Purity, NR Feed 0, 1, 2, 4 g/kg bw per day 60, 60, 60, 60 60, 60, 60, 60	*Lung* Bronchioloalveolar adenoma 0/60 (1.7%), 1/60 (1.7%), 3/60 (5%), 3/60 (5%) *Adrenal gland* Benign pheochromocytoma 1/60 (1.7%), 1/60 (1.7%), 6/59 (10.2%), 2/60 (3.3%) Malignant pheochromocytoma 0/60, 0/60, 1/59 (1.7%), 1/60 (1.7%)	 NS NS NS	*Principal strengths*: males and females used; multiple-dose study. *Principal limitations*: purity, NR. *Other comments*: histological reanalysis of Ishii et al. (1981) by Shibui et al. (2019), also reported by EFSA_UA04 (2011).

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, Charles River albino (M) Weanling 104 wk EFSA_E34 (2011)	Oral administration (feed) Purity, NR (SC-18862; impurities, ≤ 1.5% of conversion product diketopiperazine, SC-191912) Feed 0, 1, 2, 4, 8 g/kg bw per day 60, 40, 40, 40, 40 23, 18, 21, 23, 21	*Brain* Glioma (astrocytoma, oligodendroglioma, combined) 0/60, 2/40 (5%), 1/40 (2.5%), 2/40 (5%), 0/40	NS	*Principal strengths*: 104-wk study; males and females used; multiple-dose study; adequate number of animals per group. *Principal limitations*: 35% and 25% survival in females at 4 g/kg bw and 8 g/kg bw; survival was about 50% in most groups; chronic murine pneumonia complex in control and treated animals; only 25% of animals examined microscopically in groups at 1 g/kg bw and 2 g/kg bw; study from pre-GLP era.
Full carcinogenicity Rat, Charles River albino (F) Weanling 104 wk EFSA_E34 (2011)	Oral administration (feed) Purity, NR (SC-18862; impurities, up to 1.5% of conversion product diketopiperazine, SC-191912) Feed 0, 1, 2, 4, 8 g/kg bw per day 60, 40, 40, 40, 40 26, 23, 20, 14, 10	*Brain* Glioma (astrocytoma, oligodendroglioma, glioma unclassified, combined) 0/60, 2/40 (5%), 0/40, 0/40, 1/40 (2.5%)	NS	*Principal strengths*: 104-wk study; males and females used; multiple-dose study; adequate number of animals per group. *Principal limitations*: 35% and 25% survival in females at 4 g/kg bw and 8 g/kg bw; survival was about 50% in most groups, chronic murine pneumonia complex in control and treated animals, only 25% of animals examined microscopically in 1 g/kg bw and 2 g/kg bw groups, study from the pre-GLP era.

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, Sprague-Dawley (M) Day 12 of gestation Lifetime Soffritti et al. (2007)	Oral administration (feed) Purity, > 98.7% (impurities, diketopiperazine, < 0.3%, and L-phenylalanine, < 0.5%) Feed 0, 400, 2000 ppm, daily 95, 70, 70 95, 70, 70	*Systemic (multiple organs)* Lymphoma or leukaemia (combined) 9/95 (9.5%), 11/70 (15.7%), 12/70 (17.1%)* *Renal pelvis* Papilloma 0, 2/70 (2.9%), 1/70 (1.4%) *Mammary gland* Carcinoma 0, 0, 2/70 (2.9%)	*$P \leq 0.05$, Cox regression model [$P = 0.0030$, Fisher exact test] NS NS	*Principal strengths*: males and females used; prenatal exposure; large group size (70–95); lifetime exposure (> 2 yr); two doses, with the lower dose being relevant to human exposure. *Principal limitations*: details of pathology descriptions were not available. *Other comments*: no information on the incidence, distribution, and histotype of the lymphomas and leukaemias presented. See the text for details on the histological reanalysis by Tibaldi et al. (2020) and the statistical reanalysis by Gnudi et al. (2023). Historical controls: lymphoma and leukaemia (combined), 2265 males, 20.6% (range, 8.0–30.9%).

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, Sprague-Dawley (F) Day 12 of gestation Lifetime Soffritti et al. (2007)	Oral administration (feed) Purity, > 98.7% (impurities, diketopiperazine, < 0.3%, and L-phenylalanine, < 0.5%) Feed 0, 400, 2000 ppm, daily 95, 70, 70 95, 70, 70	*Systemic (multiple organs)* Lymphoma or leukaemia 12/95 (12.6%), 12/70 (17.6%), 22/70 (31.4%)*	$P \leq 0.01$, Cox regression model (trend) *$P \leq 0.01$, Cox regression model; [$P = 0.0030$, Fisher exact test]	*Principal strengths*: males and females used; prenatal exposure; large group size (70–95); lifetime exposure (> 2 yr); two doses, with the lower dose being relevant to human exposure. *Principal limitations*: details of pathology descriptions were not available. *Other comments*: no information on the incidence, distribution, and histotype of the lymphomas and leukaemias was presented. Historical controls: lymphoma and leukaemia (combined), 2274 females, 13.3% (range, 4.0–25.0%); mammary gland carcinoma, 2274 females, 9.2% (range, 4.0–14.2%); a statistical reanalysis by Gnudi et al. (2023) showed positive significant trends in the incidence of total lymphoid tumours, myeloid leukaemia, and total myeloid tumours, furthermore, significant increases were shown in the incidence of total lymphoid tumours, lymphoma (all types), and leukaemia (all types).
		Renal pelvis Papilloma 0/95, 6/70 (8.6%)*, 0/70	[*$P = 0.0051$, Fisher exact test]	
		Mammary gland Carcinoma 5/95 (5.3%), 5/70 (7.1%), 11/70 (15.7%)*	$P \leq 0.05$, Cox regression model (trend) *$P \leq 0.05$, Cox regression model; [$P = 0.0245$, Fisher exact test]	

Table 3.2 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, Charles River albino (M) F_1 rats were obtained from parents exposed 60 days before mating 104 wk EFSA E70 (2011)	Oral administration (feed) Purity, > 98.5% (SC-18862; impurities, conversion product diketopiperazine, SC-191912, 0.4–1.5%) Feed 0, 2, 4 g/kg bw per day 60, 40, 40 25, 20, 23	*Brain* Astrocytoma 3/60 (5%), 1/40 (2.5%), 1/40 (2.5%)	NS	*Principal strengths*: 104-wk study; males and females used; multiple-dose study; adequate duration of exposure and observation. *Principal limitations*: survival was about 50% in most groups; chronic murine pneumonia complex in control and treated animals; study did not adjust for litter effects.
Full carcinogenicity Rat, Charles River albino (F) F_1 rats were obtained from parents exposed 60 days before mating 104 wk EFSA E70 (2011)	Oral administration (feed) Purity, > 98.5% (SC-18862; impurities, conversion product diketopiperazine, SC-191912, 0.4–1.5%) Feed 0, 2, 4 g/kg bw per day 60, 40, 40 28, 18, 21	*Brain* Astrocytoma 1/60 (1.7%), 1/40 (2.5%), 0/40	NS	*Principal strengths*: 104-wk study; males and females used; multiple-dose study; adequate duration of exposure and observation. *Principal limitations*: survival was about 50% in most groups; chronic murine pneumonia complex in control and treated animals; study did not adjust for litter effects.

approx., approximately; bw, body weight; F, female; GLP, Good Laboratory Practice; M, male; NR, not reported; NS, not significant; ppm, parts per million; wk, week(s); yr, year(s).

In female rats, an overall increase in the incidence of animals bearing malignant tumours was observed, with a significant positive trend ($P \leq 0.01$, Cochran–Armitage trend test); the incidence in rats at 50 000 ppm was significantly higher than that in the control group ($P \leq 0.01$, poly-3 test). There was a significant positive trend ($P \leq 0.01$, Cochran–Armitage trend test; $P \leq 0.05$, poly-3 trend test) in the incidence of lymphoma and leukaemia (combined) (multiple organs) – 13/150 (9%), 22/150 (15%), 30/150 (20%), 28/150 (19%), 19/100 (19%), 25/100 (25%), and 25/100 (25%) at 0 (control), 80, 400, 2000, 10 000, 50 000, and 100 000 ppm, respectively – with the incidence being statistically significant at 2000 and 10 000 ppm ($P \leq 0.05$, poly-3 test; [$P \leq 0.01$, Fisher exact test]) and at 50 000 and 100 000 ppm ($P \leq 0.01$, poly-3 test; [$P \leq 0.01$, Fisher exact test]), and within the upper bound of the range observed in historical controls (2274 males, 13.3%; range, 4.0–25.0%) from this laboratory (Soffritti et al., 2005, 2006). [The Working Group noted that the lymphoid and myeloid tumour classification was originally performed according to the IARC Classification of Rodent Tumours (IARC, 1993) and subsequently according to the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions Project (INHAND), both on the basis of haematoxylin-and-eosin–stained sections only, defining lymphoid proliferations (lymphoblastic leukaemia, lymphocytic lymphoma, lymphoimmunoblastic lymphoma) and myeloid proliferations (histiocytic sarcoma, monocytic leukaemia, myeloid leukaemia). The main types reported were lymphocytic lymphomas and lymphoimmunoblastic lymphomas that involved the thymus, lung, spleen, and peripheral lymph nodes. Although details on the incidence of the different morphologies or localization were not reported in Soffritti et al. (2006), they were subsequently provided in Gnudi et al. (2023), at the request of the Working Group. The Working Group members were in agreement on the combinations of lymphoid tumours and lymphoid leukaemias, and combinations of myeloid leukaemias, but not on the combinations of lymphoid and myeloid tumours, and combinations of lymphoid leukaemias and myeloid leukaemias.] The incidence of peripheral nerve malignant schwannoma (cranial and other) – 0/150, 2/150 (1.3%), 0/150, 3/150 (2%), 1/100 (1%), 1/100 (1%), 2/100 (2%), at 0 (control), 80, 400, 2000, 10 000, 50 000, and 100 000 ppm, respectively – was within the upper bound of the range observed in historical controls (2274 females, 0.1%; range, 0–2.0%) from this laboratory (Soffritti et al., 2005, 2006). [The Working Group noted that all the schwannoma diagnoses were supported by positive immunohistochemical staining for S100 protein.] There were significant exposure-specific increases in the incidence of proliferative urothelial lesions within the renal pelvis and ureter in female rats. There was a significant positive trend ($P \leq 0.05$, Cochran–Armitage trend test) in the incidence of dysplastic papilloma – 0/150, 1/150 (0.7%), 1/150 (0.7%), 1/150 (0.7%), 1/100 (1%), 1/100 (1%), 3/100 (3%), at 0 (control), 80, 400, 2000, 10 000, 50 000, and 100 000 ppm, respectively. The incidence of carcinoma of the renal pelvis and ureter – 0/150, 1/150 (0.7%), 3/150 (2%), 3/150 (2%), 3/100 (3%), 3/100 (3%), 4/100 (4%), at 0 (control), 80, 400, 2000, 10 000, 50 000, and 100 000 ppm, respectively – was significantly increased at 100 000 ppm ($P \leq 0.05$, poly-3 test; [$P = 0.0247$, Fisher exact test]), and exceeded the upper bound of the range observed in historical controls (2274 females, 0.04%; range, 0–1.0%) from this laboratory at all doses except for 80 ppm. There was a significant positive trend in the incidence of papilloma or carcinoma (combined) of the renal pelvis and ureter [$P = 0.041$, Cochran–Armitage trend test], with the incidence – 0/150, 2/150 (1.3%), 4/150 (2.7%), 4/150 (2.7%), 4/100 (4%), 4/100 (4%), 7/100 (7%), at 0 (control), 80, 400, 2000, 10 000, 50 000, and 100 000 ppm, respectively – being significantly increased at 10 000 and 50 000 ppm [$P = 0.0247$, Fisher exact test] and 100 000 ppm [$P = 0.0014$,

Fisher exact test]. There was a significant increase [P = 0.0015, Fisher exact test] in the incidence of mammary gland carcinoma in the group at 50 000 ppm – 18/100 (18%) compared with 8/150 (5%) in controls. In addition, one transitional cell carcinoma of the urinary bladder was reported in female rats at 2000 ppm. There was a single incidence each of adenoma and of olfactory neuroblastoma in females at 100 000 ppm.

Exposure to aspartame increased the incidence of malignant brain tumours (10 gliomas, 1 medulloblastoma, and 1 malignant meningioma) in male and female rats, with none being reported in concurrent controls (Soffritti et al., 2006). [The Working Group noted that gliomas are rare tumours (incidence, approximately 0.5–1%) among historical controls in this colony of Sprague-Dawley rats.] The incidence of glioma in males and females in the study by Belpoggi et al. (2006) was reported as follows: males, 0/150, 2/150, 0/150, 1/150, 0/100, 2/100, 1/100; females, 0/150, 1/150, 0/150, 1/150, 0/100, 1/100, 1/100 at 0 (control), 80, 400, 2000, 10 000, 50 000, and 100 000 ppm, respectively. The incidence of brain tumours exhibited no dose–response trends. The data on brain neoplasms were based on the examination of three sagittal sections rather than the seven or eight coronal sections used in other studies. [The Working Group noted that males and females were combined when counting malignant brain tumours. The Working Group also noted that incidence was reported separately for each sex in the study by Belpoggi et al. (2006) reported above.]

Regarding non-neoplastic lesions, there was also an increase in the incidence of dysplastic hyperplasia of the transitional cell epithelium of the renal pelvis and ureter in males (no significant trend) and in females (significant positive trend). There was an increase in the incidence of calcification (mineralization) within the renal pelvis and ureter of female rats, especially at the higher doses (10 000 ppm, 19%; 50 000 ppm, 25%; and 100 000 ppm, 39%) compared with the concurrent controls (8%). A similar effect was not reported in the male rat kidney. Information on the severity and distribution of this mineralization was not available, and its contribution to the proliferative lesions remains to be determined. The incidence of hyperplasia of the olfactory epithelium exhibited a significant positive trend in males, and was significantly increased at 10 000, 50 000, and 100 000 ppm in males and females, and at 400 ppm in males. [The Working Group noted that this was a well-conducted long-term study that used multiple dose groups, both sexes, a large group size, and with a duration that covered most of the rat lifespan. However, it was limited by the lack of detailed pathology descriptions for some haematolymphoid lesions, the combination of non-neoplastic and neoplastic proliferative lesions of the renal pelvis and ureter for statistical analyses, and the use of combinations of lymphoid and myeloid neoplastic lesions that are not common. The Working Group noted that the practice of combining lymphoid and myeloid proliferations was unwarranted since these lesions derive from different cell lineages. It was noted that lung lymphomas (primary pulmonary lymphomas, with isolated or dominant lung involvement) are rare in all species, including rats, and are difficult to distinguish from non-neoplastic proliferative lesions. However, in the present study, this presentation and diagnosis was highly prevalent. The Working Group also noted that the arguments used for some lymphoma diagnoses reported in Soffritti et al. (2006) and the histological reanalysis by Tibaldi et al. (2020) were not sufficient to allow differentiation between inflammatory or neoplastic proliferations, especially those in the lung (see Section 3.2.2). The diagnostic criteria were adequate for myeloid and lymphoid tumours and leukaemia, with the exception of immunoblastic lymphoma of the lung, for which the differentiation between reactive hyperplasia and neoplasia has been recognized as challenging by the NTP, Ramazzini Institute, and pathologists

from other laboratories (Malarkey and Bucher, 2011; Tibaldi et al., 2020).]

Gnudi et al. (2023) reported on the details of diagnoses of haemolymphoreticular tumours and related statistics for the study by Soffritti et al. (2006) in rats treated with aspartame. In males, this reanalysis showed a significant positive trend in the incidence of immunoblastic lymphoma (P = 0.0006, Cochran–Armitage trend test) and total lymphoid tumours (P = 0.0022, Cochran–Armitage trend test). There was a significant increase in the incidence of lymphoma (all types) (P = 0.016, Fisher exact test) at 100 000 ppm, immunoblastic lymphoma (P = 0.016, Fisher exact test) at 100 000 ppm, and total lymphoid tumours (P = 0.023, Fisher exact test) at 100 000 ppm.

In females, there was a significant positive trend in the incidence of immunoblastic lymphoma (P = 0.0015, Cochran–Armitage trend test), myeloid leukaemia (P = 0.0060, Cochran–Armitage trend test), and total myeloid tumours (P = 0.0473, Cochran–Armitage trend test). There was a significant increase in the incidence of lymphoma (all types) at 100 000 ppm (P = 0.028, Fisher exact test), of immunoblastic lymphoma at 50 000 and 100 000 ppm (P = 0.030, P = 0.016, respectively, Fisher exact test), of total lymphoid tumours at 100 000 ppm (P = 0.028, Fisher exact test), of monocytic leukaemia at 50 000 ppm and 400 ppm (P = 0.025, P = 0.030, respectively, Fisher exact test), of histiocytic sarcoma at 10 000 and 50 000 ppm (P = 0.015, P = 0.053, respectively, Fisher exact test), and of total myeloid tumours at 400, 2000, 10 000, 50 000, and 100 000 ppm (P = 0.013, P = 0.021, P = 0.004, P = 0.002, P = 0.008, respectively, Fisher exact test). [The Working Group acknowledged that histological diagnoses of the original study were retained for this analysis. The diagnostic criteria were adequate for myeloid and lymphoid tumours and leukaemia, with the exception of immunoblastic lymphoma of the lung, for which the differentiation between reactive hyperplasia and neoplasia has been recognized as challenging by the NTP, the Ramazzini Institute, and pathologists from other institutes (Malarkey and Bucher, 2011; Tibaldi et al., 2020). The Working Group noted that the histological diagnoses of immunoblastic lymphoma of the lung remained inconclusive. Although the majority of the Working Group considered that the histological diagnoses in autolytic tissues were not reliable, a minority of the Working Group considered that these bore no significance, because the moribund animals were monitored two to three times daily to avoid such concerns.]

In a study by Ishii (1981), also reported by Ishii et al. (1981), groups of 60 male and 60 female SLC Wistar rats (age, 6 weeks) were treated with feed containing aspartame (purity not reported) at a concentration of 0 (control), 1, 2, or 4 g/kg bw per day for 104 weeks. All surviving rats in these groups were killed after 104 weeks. Two additional groups of 16 males and 16 females or 10 males and 10 females were scheduled for interim evaluation at experimental weeks 52 and 26, respectively. Survival at 104 weeks was lower in males (26/60, 16/60, 28/60, 25/60) than in females (49/60, 40/60, 51/60, 43/60) at 0 (control), 1, 2, and 4 g/kg bw per day, respectively. Mean feed consumption was lower in all dosed rats than in controls. A dose-dependent decrease in body-weight gain was observed in groups of males at 2 and 4 g/kg bw per day, and in all dosed groups of females. At 52 weeks, an increase in relative spleen weight was reported only in males at 2 and 4 g/kg bw per day, but no histological correlates were described (Ishii, 1981; also reported by Ishii et al., 1981). Dietary administration of aspartame did not cause a significant increase in the incidence of any type of neoplasm in male or female rats at either interim time of 26 or 52 weeks Ishii (1981).

At 104 weeks, dietary administration of aspartame did not cause a significant increase in the incidence of any type of neoplasm in male or female rats Ishii et al. (1981). There were no

differences in the incidence of glioma across all groups in males – 0/60, 1/60 (1.7%), 0/60, 1/60 (1.7%) or females, 1/60 (1.7%), 0/60, 1/60 (1.7%), 0/60, at 0 (control), 1, 2, and 4 g/kg bw, respectively (Ishii, 1981).

Shibui et al. (2019), also reported by EFSA_UA04 (2011), subsequently re-evaluated the rat tissues from groups at 0 (control), 1, 2, and 4 g/kg bw per day from the study by Ishii et al. (1981). [The Working Group noted that the study by Shibui et al. (2019) was a comprehensive re-evaluation of the study by Ishii et al. (1981), carried out by cutting new slides.] In males, there was a non-significant increase in the incidence of bronchioloalveolar adenoma – 1/60 (1.7%), 3/60 (5%), 0%, 2/60 (3.3%) at 0 (control), 1, 2, and 4 g/kg bw per day, respectively. In addition, there were two bronchioloalveolar carcinomas reported in the group at 2 g/kg bw per day. When the bronchioloalveolar adenomas and carcinomas were combined, their incidence was 1/60 (1.7%), 3/60 (5%), 2/60 (3.3%), 2/60 (3.3%) at 0 (control), 1, 2, and 4 g/kg bw per day, respectively. There were non-significant exposure-related increases in the incidence of adenoma in the pars distalis of the pituitary gland – 5/48 (10.4%), 8/48 (16.7%), 12/53 (22.6%), 5/53 (9.4%) at 0 (control), 1, 2, and 4 g/kg bw per day, respectively. In addition, there was one carcinoma in the pars distalis of the pituitary gland in the group at 1 g/kg bw per day. There were non-significant increases in the incidence of benign pheochromocytoma – 4/59 (6.8%), 9/59 (15.3%), 4/60 (6.7%), 10/60 (16.7%) at 0 (control), 1, 2, and 4 g/kg bw per day, respectively.

In females, there was a non-significant increase in the incidence of bronchioloalveolar adenoma – 0%, 1/60 (1.7%), 3/60 (5%), 3/60 (5%) – that approached statistical significance. There were no differences in the incidence of pituitary tumours in female rats across all groups. There were non-significant increases in the incidence of benign pheochromocytoma in females – 1/60 (1.7%), 1/60 (1.7%), 6/59 (10.2%), 2/60 (3.3%) at 0 (control), 1, 2, and 4 g/kg bw per day, respectively. There were single instances of malignant pheochromocytoma in the groups at 2 and 4 g/kg bw per day.

Regarding the non-neoplastic lesions, renal pelvis mineralization was observed in males and females. Mineralization in the renal pelvis did not correlate with transitional cell hyperplasia in the kidney in either male or female rats (Shibui et al., 2019). [The Working Group noted that the study by Ishii (1981), also reported by Ishii et al. (1981), used both sexes, multiple doses, an adequate number of animals per group, and an adequate duration of exposure and observation. However, it was a pre-GLP study, although aligned to OECD TG451 (OECD, 2018). It was limited by the lack of information on purity and the sparse details on histopathology in the original study. These data showed increases in the incidence of bronchioloalveolar tumours that approached statistical significance in female rats and non-significant increases in the incidence of benign pheochromocytoma in male and female rats, suggesting a potential effect of chronic exposure to aspartame.]

The first studies on aspartame (SC-18862 compound) were completed in 1973 (EFSA_E33, 2011; EFSA_E34, 2011; EFSA_E87, 2011) and 1974 (EFSA_E70, 2011).

In the 1973 study (EFSA_E34, 2011; also reported by EFSA_E33, 2011; and EFSA_E87, 2011), groups of 40 male and 40 female Charles River Albino rats (60 males and 60 females for the control group) were treated with feed containing aspartame [SC-18862 compound, purity not reported; impurities, 0–1.5% of conversion product SC-191912] at a dose of 0 (control), 1, 2, 4, or 8 g/kg bw per day for 104 weeks. The overall survival was around 50% in both male and female rats but significantly lower (25%) in females at 8 g/kg bw per day. Body-weight gains were significantly lower in the groups of males and females at 8 g/kg bw per day than in the controls, owing to lower feed consumption. Relative kidney weights were increased in male

rats at 2, 4, and 8 mg/kg bw per day, and relative uterus weights were increased in female rats at 2 and 4 mg/kg bw per day, but there were no histological correlates.

There were no significant differences in the incidence of grossly detected tumours in controls and all the exposed dose groups in males and females. Histopathology examinations were performed on all gross lesions identified at necropsy, as well as on 20–25 tissues in the control group and groups at 4 and 8 g/kg bw per day and about 25% of the animals in the groups at 1 and 2 mg/kg bw per day. There was a non-significant increase in the incidence of glioma (astrocytoma, oligodendroglioma, glioma not otherwise specified) in the treated groups of males and females (EFSA E87, 2011). There were two ependymomas in male rats at 4 g/kg bw per day and one ependymoma and one meningeal sarcoma in female rats at 4 g/kg and 8 g/kg bw per day, respectively. There were no other major differences in tumour incidence between controls and all the exposed groups.

Regarding non-neoplastic lesions, renal pelvis epithelial hyperplasia was observed in males. Nodular hyperplasia in the pancreas, a non-neoplastic proliferative lesion, was observed in female rats. [The Working Group noted that this study used both sexes, multiple doses, and an adequate duration of exposure and observation. However, this was a pre-GLP study, and was limited by the lack of information on purity, and an infection (murine pneumonia) observed in controls and treated animals.]

3.2.2 Transplacental and perinatal administration followed by oral administration (feed)

In a study published by Soffritti et al. (2007), also reported by Chiozzotto et al. (2011), groups of 70–95 male and 70–95 female Sprague-Dawley rats were treated with feed containing aspartame (purity, > 98.7%; impurities, diketopiperazine < 0.3%, and L-phenylalanine < 0.5%) for pregnant dams starting at day 12 of gestation and continuing in the pups until their natural death. This study focused on human-relevant aspartame dosage levels at 0, 400, and 2000 ppm that simulated a daily intake of 0, 20, and 100 mg/kg bw. At age 4–5 weeks, the pups were assigned to dose groups that reflected the exposure of their respective dams. The Sprague-Dawley rats were obtained from the breeding colony maintained at the Ramazzini Institute. The exposure lasted until the natural death of the animals, and all moribund or found dead animals were promptly necropsied for gross and microscopic evaluation of an extensive list of tissues identified in the study protocol. The in-life phase of the study lasted until 147 weeks. There appeared to a slight reduction (significance not reported) in survival in dosed groups compared with controls. There were no significant differences in water consumption, in feed consumption, and in body-weight gains (Soffritti et al., 2007; also reported by Chiozzotto et al., 2011).

In male rats, there was a significant positive trend ($P \leq 0.01$, Cox regression model) in the incidence of animals bearing malignant tumours, with the incidence – 23/95 (24.2%), 18/70 (25.7%), 28/70 (40%), for 0 (control), 400 and 2000 ppm, respectively – being significantly increased ($P \leq 0.01$, Cox regression model; [$P = 0.0231$, Fisher exact test]) at 2000 ppm. There was an increase in the incidence of lymphoma/leukaemia (combined) – 9/95 (9.5%), 11/70 (15.7%), 12/70 (17.1%), at 0 (control), 400, and 2000 ppm, respectively – with the incidence being significant ($P \leq 0.05$, Cox regression model, [$P = 0.003$, Fisher exact test]) at 2000 ppm, and these increases were within the upper bound of the range observed in historical controls (2265 males, 20.6%; range, 8–30.9%) from this laboratory (Soffritti et al., 2005, 2006). [The Working Group noted that lung lymphomas (primary lymphomas presenting in the lung, with isolated or dominant lung involvement) are rare in all species, including rats,

and notoriously difficult to differentiate from inflammatory conditions. In the present studies, however, this presentation and diagnosis was a predominant finding. The results of the subsequent study (Tibaldi et al., 2020) were insufficient to differentiate between inflammatory or neoplastic proliferations, since the immunohistochemical markers used were not appropriate for this purpose and the staining was of insufficient technical quality, on the basis of the figures provided. Additional supportive evidence for neoplasia using flow cytometry or molecular techniques such as polymerase chain reaction or Southern blot were not provided. Therefore, these diagnoses remain largely unconfirmed. The Working Group noted that the review of slides in this study confirmed the diagnoses in 72 out of 78 cases and revised the diagnoses from malignant to benign in 8% of evaluable cases.] No significant difference was observed in the incidence of renal pelvis papilloma and mammary gland carcinoma compared with concurrent controls.

In female rats, there was a significant positive trend ($P \leq 0.01$, Cox regression model) in the incidence of lymphoma/leukaemia (combined), with the incidence – 12/95 (12.6%), 12/70 (17.1%), 22/70 (31.4%), at 0 (control), 400, and 2000 ppm, respectively – being significantly increased at the highest dose ($P \leq 0.01$, Cox regression model; [$P = 0.0030$, Fisher exact test]), and exceeding the upper bound of the range in historical controls at the highest dose (2274 females, 13.3%; range, 4.0–25%) from this laboratory (Soffritti et al., 2005, 2006). There was a significant positive trend ($P \leq 0.05$, Cox regression model) in the incidence of carcinoma of the mammary gland, with the incidence – 5/95 (5.3%), 5/70 (7.1%), 11/70 (15.7%), at 0 (control), 400, and 2000 ppm, respectively – being significantly increased at the highest dose ($P \leq 0.05$, Cox regression model; [$P = 0.0245$, Fisher exact test]), and exceeding the upper bound of the range in historical controls at this dose (2274 females, 9.2%; range, 4.0–14.2%) from this laboratory (Soffritti et al., 2005, 2006). The incidence of renal pelvis papilloma was significantly increased [$P = 0.0051$, Fisher exact test] at the lowest dose – 0/95, 6/70 (8.6%), 0/70, at 0 (control), 400, and 2000 ppm, respectively (Soffritti et al., 2007; also reported by Chiozzotto et al., 2011). There was a significant increase in the incidence of uterus polyps and of head osteoma in the group at 400 ppm (Soffritti et al., 2007; also reported by Chiozzotto et al., 2011).

Regarding non-neoplastic lesions, no data were available to the Working Group.

[The Working Group noted that this study used both sexes, a low dose relevant to human exposure, a high number of animals per group, and an adequate duration of exposure and observation (prenatal and lifetime exposure). The study was not conducted under GLP. The Working Group expressed concerns regarding the lack of adjustment for potential litter effects (e.g. there was no information regarding the number of litters per treatment group, the number of pups per litter per treatment group, or whether the treatments were loaded in a balance fashion), which could lead to false-positive results. A minority of the Working Group considered that these concerns might have been overcome because the large sample size per group (n = 70–150), significant dose–response trend, and pairwise testing of multiple tumour types suggested the absence of an potential bias due to litter effects. The diagnostic criteria were adequate for myeloid and lymphoid tumours and leukaemia, with the exception of immunoblastic lymphoma of the lung, for which the differentiation between reactive hyperplasia and neoplasia has been recognized as challenging by the NTP, the Ramazzini Institute, and other pathologists (Malarkey and Bucher, 2011; Tibaldi et al., 2020).]

Subsequently, all the haematopoietic and lymphoid tissue tumours from the prenatal exposure study (Soffritti et al., 2007) were re-evaluated according to updated pathological criteria and with the use of immunohistochemistry (Tibaldi et al., 2020). The analysis confirmed the

tumour diagnoses in 72 cases out of 78, identified 3 cases of lymphoid hyperplasia and categorized 3 cases as inflammatory lesions. A statistically significant increase in the incidence of total haematopoietic and lymphoid tissue tumours (P = 0.006, Fisher exact test), total lymphomas (P = 0.032, Fisher exact test), and total leukaemia (P = 0.031, Fisher exact test) in treated female rats was confirmed (high dose versus controls), and a statistically significant linear trend for each haematolymphoid tumour type was also observed. [The Working Group noted that the immunohistochemistry study by Tibaldi et al. (2020) provided immunophenotypical characterization of the constituent cell populations in the proliferative haematolymphoid lesions, but this information did not provide definitive data to support or refute whether a haematolymphoid proliferative lesion in the lung was neoplastic or reactive. The Working Group agreed that determining clonality, for establishing the neoplastic nature of haematolymphoid proliferative lesions in the lung, was beyond the scope of routine rodent carcinogenicity assays.]

The statistical reanalysis performed by Gnudi et al. (2023) reported positive significant trends in females: total lymphoid tumours (P = 0.0368, Cochran–Armitage trend test), myeloid leukaemia (P = 0.0324, Cochran–Armitage trend test), total myeloid tumours (P = 0.0485, Cochran–Armitage trend test), and lymphoma all types (P = 0.0368, Cochran–Armitage trend test). Furthermore, statistically significant increases in the incidence of total lymphoid tumours (P = 0.027, Fisher exact test), and leukaemia all types (P = 0.007, Fisher exact test) were observed in females at 2000 ppm females compared with their respective controls. The constituent lesions comprising the lymphoma or leukaemia (combined) diagnosis included various lymphoid tumours (lymphoblastic lymphoma, lymphocytic lymphoma, lymphoimmunoblastic lymphoma) and myeloid tumours (histiocytic sarcoma, monocytic leukaemia, myeloid leukaemia). [The Working Group acknowledged that revised histological diagnoses of the Tibaldi et al. (2020) study were retained for this analysis. The diagnostic criteria were adequate for myeloid and lymphoid tumours and leukaemia, with the exception of immunoblastic lymphoma of the lung, for which the differentiation between reactive hyperplasia and neoplasia has been recognized as challenging by the NTP, the Ramazzini Institute, and pathologists from other institutes (Malarkey and Bucher, 2011; Tibaldi et al., 2020).]

The 1974 study (EFSA_E70, 2011; also reported by EFSA_E87, 2011) included prenatal exposure to aspartame (SC-18862 compound; impurities, conversion product SC-191912, 0.4–1.5%) of at a dose of 0 (control), 2, or 4 g/kg bw per day (60 days before mating) and subsequent exposure for 104 weeks through maternal milk and through feed after weaning in male and female Charles River Albino rats per day. The controls included 60 males and 60 females, and the treatment groups included 40 males and 40 females. Compared with controls, body-weight gains were significantly lower in males at 4 g/kg bw and were related to lower feed consumption in this group. Survival was around 50% in both the control and exposed groups. Histopathology was performed on all gross lesions, as well as on 20–25 tissues in the control and treated groups.

The incidence of astrocytoma was 3/60 (5%), 1/40 (2.5%), 1/40 (2.5%) in male rats and 1/60 (1.7%), 1/40 (2.5%) and 0/40 in female rats at 0 (control), 2, and 4 g/kg bw, respectively (EFSA_E87, 2011). There was one ependymoma in male rats at 2 g/kg bw and one meningioma in female rats at 4 g/kg bw. No significant differences in tumour incidence were observed between control and treated groups.

Regarding non-neoplastic lesions, adrenal nodular hyperplasia was observed in males, and liver hyperplastic nodules were observed in females. [The Working Group noted that this study used both sexes, multiple doses, and an adequate duration of exposure and observation.

However, there was no adjustment for litter effects and the study was limited by low survival, and an infection (murine pneumonia) observed in controls and treated animals. In addition, this study was performed in a pre-GLP era, and the pathology diagnoses and criteria may not satisfy current practices.]

3.2.3 *Urinary bladder tumour initiation with* N-*butyl*-N-*(4-hydroxybutyl) nitrosamine and promotion by aspartame*

In the study by Hagiwara et al. (1984), also reported by Ito et al. (1984), groups of 30 male F344/DuCrj rats (age, 6 weeks) were treated with drinking-water containing 0.01% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) for 4 weeks followed by feed containing 5% aspartame for 32 weeks, to determine the tumour promotion effect of aspartame. [No information on the purity of BBN or aspartame was provided.] Controls received BBN alone for 4 weeks or aspartame alone for 32 weeks. At necropsy, urinary bladders were examined for evidence of proliferative urothelial lesions such as papillary or nodular hyperplasia, papilloma, or carcinoma.

There was no evidence of increased proliferative urothelial lesions that could be attributed to aspartame exposure. [The Working Group noted that the study by Hagiwara et al. (1984) used an adequate number of animals per group. It was limited by the use of a single sex and a single dose, pathology examinations on one tissue only, the lack of information on purity, and the inadequate duration of exposure and observation for this initiation–promotion model. This study was judged inadequate for the evaluation of the carcinogenicity of aspartame in experimental animals.]

3.3 Hamster

See Table 3.3.

Oral administration (diet)

Groups of 35 male and 35 female Syrian Golden hamsters (weanling; age, 21 days), housed individually, were treated with feed containing aspartame (purity not reported; diketopiperazine, < 1%) at a dose of 1, 2, 4, or 12 g/kg bw per day for 46 weeks (EFSA_E27, 2011; EFSA_E35, 2011; EFSA_E36, 2011). The control group (aspartame, 0 g/kg bw per day) contained 70 males and 70 females. Although the bioassay was scheduled to last 104 weeks, it was terminated after 46 weeks because of extensive mortality (males, 57–77%; females, 69–80%). The deaths occurred across all treatment groups, were not related to aspartame exposure, and were attributed to an unidentified infection, possibly "wet tail" syndrome, a condition to which hamsters are quite susceptible. Body weights were typically within 10% of that of the control group for both males and females. Food consumption did not appear to be affected by treatment with aspartame. The measured consumption of aspartame was 1.1, 2.4, 4.9, and 8.4 g/kg bw per day in males and 1.2, 2.5, 4.4, and 8.1 g/kg bw per day in females. The dietary intake of aspartame in the highest dose group was less than the target of 12 g/kg bw per day in both males and females because the hamsters were fed aspartame at 8 g/kg bw per day during weeks 1–14, 8 g/kg bw per day during weeks 15–18, 10 g/kg bw per day during weeks 19–22, and 12 g/kg bw per day from week 23 onward. Gross and microscopic examinations were conducted on all dead and surviving hamsters.

Dietary administration of aspartame did not cause a significant increase in the incidence of any type of malignant neoplasm in hamsters removed before 46 weeks. A low incidence of adrenal cortex adenoma, which was not related to

Table 3.3 Studies of carcinogenicity with aspartame in hamsters and dogs

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Hamster, Syrian golden (M) Weanling (age, 21 days) 46 wk EFSA E27 (2011)	Oral administration (feed) Purity, NR (< 1% diketopiperazine) Feed 0, 1.1, 2.4, 4.9, 8.4 g/kg bw per day 70, 35, 35, 35, 35 24, 15, 13, 14, 8	*Adrenal cortex* Adenoma 1/22, 0/8, 0/13, 1/13, 1/15	NS	*Principal strengths*: appropriate experimental design, including pathological analysis; sufficient number of animals per group, randomly allocated. *Principal limitations*: extensive mortality due to infection; insufficient duration of exposure; stability and homogeneity of aspartame in the diet not measured; uncertainty regarding purity. *Other comments*: incidence of adrenal cortex adenoma was based upon surviving animals.
Full carcinogenicity Hamster, Syrian golden (F) Weanling (age, 21 days) 46 wk EFSA E27 (2011)	Oral administration (feed) Purity, NR (< 1% diketopiperazine) Feed 0, 1.2, 2.5, 4.4, 8.1 g/kg bw per day 70, 35, 35, 35, 35 14, 7, 6, 11, 11	No significant increase in tumour incidence in treated animals		*Principal strengths*: appropriate experimental design, including pathological analysis; sufficient number of animals per group, randomly allocated. *Principal limitations*: extensive mortality due to infection; insufficient duration of exposure; stability and homogeneity of aspartame in the diet not measured; uncertainty regarding purity.
Full carcinogenicity Dog, Beagle (M) 150–160 days 106 wk EFSA E28 (2011)	Oral administration (feed) Purity, NR (0.1–1% diketopiperazine) from week 20 onwards Feed 0, 1, 2, 4 mg/kg bw per day 5, 5, 5, 5 5, 5, 5, 5	No significant increase in tumour incidence in treated animals		*Principal strengths*: appropriate pathological analyses. *Principal limitations*: limited number of animals per dose group; insufficient duration of exposure; uncertainty regarding purity, from week 20 until the end of week 106 of the feeding period, the test material contained 0.1–1% diketopiperazine (by weight).
Full carcinogenicity Dog, Beagle (F) 150–160 days 106 wk EFSA E28 (2011)	Oral administration (feed) Purity, NR (0.1–1% diketopiperazine) from week 20 onwards Feed 0, 1, 2, 4 mg/kg bw per day 5, 5, 5, 5 5, 5, 5, 5	No significant increase in tumour incidence in treated animals		*Principal strengths*: appropriate pathological analyses. *Principal limitations*: limited number of animals per dose group; insufficient duration of exposure; uncertainty regarding purity, from week 20 until the end of week 106 of the feeding period, the test material contained 0.1–1% diketopiperazine (by weight).

approx., approximately; bw, body weight; F, female; M, male; NR, not reported; NS, not significant; ppm, parts per million.

treatment, was found in surviving male hamsters (EFSA_E27, 2011; EFSA_E35, 2011; EFSA_E36, 2011).

[The Working Group noted that this study used an adequate number of animals per group (randomly allocated) and an appropriate experimental design, including pathological analysis. However, the study was limited by the lack of information on purity, the lack of measurement of the stability and homogeneity of aspartame in the diet, and the extensive mortality due to infection. The Working Group also noted that the 46-week study period was insufficient for tumour induction on the basis of the lifespan of a hamster, which is 2–3 years.]

3.4 Dog

Oral administration (diet)

Groups of five male and five female Beagle dogs (age, 150–160 days), randomized and housed individually, were treated with feed containing aspartame at a dose of 0, 1, 2, or 4 g/kg bw per day for 106 weeks. Although the purity of the aspartame was not indicated, the test material contained 0.1–1.0% diketopiperazine (by weight) from week 20 until the end of the 106-week feeding period (EFSA_E28, 2011; EFSA_E86, 2011). In males, the geometric mean body weight in the group at 2 g/kg bw per day from approximately week 14 was ≤ 90% that of the control group; by week 106, it was 79% that of the control group. The body weight of males in the group at 4 g/kg bw per day was 90% (geometric mean) that of males in the control group from week 88 onward. The geometric mean body weight of treated females was ≥ 90% that of the control group. At the end of the 106-week feeding period, the dogs were killed and a complete macroscopic and microscopic (histological) evaluation was conducted. Subsequently, selected sections from the brain were subjected to an additional histopathological evaluation with an emphasis on ependymal neoplasms because brain ependymal glial proliferation was observed in two animals at 2 g/kg bw per day.

Dietary administration of aspartame did not cause a significant increase in the incidence of any type of neoplasm in this study (EFSA_E28, 2011; EFSA_E86, 2011). [The Working Group noted that this study was limited by the number of dogs used, the lack of information on purity, and the lack of measurement of the stability and homogeneity of aspartame in the diet. The Working Group also noted that the 106-week study period represents < 20% of the lifespan of a Beagle dog and that the induction of tumours in bioassays in Beagle dogs typically takes more than 3 years. Based on the limitations noted, the Working Group considered that the absence of tumour induction was insufficient to assess the carcinogenicity of aspartame.]

3.5 Evidence synthesis for cancer in experimental animals

There were numerous studies available to the Working Group regarding cancer in multiple species (mouse, rat, dog, and hamster). The carcinogenicity of aspartame was assessed in well-conducted GLP studies on oral administration (feed) in male and female transgenic mice in three models: a FVB/N-TgN(v-Ha-*ras*) Led (Tg.AC) hemizygous mouse model (NTP, 2005); a B6.129-*Trp53*^{tm1Brd} (N5) haploinsufficient mouse model (NTP, 2005); and a B6.129-*Cdkn2a*^{tm1Rdp} (N2) deficient mouse model (NTP, 2005). No significant increase in the incidence of tumours was observed. The NTP concluded and the Working Group noted that these were new transgenic mouse models, and the duration of exposure may not have been sufficiently sensitive to detect a carcinogenic effect after chronic exposure to aspartame.

Additional studies included oral administration (feed) in male and female HAM-ICR Swiss mice (EFSA_E75, 2011), in male and female SLC Wistar rats (Ishii, 1981, also reported by Ishii et al., 1981, and reanalysed histologically by Shibui et al., 2019), in male and female Charles River Albino rats (EFSA_E34, 2011; also reported by EFSA_E33, 2011; and EFSA_E87, 2011), and in male and female Charles River Albino rats (EFSA_E70, 2011; also reported by EFSA_E87, 2011); in utero exposure followed by oral administration (drinking-water) in C57BL/6 Ela1-Tag mice (Dooley et al., 2017); and intravesical pellet implantation in female Swiss albino mice (EFSA_E58, 2011; EFSA_E72, 2011 both reported by Bryan, 1984). In addition, initiation–promotion studies were conducted in male Fischer 344 rats (Hagiwara et al., 1984; also reported by Ito et al., 1984).

The Working Group noted that the studies with negative results in mice (EFSA_E75, 2011), rats (EFSA_E34, 2011, and EFSA_E70, 2011); hamsters (EFSA_E27, 2011), and dogs (EFSA_E28, 2011) and by Ishii et al. (1981) were conducted before the advent of GLP guidelines and had some limitations, e.g. lack of information on test substance purity and selective histopathology. Studies on in utero exposure followed by oral administration (drinking-water) in C57BL/6 Ela1-Tag mice (Dooley et al., 2017), by intravesical pellet implantation in female Swiss albino mice (EFSA_E58, 2011; EFSA_E72, 2011; both reported by Bryan, 1984), and the one initiation–promotion study in male Fischer 344 rats (Hagiwara et al., 1984; also reported by Ito et al., 1984) were judged to be inadequate for the evaluation of the carcinogenicity of aspartame in experimental animals.

The carcinogenicity of aspartame has been also assessed by other routes of exposure in studies that did not comply with GLP. Specifically, there were studies on oral administration (feed) in male and female Sprague-Dawley rats (Soffritti et al., 2005, 2006; also reported by Belpoggi et al., 2006; statistical reanalysis by Gnudi et al., 2023) and on transplacental and perinatal exposure followed by oral administration (feed) in male and female Swiss mice (Soffritti et al., 2010; statistical reanalysis by Gnudi et al., 2023) and in male and female Sprague-Dawley rats (Soffritti et al., 2007; reanalysis by Tibaldi et al., 2020, and Gnudi et al., 2023; also reported by Chiozzotto et al., 2011).

During the review of the set of studies conducted by the Ramazzini Institute, Italy (Soffritti et al., 2005, 2006, 2007, 2010, and subsequent reanalyses), the Working Group raised concerns regarding diagnoses of lymphomas located predominantly, but not exclusively, in the lung. Therefore, the Working Group focused its evaluation of the potential carcinogenicity of aspartame in experimental animals on all neoplastic lesions (all solid tumours, myeloid tumours, and leukaemia) except lymphoid tumours and related combinations.

In a study on transplacental and perinatal followed by oral administration (feed) in male and female Swiss mice (Soffritti et al., 2010), there was a significant positive trend in the incidence of hepatocellular carcinoma in males, with the incidence being significantly increased at all doses except the lowest (2000 ppm). There was a significant increase in the incidence of hepatocellular adenoma or carcinoma (combined) at the higher intermediate dose (16 000 ppm) in males. There was a significant positive trend in the incidence of bronchioloalveolar carcinoma in males, with the incidence being significantly increased at the highest dose. There was a significant positive trend in the incidence of bronchioloalveolar adenoma or carcinoma (combined) in males, with incidence being significantly increased at the highest dose. In female mice, dietary administration of aspartame did not cause a significant increase in the incidence of any type of neoplasm (Soffritti et al., 2010). The reanalysis by Gnudi et al. (2023) reported a significant positive trend for leukaemia (all types), with incidence being

significantly increased at all doses in males. Also in males, there was a significant increase in the incidence of lymphoblastic leukaemia at the highest three doses, and a significant increase in the incidence of monocytic leukaemia and total myeloid tumours at the higher intermediate dose (16 000 ppm). In females, there was a significant increase in the incidence of lymphoblastic leukaemia and leukaemia (all types) at the lowest dose.

In the study on oral administration (feed) in male and female Sprague-Dawley rats (Soffritti et al., 2005, 2006; also reported by Belpoggi et al., 2006), there was an overall increase in the incidence of rats bearing malignant tumours, with a significant positive trend for malignant schwannoma of the peripheral nerve, a rare type of tumour, in males. In females, there was an overall increase in the incidence of rats bearing malignant tumours, with a significant positive trend. There was a significant positive trend in the incidence of dysplastic papilloma of the renal pelvis and ureter, a rare type of tumour. The incidence of carcinoma of the renal pelvis and ureter was significantly increased at the highest dose. There was a significant positive trend in the incidence of papilloma and carcinoma (combined) of the renal pelvis and ureter, with incidence being significantly increased at the highest dose. There was a significant increase in the incidence of mammary gland carcinoma in the highest intermediate dose group (50 000 ppm). In addition, one transitional cell carcinoma of the urinary bladder, a rare type of tumour, was reported in female rats at 2000 ppm. The reanalysis by Gnudi et al. (2023) reported a significant positive trend for myeloid leukaemia and total myeloid tumours in females. There was a significant increase in the incidence of monocytic leukaemia at 400 and 50 000 ppm, histiocytic sarcoma at 10 000 and 50 000 ppm, and total myeloid tumours at all doses except the lowest (80 ppm).

In the study on transplacental and perinatal exposure followed by oral administration (feed) in male and female Sprague-Dawley rats (Soffritti et al., 2007; also reported by Chiozzotto et al., 2011), there was a significant positive trend in the incidence of carcinoma of the mammary gland in females, with the incidence being significantly increased at the highest dose (2000 ppm). The incidence of renal pelvis papilloma was significantly increased at the lowest dose (400 ppm). A reanalysis of these data by Gnudi et al. (2023) reported positive significant trends in incidence for leukaemia (all types), myeloid leukaemia, and total myeloid tumours in females. There was a significant increase in the incidence of leukaemia (all types) at 2000 ppm.

Overall, the Working Group considered that there were unresolved questions about the adequacy of the design, conduct, interpretation, and/or reporting of the available studies (Soffritti et al., 2006, 2007, 2010, and subsequent reanalyses). For example, no adjustments were made for litter effects, which can lead to false-positive results for incidence and trend. A minority of the Working Group considered that these limitations were minor and would not affect the overall interpretation of these studies.

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Humans

The absorption, distribution, metabolism, and excretion of aspartame (Chemical Abstracts Service, CAS No. 22839-47-0; α-aspartame; L-α-aspartyl-L-phenylalanine methyl ester) have been intensively investigated in humans after dietary exposure.

Aspartame is normally hydrolysed in the gastrointestinal tract to its major constituents: aspartate, phenylalanine, and methanol (EFSA_E15, 2011a) (Fig. 1.1). It has also been reported that

esterases and peptidases (e.g. aminopeptidase A) in the gastrointestinal tract yield equimolar amounts of the three constituents, as shown in microvillar membranes prepared from human duodenum, jejunum, and ileum in vitro. The jejunal section of the human intestinal microvillar membranes has been reported to be more than twice as active as the duodenal sections at hydrolysing aspartame (Hooper et al., 1994).

The three hydrolytes undergo absorption from the intestinal lumen and reach the systemic circulation, similarly to amino acids and methanol obtained from dietary sources (Stegink, 1987; Butchko et al., 2002; Magnuson et al., 2007). Alternatively, ester hydrolysis in the lumen may yield methanol and the dipeptide, aspartyl-phenylalanine, which is also absorbed into intestinal mucosal cells via a peptide transport mechanism and subsequently undergoes hydrolysis to aspartic acid and phenylalanine in the enterocyte (Stegink, 1984, 1987; Tobey and Heizer, 1986). The hydrolysis of aspartame has been considered efficient, with the three metabolites being absorbed, entering the bloodstream, distributed, and finally excreted through expired air, faeces, or urine, or entering the amino acid pool.

Several studies have failed to detect free aspartame or aspartyl-phenylalanine in the plasma (Ranney et al., 1976; Stegink et al., 1981a; Zhang et al., 2016). Ranney et al. reported that between 10–24% of ^{14}C from radiolabelled aspartame was expired in 24 hours, with 0.7–11% being excreted in the faeces or urine and 10% incorporated into the plasma amino acid pool (Ranney et al., 1976). Only one study, in rodents (Creppy et al., 1998), has measured absorption of aspartame as the intact compound (by high-performance liquid chromatography, HPLC) (see Section 4.1.2).

More recently, in humans, Zhang et al. (2016) used highly sensitive HPLC coupled with isotope dilution tandem mass spectrometry to quantify aspartame and some other artificial sweeteners (acesulfame-K, saccharin, cyclamate) in paired samples of blood and urine from healthy adults and in liver samples from liver cancer patients. Aspartame was not found in any analysed sample, although acesulfame-K, saccharin, and cyclamate were reported to be within the limits of quantification (LOQs) at 0.001–0.01 ng/mL in urine, 0.01–0.1 ng/mL in blood, and 0.01–0.1 ng/g in liver tissue. [The Working Group noted that the specific LOQs for each analyte were not reported.] In contrast, Liu et al. (2022a) recently reported the detection of aspartame in serum samples from 218 pregnant women with and without gestational diabetes. Using ultra-performance liquid chromatography coupled with tandem mass spectrometry, they found serum aspartame levels in the range of 0.0459 to 0.0692 nmol/L (median, 0.0563 nmol/L [16.57 ng/L]), with no significant differences between the two groups. [The Working Group noted that the aspartame levels reported by Liu et al. (2022a) were several orders of magnitude lower than the reported increases in serum levels of aspartame-derived phenylalanine after aspartame intake; however, the Working Group expressed concerns about the quality of this study (see Section 1.4.3).]

Aspartame has not been detected in breast milk from lactating women (Stegink et al., 1979a; Sylvetsky et al., 2015). [The Working Group noted that in the studies from Stegink et al., aspartame was mainly provided in orange juice.]

After dosing with aspartame (34 mg/kg body weight (bw), in orange juice), absorbed phenylalanine was shown to enter the plasma pool of free amino acids from the portal blood, after partial conversion to tyrosine in the liver mediated by phenylalanine hydroxylase. Aspartic acid underwent transamination in the enterocyte, producing oxaloacetate, an intermediate in the citric acid cycle, thus decreasing the amount of aspartate entering the portal circulation (Filer and Stegink, 1989). Methanol does not undergo metabolism in the enterocyte but enters the portal circulation and is oxidized to formaldehyde by hepatic alcohol dehydrogenase.

Formaldehyde quickly undergoes formaldehyde dehydrogenase-mediated oxidation to formic acid ($t_{1/2}$ = 1–2 minutes) in the portal circulation (reviewed by Magnuson et al., 2007). Ultimately, formic acid has been reported to convert to carbon dioxide and water via formation of 10-formyl tetrahydrofolate (Barceloux et al., 2002). [The Working Group noted that methanol released from aspartame hydrolysis corresponds to 10% of aspartame by weight. It has been estimated that the overall exposure to methanol from aspartame at 40 mg/kg bw per day (the established acceptable daily intake, ADI) could range from 1% to 10% in the general population, with exposure from other food sources accounting for < 10% and exposure to endogenous pathways accounting for > 80% (EFSA, 2013). Although the Working Group also noted that formaldehyde has been classified by IARC as *carcinogenic to humans* (Group 1), no evidence was available as to whether exposure to formaldehyde at the levels arising from aspartame consumption would significantly alter normal endogenous formaldehyde concentrations.]

The metabolism of aspartame and of each of its components has been studied in healthy adults and infants, lactating women, adults who are heterozygous and homozygous for phenylketonuria, and in people with diabetes, high body mass index (BMI), or non-fatty liver disease. These studies have included a broad range of doses, single, repeated, or long-term dosing, and co-administration with meals (Stegink et al., 1982, 1983a, 1987b, 1991; Romano et al., 1989; Burns et al., 1991). There were no significant effects of aspartame on the plasma concentrations of any amino acids. The following paragraphs summarize the relevant information.

(a) Metabolism in healthy volunteers

In an early study from the 1970s by the manufacturer, for example, the absorption, distribution, and metabolism of aspartame were determined in three men given a single oral dose of 500 mg of [^{14}C-Phe]aspartame (18.7 μCi) in water (EFSA E15, 2011a). As a result of rapid hydrolysis of aspartame in the gut, plasma radiolabel levels rose quickly; half-maximal levels were reached after about 15 minutes and peak levels after 4–8 hours after dosing. The elimination half-life from the plasma was relatively slow ($t_{1/2}$ = 47.5 hours), consistent with incorporation into body constituents; plasma radiolabel was detected in naturally occurring polar compounds that included proteins, peptides, and free amino acids (phenylalanine and tyrosine). Total urinary excretion of the radiolabel represented a very small fraction of the administered dose (0.46 ± 0.04%, measured 0–48 hours after dosing) and occurred primarily in the first 4 hours (EFSA E15, 2011a).

Plasma or erythrocyte aspartate concentrations were not significantly affected by single doses of aspartame, except for slight increases in mean peak plasma concentrations at higher doses (Stegink et al., 1977, 1979a, b, 1987a, 1980, 1981a). In lactating women, a small, but statistically significant, difference in overall milk aspartate levels was noted 4 hours after dosing but did not persist over 24 hours (Stegink et al., 1979a). [The Working Group noted that aspartame was provided in orange juice, and a clear negative control was not used in the study.]

Plasma phenylalanine concentrations were not significantly increased after a single dose of aspartame (about 4 mg/kg bw) (Wolf-Novak et al., 1990), although there were brief significant increases at higher doses (10 mg/kg bw) (Stegink et al., 1979b, 1987a). Erythrocyte phenylalanine levels exhibited similar, but smaller changes (Stegink et al., 1979b). After very high doses of aspartame (100, 150, and 200 mg/kg bw), mean peak plasma phenylalanine concentrations were approximately proportional to the administered dose, and considerably higher than the normal postprandial range (Stegink et al., 1980, 1981a). Erythrocyte phenylalanine concentrations showed similar changes (Stegink et al., 1981a).

[The Working Group noted that the aspartame was provided in orange juice, and a clear negative control was not used in several of the above studies.]

Like aspartate in the same study, a small, but statistically significant, difference in overall milk phenylalanine levels was noted 4 hours after dosing but did not persist over 24 hours (Stegink et al., 1979a).

Regarding methanol metabolism, in men and women given a single dose of aspartame (34, 100, 150, or 200 mg/kg bw), blood methanol concentrations were below the limit of detection (0.4 mg/dL) at the lowest dose [the Working Group noted that currently available methods would have better sensitivity] but significantly increased after each higher dose, and mean peak blood methanol concentrations and blood methanol AUCs increased proportionally to dose (Stegink et al., 1981b). Concentrations returned to baseline by 24 hours after aspartame administration for all dosing levels (Stegink et al., 1981b). Formate analyses for participants at the highest dose showed no significant changes in blood formate concentration, but urinary formate excretion was significantly increased in urine samples taken 0–4 hours and 4–8 hours after aspartame ingestion, returning to baseline values by 8–24 hours after aspartame loading. [The Working Group noted that the urinary excretion data were consistent with the conversion of methanol to formate, with the rate of formate production not exceeding the rate of formate excretion. The Working Group further noted that the data suggest that the metabolism of methanol to formate and the renal excretion of formate are efficient.]

The effects of long-term dosing have been assessed in a 24-week randomized, double-blind, placebo-controlled, parallel-group study (Leon et al., 1989). After a large daily dose of aspartame (75 mg/kg bw), no persistent changes were noted in clinical and standard laboratory tests throughout the study. Over time, mean fasting plasma concentrations of aspartate and phenylalanine did not show any statistically significant differences from baseline. Likewise, plasma levels of all other amino acids did not indicate any consistent pattern of change in either group, except for tyrosine, for which plasma levels were slightly higher (although within the normal range) at weeks 3 and 24 in the aspartame group compared with controls ($P < 0.05$). Likewise, the ratio of plasma levels of phenylalanine to other large neutral amino acids (Phe/LNAA) did not change in either group over the course of the study. Most blood methanol concentrations were below the limit of detection (0.31 mmol/L) and there was no indication of aspartame-related accumulation of methanol. There were no significant differences in blood formate levels, in mean 24-hour concentrations of urinary formate, or in urinary formate to creatinine ratios, consistent with no significant increase in formate production after a long-term, high-dose aspartate regimen.

Aspartame metabolism in infants was reported to be generally similar to that in adults receiving aspartame at the same dose levels (Filer et al., 1983; Stegink et al., 1983b). In older people, the volume of distribution and clearance of phenylalanine were significantly decreased, but the elimination half-life ($t_{1/2}$) was not significantly different from that in younger adults (Puthrasingam et al., 1996).

(b) Metabolism in people with certain health conditions

(i) Phenylketonuria

Phenylketonuria (PKU) is an autosomal recessive disorder of phenylalanine metabolism characterized by mutations in the gene for hepatic phenylalanine hydroxylase that decrease or inactivate its function. Since this enzyme is necessary for the metabolism of phenylalanine to tyrosine, homozygous PKU subjects are at risk for build-up of dietary phenylalanine to potentially

toxic levels, ultimately leading to brain dysfunction (van Spronsen et al., 2021).

The ability to metabolize aspartame-derived phenylalanine has been studied in individuals who are PKU homozygous (e.g. EFSA_E26, 2011; Koch et al., 1976; Caballero et al., 1986) (on low phenylalanine diets or otherwise, although these individuals are not expected to ingest aspartame-sweetened products, which carry a warning label) and particularly in PKU heterozygous (e.g. single doses: Stegink et al., 1979b, 1980, 1981c, 1987a; Caballero et al., 1986; Filer and Stegink, 1989; Curtius et al., 1994; da Silva et al., 2000; EFSA_E108, 2011; EFSA_E109, 2011; repeated doses: Stegink et al., 1989, 1990; and with meals: EFSA_E25, 2011; EFSA_E67, 2011). In general, plasma phenylalanine levels and AUCs were significantly higher in the PKU heterozygotes than in the normal participants, and one study suggested that PKU heterozygotes metabolized aspartame-derived phenylalanine approximately twice as slowly as do normal individuals (Stegink et al., 1980; EFSA_E109, 2011). Plasma levels of aspartate remained within the normal range and no other significant differences were observed.

(ii) Diabetes, high BMI, and alcoholic liver disease

Aspartame metabolism has also been investigated in diabetic patients (Stern et al., 1976; EFSA_E65, 2011; Gupta et al., 1989), adolescents (Knopp et al., 1976) and adults (EFSA_E65, 2011) with a high BMI, and patients with alcoholic liver disease (Hertelendy et al., 1993; EFSA_UN09, 2011). Despite the different aspartame dosages and regimens used in the various settings, no significant effects were observed in the disposition of the three aspartame metabolites or in plasma phenylalanine and tyrosine levels.

(c) Absorption, distribution, metabolism, and excretion of impurities of aspartame

(i) Diketopiperazine

5-Benzyl-3,6-dioxo-2-piperazine acetic acid (or diketopiperazine, DKP; CAS No. 55102-13-1) is an impurity that may be found in commercial preparations of aspartame for use as a sweetener. DKP is formed via the intramolecular reaction of the primary amine with the methyl ester group of aspartame, which can be formed during the manufacturing process, resulting in the liberation of methanol (Lin and Cheng, 2000; EFSA, 2013) (Fig. 1.1). DKP content may increase substantially in aspartame-containing food and drinks during processing and storage, depending on the temperature and duration of storage, and is typically present at concentrations ranging from 0.1% to 4% (EFSA, 2013).

DKP is poorly absorbed from the gut and is found in the faeces, primarily as unchanged DKP. In vitro, DKP is not hydrolysed by human intestinal microvillar membrane preparations or by purified preparations of aminopeptidases A and W (Hooper et al., 1994). After oral administration, DKP is metabolized in the gastrointestinal tract to phenylacetic acid, which undergoes absorption and is then rapidly excreted in the urine, both as intact phenylacetic acid and as phenylacetylglutamine, a naturally occurring urinary metabolite, after conjugation with glutamate. In humans, the combined urinary excretion of phenylacetic acid and phenylacetylglutamine accounts for about 50% of orally administered DKP (EFSA, 2013).

Cho et al. (1987) measured plasma and urinary concentrations of DKP (by HPLC) in six adults who ingested a dose of 200 mg/kg bw of aspartame containing 1.1% DKP. Over 24 hours, DKP was detected in all collected samples of urine but was below the limit of detection (< 1 μg/mL) in all plasma samples. Mean total urinary DKP excreted over the 24 hours after dosing was 6.68 ± 1.30 mg (corresponding to 4.83 ± 0.23% of

the ingested DKP), and about 44% of total urinary excretion of DKP occurred in the first 4 hours after dosing (EFSA_E15, 2011b), consistent with the data reported above.

(ii) β-Aspartame

β-Aspartame (β-L-aspartyl-L-phenylalanine methyl ester) (Fig. 1.1) is another aspartame impurity that is formed in small quantities during the manufacture and storage of aspartame-sweetened beverages. The pharmacokinetics and metabolism of β-aspartame (administered as [^{14}C-Phe]β-aspartame) in humans were studied in the late 1980s (EFSA, 2013). It was estimated that more than 90% of the administered radiolabel was absorbed: over 7 days, 9.6% of the administered dose was excreted in the faeces, parent radiolabelled β-aspartame was not detected in the plasma, and less than 0.15% was excreted unchanged in the urine. Hydrolysis of the methyl ester yielded the major metabolite [^{14}C]β-L-aspartyl-L-phenylalanine) (a normal constituent of human plasma and urine; Burton et al., 1989), which was detected in the plasma (t_{max} = 1.5 hours; $t_{1/2}$ = 1.1 hours) and in the urine, where it accounted for 7% of the administered radiolabel. In plasma, phenylacetylglutamine was another major metabolite, and free ^{14}C-labelled phenylalanine was a minor one. Urine was the major route of excretion of the radiolabel (42% of the administered dose), and phenylacetylglutamine was the major urinary metabolite (30.8% of the administered dose). The only metabolite recovered in the faeces was ^{14}C-labelled phenylalanine. No clinical effects associated with the administration of [^{14}C-Phe]β-aspartame were observed in the study participants.

4.1.2 Absorption, distribution, metabolism, and excretion in experimental systems

This section describes the available evidence on the absorption, distribution, metabolism, and excretion of aspartame in experimental systems. The disposition of aspartame and its metabolites in experimental systems was originally studied in rats, mice, rabbits, dogs, and monkeys in the early 1970s (Oppermann et al., 1973a, b; Ranney et al., 1976; EFSA_E15, 2011a; EFSA_E17, 2011; EFSA_E18, 2011; EFSA_E80, 2011). The available studies used various radiolabelled versions of aspartame (Fig. 4.1) and of its metabolites (i.e. methanol, phenylalanine methyl ester, aspartyl-phenylalanine, phenylalanine, and aspartic acid). As briefly mentioned in Section 4.1.1, evidence for the absorption of aspartame as intact compound was reported in one study from the late 1990s in rodents (Creppy et al., 1998). The authors reported that 10–12% of the administered dose of aspartame (25 mg/kg), as measured by HPLC, was absorbed as free aspartame and distributed to various tissues (kidney, 73 ± 6 μg/g; liver, 1.8 ± 0.1 μg/g; brain, 156 ± 9 μg/g; testis, 34 ± 2 μg/g; urine, 66 ± 5 μg/mL; and serum, 19.2 ± 2 μg/mL).

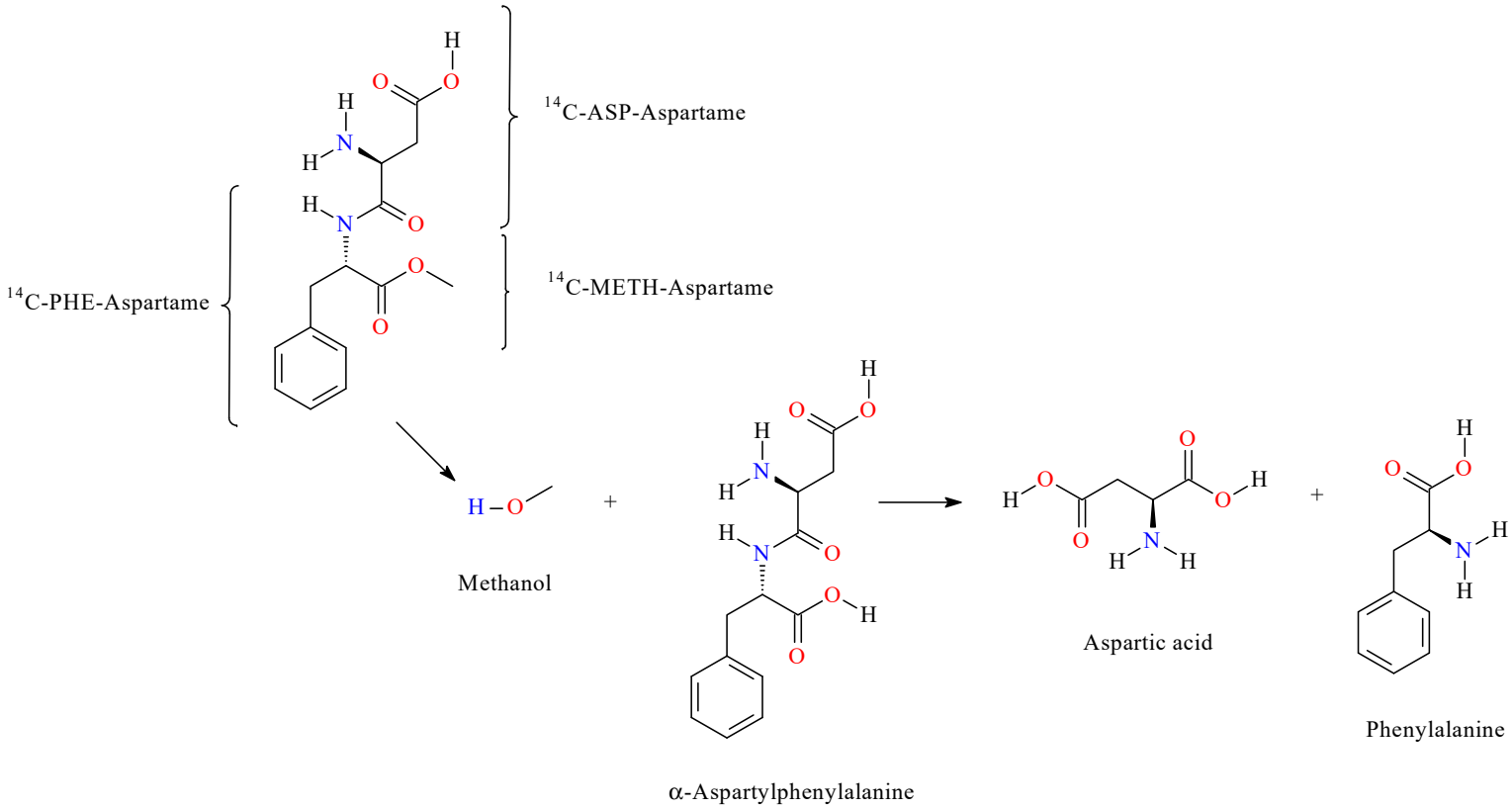
Many of the studies were conducted before the establishment of Good Laboratory Practice (GLP) and internationally recognized guidelines for testing. [The Working Group noted that although relatively minor deficiencies in reporting have been highlighted compared with standards established for modern GLP-compliant studies, independent reviews of the toxicokinetic studies on aspartame concluded that there were no significant issues concerning how the results were reported (EFSA_E91, 2011; Stanley, 2013).]

(a) Non-human mammals in vivo

(i) Kinetics of methanol

The metabolism of methanol derived from aspartame has been studied in rats, pigs, and monkeys. Based on the available evidence, methanol is completely cleaved from aspartame in the small intestine (Burgert et al., 1991) and rapidly excreted in expired air (EFSA_E15, 2011a). Low levels of methanol from aspartame are retained (EFSA_E15, 2011a). Davoli et al. (1986) showed

Fig. 4.1 Chemical structure of aspartame and diagram of the various radiolabelled compounds used in the toxicokinetic studies



Structure of aspartame and its metabolites: radiolabelled methyl component of aspartame (^{14}C-METH-aspartame); radiolabelled aspartic acid component of aspartame (^{14}C-ASP-aspartame); radiolabelled phenylalanine component of aspartame (^{14}C-PHE-aspartame).
Created by the Working Group.

that a single dose of aspartame (34 mg/kg bw) causes a briefly increase in endogenous serum concentrations of methanol in rats, and Trocho et al. (1998) has suggested that methanol from aspartame is capable of binding with proteins and nucleic acids in the liver and blood, or of being incorporated into these molecules via the one-carbon metabolic pool.

When comparing the disposition of ^{14}C-METH-aspartame (aspartame labelled with ^{14}C in the methyl component) versus that of ^{14}C-methanol (oral exposure, 0.068 mmol/kg bw) in young female Rhesus monkeys (weight, 2–3 kg), the proportion of radiolabel in the cumulative expired air of monkeys exposed to ^{14}C-METH-aspartame (67.12% after 8 hours) was very similar to that in monkeys exposed to ^{14}C-methanol (73.02% after 8 hours; see Fig. 4.1 and Fig. 4.2). Plasma radioactivity in monkeys exposed to ^{14}C-METH-aspartame or ^{14}C-methanol was low, and the clearance of radioactivity was slow. The amount of radiolabel recovered from the urine 8 hours after dosing was low in both groups (^{14}C-METH-aspartame, 1.57%; ^{14}C-methanol, 3.17%). The amounts of radiolabel estimated to remain in the carcass of monkeys exposed to ^{14}C-METH-aspartame or ^{14}C-methanol were not significantly different ($P > 0.05$). No faeces samples were produced within the 8-hour observation interval (Oppermann et al., 1973a; EFSA_E15, 2011a).

(ii) Kinetics of aspartic acid

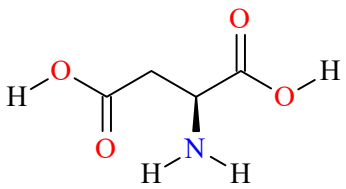
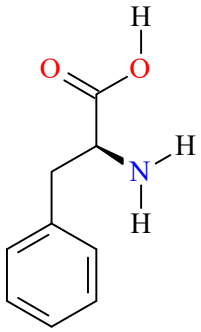
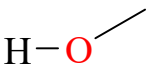
The metabolism of aspartic acid derived from aspartame has been studied in rats (Matsuzawa and O'Hara, 1984; EFSA_E15, 2011a), pigs (Burgert et al., 1991), and monkeys (Oppermann et al., 1973a; EFSA_E17, 2011). Based on the available evidence, the aspartame peptide bond is completely cleaved in the small intestine (Burgert et al., 1991). Some of the resulting aspartic acid enters cellular metabolism, but most is rapidly excreted in the expired air (EFSA_E15, 2011a; EFSA_E17, 2011).

Female Rhesus monkeys (*Macaca mulatta*) (four per group; weight, 5–7 kg) were pre-exposed to aspartame or aspartic acid (approximately 0.068 mmol/kg bw) mixed with peanut butter and spread on a quarter slice of bread for 5 days. On day 6, the monkeys were exposed to aspartame labelled on the aspartyl component, ^{14}C-ASP-aspartame, or ^{14}C-aspartic acid at approximately 0.068 mmol/kg bw by gavage, and expired air, plasma, urine, and faeces were collected and analysed for radioactivity. Most of the radiolabel was rapidly (peak, 1 hour) detected in expired air as CO_2 after exposure to either compound (after 12 hours: ^{14}C-ASP-aspartame, 77%; and ^{14}C-aspartic acid, 67%). The plasma radioactivity time curves appeared biphasic in animals exposed to ^{14}C-ASP-aspartame or ^{14}C-aspartic acid. Urinary and faecal analysis showed low radioactivity for both ^{14}C-ASP-aspartame and ^{14}C-aspartic acid (e.g. < 4 and < 2%, respectively) (Fig. 4.2). The authors noted that there appeared to be considerable individual variation in levels of radioactivity in the plasma and CO_2 after exposure to ^{14}C-ASP-aspartame and ^{14}C-aspartic acid (Oppermann et al., 1973a; EFSA_E17, 2011).

(iii) Kinetics of phenylalanine

The metabolism of phenylalanine derived from aspartame has been studied in mice (EFSA_E18, 2011; Hjelle et al., 1992), rats (Oppermann, 1984; EFSA_E15, 2011a; EFSA_E17, 2011; EFSA_E18, 2011; Hjelle et al., 1992; Romano et al., 1989, 1990; Reilly and Lajtha, 1995; Matsuzawa and O'Hara, 1984), rabbits (EFSA_E18, 2011; EFSA_E80, 2011), dogs (Oppermann, 1984; EFSA_E17, 2011), pigs (Burgert et al., 1991), and monkeys (Reynolds et al., 1980; Oppermann, 1984; Oppermann et al., 1973a, b; EFSA_E15, 2011a; EFSA_E17, 2011). On the basis of the available evidence, phenylalanine from aspartame is completely cleaved in the small intestine (Burgert et al., 1991) and primarily excreted in expired air (EFSA_E15, 2011a; EFSA_E17, 2011; EFSA_E18,

Fig. 4.2 Distribution of metabolites of aspartame

Metabolites	Expired air/breath (as CO_2)	Carcass	Excreta (urine)
Aspartate 	77% (65%)	19% (30%)	4% (5%)
Phenylalanine 	18% (18%)	78% (74%)	4% (8%)
Methanol 	67% (73%)	31% (24%)	2% (3%)

Distribution of metabolites of aspartame (measured as a percentage of ^{14}C) in various body compartments in monkeys exposed to radiolabelled aspartame. Up to 78% of phenylalanine is retained in the body (carcass). Aspartate and methanol are mainly excreted as CO_2 in expired air or breath. The percentages in parentheses refer to the distribution of these metabolites when they are administered directly. The highest percentages of distribution are in red.
Data were extracted from Oppermann et al. (1973a) and EFSA_E15 (2011a).
Adapted from EFSA_E15 (2011a).

2011). However, a portion of the absorbed phenylalanine is incorporated into plasma proteins (e.g. Oppermann, 1984; EFSA_E17, 2011; EFSA_E80, 2011) and exhibits a half-life of about 25 hours in rats (EFSA_E15, 2011a).

The disposition of aspartame labelled on the phenyl component ^{14}C-PHE-aspartame or ^{14}C-phenylalanine in young female Rhesus monkeys (weight, 3–7 kg) was examined after exposure to an oral dose of approximately 0.068 mmol/kg bw (equivalent to ~20 mg/kg bw for ^{14}C-PHE-aspartame or ~11 mg/kg bw

for ^{14}C-phenylalanine). Animals exposed to ^{14}C-PHE-aspartame showed similar amounts of radiolabel total recovery in the expired air, urine, and faeces (i.e. 21.89%) compared with ^{14}C-phenylalanine exposed animals (26.56%), with most of the recovered radiolabel being detected as CO_2 in the expired air (e.g. 17.52% versus 18.00%). Detection of radioactivity in the plasma after exposure to ^{14}C-PHE-aspartame or ^{14}C-phenylalanine peaked at 5 hours, showed an apparent biphasic decay, and a levelling off 48 hours after exposure. On thin-layer chromatography, the majority (~95%) of radioactivity in the plasma of monkeys exposed orally to ^{14}C-PHE-aspartame was present at the origin (i.e. there was very little migration to the mobile phase) (Oppermann et al., 1973a; EFSA E15, 2011a).

To determine whether repeated doses of aspartame had any effect on the normal metabolism of phenylalanine, a study was conducted in monkeys exposed to unlabelled aspartame or ^{14}C-phenylalanine (Oppermann et al., 1973b; EFSA E17, 2011). Female Rhesus monkeys (*Macaca mulatta*) (three per dose; weight, 4.2–6.4 kg) were pre-exposed to unlabelled aspartame at 0, 15, or 60 mg/kg bw, with a mixture of peanut butter on a quarter slice of bread, for 10 days. On day 11, ^{14}C-phenylalanine at a dose of approximately 11 mg/kg bw (0.068 mmol/kg bw) was administered via intravenous injection. The disposition of the radiolabelled portion of ^{14}C-phenylalanine was determined in expired air and in the plasma. Additionally, the concentrations of phenylalanine and tyrosine in the plasma and the level of radiolabel incorporated into protein precipitate were determined. No significant differences in any of the parameters measured were noted between aspartame-exposed and non-exposed monkeys; however, a slightly lower amount of total radioactivity was observed in the plasma of control animals compared with aspartame-exposed animals. Also, small amounts of aspartame were detected in the faeces of Rhesus monkeys exposed to aspartame at 20 mg/kg (Oppermann et al., 1973a).

Reynolds et al. (1980) exposed groups of neonatal Rhesus monkeys (three species: *Macaca mulatta*, *M. fascicularis*, and *M. arctoides*) (age 1–22 days; weight, 280–820 g) to aspartame as a single dose at 2000 mg/kg bw by gavage (2 males; 6 females) or a combination of aspartame at 2000 mg/kg bw and monosodium glutamate at 1000 mg/kg bw (3 males; 3 females). A vehicle control group (water; 2 males; 3 females) and non-exposed control group (5 males; 1 female) were also investigated. Blood samples were taken at various time points between 4 and 5 hours after dosing, and concentrations of aspartic acid, phenylalanine, and tyrosine were determined using an automated amino acid analyser. After dosing with aspartame, the plasma concentrations of aspartic acid, phenylalanine, and tyrosine increased significantly. Maximum mean plasma concentrations of aspartic acid and phenylalanine in the neonatal monkeys were observed 60 and 90 minutes, respectively, after exposure to aspartame. Plasma concentrations of tyrosine in the neonatal monkeys steadily increased over the observation period, and the maximum mean plasma tyrosine concentration was observed at the last observation interval of 240 minutes. [The Working Group noted that there was considerable individual variation in the levels of each amino acid, which may have been due to the dose formulation (e.g. administration as a slurry not a solution). For example, Stegink et al. (1979c) showed that dosing as a slurry in humans produced more variable results than did dosing in solution.]

(iv) Effects on enzymes

Studies in rats suggest that equimolar doses of aspartame and phenylalanine show similar changes in plasma phenylalanine and phenylalanine hydroxylase activity (EFSA E17, 2011; EFSA E80, 2011). Aspartame exposure does not appear to significantly alter gastric juice

secretion, concentration of gastric acid, acid output, or proteolytic activity in rats (Bianchi et al., 1980).

Although no significant increase in cytochrome P450 (CYP) content or the activity of any of the enzymes tested was observed in rats exposed to aspartame at 0, 40 or 4000 mg/kg bw per day for 90 days, (Tutelyan et al., 1990), Vences-Mejía et al. (2006) showed increased levels of CYPs proteins (1A1, 1A2, 2B, 3A2) and enzymatic activities (ethoxyresorufin *O*-deethylase, methoxyresorufin *O*-demethylase, pentoxyresorufin *O*-dealkylase, benzyloxyresorufin *O*-debenzylase, 4-nitrophenol hydroxylase, and erythromycin-*N*-demethylase) in the cerebrum and cerebellum after aspartame exposure (75 or 125 mg/kg bw per day by gavage) for 30 days.

(b) Non-human mammalian in vitro studies

Consistent with the in vivo data, in vitro incubation of aspartame with pepsin or dog gastric juice showed that pepsin and gastric juices had no effect on aspartame concentrations in the medium (EFSA_E15, 2011a). Bianchi et al. (1980) reported that aspartame did not affect the proteolytic activity of pepsin or the lipolytic activity of pancreatic lipase at concentrations of 143 µg/mL and 1.25 mg/mL, respectively, in vitro.

Hooper et al. (1994) reported that microvillar membranes from pig small intestines (duodenum) and kidney were able to rapidly hydrolyse aspartame in vitro.

Incubation of ^{14}C-METH-aspartame with rat plasma caused removal of the radiolabelled methyl group (EFSA_E15, 2011a). Incubation of ^{14}C-PHE-aspartame with dog plasma showed that less than 2.5% of the added radiolabel was incorporated into the high-molecular-weight fraction (EFSA_E17, 2011). This was in direct contrast to the results observed after in vivo exposure; for example, in dogs exposed orally to ^{14}C-PHE-aspartame at 20 mg/kg bw per day (0.068 mmol/kg), up to 70% of the radioactivity was incorporated into the high-molecular-weight fraction of dog plasma within 5 hours (EFSA_E17, 2011).

The potential for nitrosamide formation after incubation of aspartame with nitrite was investigated. No nitrosamide formation occurred under simulated conditions of use or simulated physiological conditions (EFSA_E71, 2011).

(c) In silico

Fatoki et al. (2020) used computer modelling to determine the binding affinity of aspartame to CYPs (1A2, 3A4, 2C9, 2C19, 2D6, 2E1) and P-glycoprotein; the results suggested that aspartame would not be substantially metabolized by the specified CYPs or transported by P-glycoprotein.

(d) Absorption, distribution, metabolism, and excretion of impurities

The kinetics of DKP have been studied in rats, rabbits, and monkeys (Lipton et al., 1991; EFSA_E15, 2011b; EFSA_E18, 2011; EFSA_E80, 2011). Some DKP is metabolized by gut bacteria to phenylacetic acid, which is then excreted in the urine as phenylacetylglutamine. Unchanged DKP is also recovered in the urine and faeces. [The Working Group noted that no toxicity (including neoplasms) was reported when DKP was administered in combination with aspartame in rats (Ishii, 1981).]

The kinetics of β-aspartame have been studied in rabbits, dogs, and monkeys (using ^{14}C-β-aspartame). β-Aspartame is readily metabolized in the gut before absorption and no intact β-aspartame has been detected in the plasma. Once cleaved, the metabolites of β-aspartame are absorbed and primarily excreted in the urine as phenylacetylglutamine and β-L-aspartyl-L-phenylalanine (EFSA, 2013). [The Working Group noted that the primary studies (i.e. E170, E169, and E171) that formed the basis for the EFSA (2013) opinion regarding the

toxicokinetics of β-aspartame were not available to the Working Group for review.]

4.2 Evidence relevant to key characteristics of carcinogens

This section reviews the mechanistic data for the key characteristics of carcinogens (Smith et al., 2016) encompassed by aspartame. Evidence was available on whether aspartame exhibits the key characteristics "is electrophilic or can be metabolically activated to an electrophile", "is genotoxic", "induces oxidative stress", "induces chronic inflammation", "is immunosuppressive", and "modulates receptor-mediated effects", "causes immortalization", and "alters cell proliferation, cell death, or nutrient supply". Insufficient data were available for the evaluation of the other key characteristics of carcinogens. In addition, other relevant evidence on the potential effects of aspartame on insulin and glucose metabolism are reported in Section 4.3. Within each section, the most informative studies are described first. The exposure assessments for mechanistic studies in humans are reported in Table S1.3 (see Annex 1, Supplementary material for Section 1, Exposure Characterization, online only, available from: https://publications.iarc.who.int/627).

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

(a) Humans

No data on DNA adducts or protein adducts were available to the Working Group.

(b) Experimental systems

(i) Non-human mammals in vivo

The only available study assessing the formation of adducts in vivo investigated the binding of formaldehyde derived from dietary aspartame to tissue components (Trocho et al., 1998). Adult male rats were exposed to aspartame radiolabelled with ^{14}C on the methanol carbon at an oral dose of 10 mg/kg. Most of the radioactivity detected (> 98% in the plasma, > 75% in the liver) was bound to protein. DNA radioactivity was essentially located on a single adduct base, not the usual bases present in DNA: after acid hydrolysis, the radiolabel incorporated into DNA migrated with a single unidentified spot near the origin in a two-dimensional thin-layer chromatogram system and not near the positions of the standards, adenine, guanosine, or thymine. [The Working Group noted that the authors described the spot as an unidentified DNA base adduct.] The accumulation of radioactivity is considered to be a direct consequence of the binding of formaldehyde to tissue structures. The chronic exposure of rats to non-labelled aspartame at 200 mg/kg for 10 days before administration of the radiolabelled dose resulted in increased accumulation of radiolabel, suggesting that there may be a cumulative increase in the number of formaldehyde adducts derived from aspartame in tissue proteins and nucleic acids (Trocho et al., 1998). [The Working Group noted that the methods used could not differentiate between formaldehyde binding and incorporation versus metabolism of methanol and metabolic incorporation of the methyl group; therefore, it was not possible to derive a clear picture of the potential to form DNA adducts.]

(ii) Non-human mammalian cells in vitro

No data were available to the Working Group.

(iii) Acellular systems in vitro

In an acellular system in vitro, the binding of aspartame to human serum albumin was studied at physiological pH using spectrophotometric spectrofluorometric competition experiments and circular dichroism techniques. The results indicated that the binding of aspartame to human serum albumin caused fluorescence quenching of the protein through a static

quenching mechanism, and the number of binding sites was approximately one. The study of molecular docking at the albumin binding site also indicated that aspartame can strongly bind to Sudlow site I (subdomain IIA) of human serum albumin, mainly by hydrophobic interaction, and that hydrogen bond interactions exist between aspartame and human serum albumin (Kheirdoosh et al., 2021).

Relative non-covalent binding affinities between calf thymus DNA (0.05 mg/mL) and aspartame (0.12–0.50 mg/mL), L-aspartic acid (0.25 mg/mL), L-phenylalanine (0.25 mg/mL), L-alanine (0.25 mg/mL), or doxorubicin (as positive control, 0.12–0.50 mg/mL) were investigated using HPLC (Karikas et al., 1998). A moderate DNA-binding affinity (39.8% DNA peak exclusion) was observed when aspartame at 0.25 mg/mL was tested with DNA. Analogous effects were exhibited by L-aspartic acid (39.3%) and L-phenylalanine (31.6%), and, to a lesser extent, by L-alanine (12.3%), whereas a 65.5% DNA-binding affinity was observed with aspartame at 0.50 mg/mL. Doxorubicin exhibited complete binding affinity (100% DNA peak exclusion) at 0.25 and 0.5 mg/mL. [The Working Group noted that several studies were available, but all described non-covalent binding to proteins, which is not relevant to electrophilicity.]

The nitrosation and alkylation reactions were investigated for three natural amino acids (alanine, glycine, aspartic acid), aspartame, and glycine ethyl ester in an acellular system (Meier et al., 1990). Aspartame showed a relatively high nitrosation rate constant (K_2 = 1.4, at pH 3.2) compared with aspartic acid (K_2 = 0.08, at pH 3.7) or glycine ethyl ester (K_2 = 0.2, at pH 2.5). Alkylation of 4-(*p*-nitrobenzyl)pyridine (used as a surrogate for pyridine base DNA and binding to DNA) by nitrosated aspartame showed rapid formation of a relatively stable alkylating product.

4.2.2 Is genotoxic

(a) Humans

(i) Exposed humans

No data in exposed humans were available to the Working Group.

(ii) Human primary cells in vitro

See Table 4.1.

Two studies were conducted using cultured human peripheral blood lymphocytes from healthy donors. Aspartame, at a single concentration of 287.342 µg/mL, equivalent to the half-maximal inhibitory concentration (IC_{50}), calculated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic activity assay, induced a significant increase in the frequency of chromosomal aberrations after 72 hours of treatment (Çadirci et al., 2020). [The Working Group noted that the limitations of the study included the use of a single concentration level, the lack of information on aberrant types and mitotic index, and the evaluation of cytotoxicity (by MTT assay), rather than cell replication.] However, in another study, a statistically significant increase in the frequency of chromosomal aberrations was also reported at all three tested concentrations (500, 1000, and 2000 µg/mL) in human lymphocytes treated with aspartame for 24 or 48 hours (Rencüzoğullari et al., 2004). A small but statistically significant increase in the frequency of micronuclei was also observed at the highest concentration (2000 µg/mL) after treatment for both 24 and 48 hours. In the same study, aspartame at concentrations of up to 2000 µg/mL did not induce sister-chromatid exchange (SCE) at 24 or 48 hours. No changes in the pH or osmolality of the treatment medium were observed (Rencüzoğullari et al., 2004). [The Working Group noted that the micronucleus study was of limited informativeness since the *t*-test employed was not corrected for multiple comparisons relative to the small increase; also, the genotoxicity

Table 4.1 End-points relevant to genotoxicity in human cells in vitro exposed to aspartame

End-point (assay)	Tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Primary cells						
Chromosomal aberrations	Human primary lymphocytes	(+)	NT	287.34 µg/mL	Single concentration (at IC_{50} by MTT assay); no information on aberrant types and mitotic index.	Çadirci et al. (2020)
Chromosomal aberrations	Human primary lymphocytes	+	NT	500 µg/mL		Rencüzoğullari et al. (2004)
Micronucleus formation	Human primary lymphocytes	(+)	NT	2000 µg/mL	Not corrected for multiple comparisons.	
Sister-chromatid exchange	Human primary lymphocytes	–	NT	2000 µg/mL		
Cell lines						
DNA strand breaks (alkaline comet assay)	Caco-2, HT-29, HEK-293 cells	–	NT	2943 µg/mL [10 mM]	No short (2–6 h) treatment.	van Eyk (2015)
DNA strand breaks (alkaline comet assay)	HL-60 cells	+	NT	15 µg/mL [0.05 mM]		Mateo-Fernández et al. (2022)
DNA strand breaks (alkaline comet assay)	HeLa cells	(+)	NT	2943 µg/mL [10 mM]	No quantitative/semiquantitative evaluation.	Pandurangan et al. (2016a)
DNA strand breaks (γH2AX)	HepG2 cells	(+)	NT	320 µg/mL	DNA fragmentation was observed at concentrations that also caused significant cytotoxicity and/or apoptosis.	Qu et al. (2019)

Caco-2, human colorectal adenocarcinoma cell line; h, hour(s); γH2AX, phosphorylated histone H2AX; HEK-293, human embryonic kidney cell line; HeLa, human cervical adenocarcinoma cell line; HepG2, human hepatoblastoma cell line; HIC, highest ineffective concentration; HL-60, human promyelocytic leukaemia cell line; HT-29, human colorectal adenocarcinoma cell line; IC_{50}, half-maximal inhibitory concentration; LEC, lowest effective concentration, MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; NT, not tested.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+) or (–), positive or negative results in a study of limited quality.

community considers results from the SCE assay to be of less relevance than other currently available genotoxicity tests.]

(iii) Human cell lines in vitro

Aspartame treatment of Caco-2 (human colorectal adenocarcinoma), HT-29 (human colorectal adenocarcinoma), and HEK-293 (human embryonic kidney) cells to aspartame at 29.4, 294.3, or 2943 µg/mL (10 mM) for 24, 48, or 72 hours did not induce DNA strand breaks according to the alkaline comet assay (van Eyk, 2015). The appearance of comets was evaluated by a score of no damage to severe damage (levels 0–4). Aspartame-treated cells yielded no or little DNA fragmentation after any incubation period in any of the cell lines tested (score, level 0 or 1) (van Eyk, 2015).

In contrast, DNA strand breaks were observed in HL-60 (human promyelocytic leukaemia) cells treated with aspartame at 15 µg/mL (0.5 mM) for 5 hours (Mateo-Fernández et al., 2022) and in HeLa (human cervical adenocarcinoma) cells treated with aspartame at 2943 µg/mL (10 mM) or more (Pandurangan et al., 2016a). [The Working Group noted that the latter study was limited by the lack of quantitative or semiquantitative evaluation.] After a 24-hour at 320 µg/mL (IC_{25}) or 480 µg/mL (IC_{50}), aspartame induced phosphorylation of histone H2AX (γH2AX), a biomarker of DNA double-strand breaks, in HepG2 (human hepatoblastoma) cells (Qu et al., 2019). [The Working Group considered that the results of this study were of limited value because of evidence of cytotoxicity and apoptosis at the concentrations at which results were positive, thus preventing the clear distinction between necrosis, apoptosis, and genotoxicity.]

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.2.

Aspartame induced a small but statistically significant increase in DNA strand breaks according to the alkaline comet assay in bone marrow of mice exposed to a single oral dose at 35 mg/kg bw by gavage (Bandyopadhyay et al., 2008). Positive findings were also obtained in the heart of Wistar albino rats exposed at 75 and 150 mg/kg bw per day by oral gavage for 4 weeks (Al-Eisa et al., 2018) and in the liver of rats exposed at 75 and 150 mg/kg bw per day by oral gavage for 30 days (Hamza et al., 2019). Histopathological observation of the liver or the heart in the groups exposed to aspartame revealed degenerative modifications (Hamza et al., 2019) or alterations in nuclear size and shape of the cardiomyocytes (Al-Eisa et al., 2018), respectively. [The Working Group noted that each study had limitations because of the poor quality of the microphotographs, which hindered the interpretation of results. In the study from Hamza et al., the rat strain was not reported.] Aspartame caused DNA damage in the sperm of male NMRI mice exposed at doses of 40, 80 or 160 mg/kg bw per day by oral gavage for 90 days (Anbara et al., 2020, 2021). On the other hand, negative findings in the comet assay were obtained in eight organs (glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow) of male ddY mice after exposure to aspartame as a single oral dose at 2000 mg/kg bw by gavage for 3 and 24 hours (Sasaki et al., 2002). [The Working Group noted that this study did not score an adequate number of cells.]

DNA fragmentation was observed in the liver of rat dams and their offspring, at birth or at 3 or 9 weeks after birth, after oral administration of aspartame to the dams at a dose of 50.4 mg/animal per day [about 265 mg/kg bw per day] by gavage during gestation or during gestation plus 3 or 9 weeks after birth (Abd Elfatah et al., 2012). [The Working Group noted that the day of gestation on which treatment began was not clearly mentioned.] DNA fragmentation was not observed in the spleen, thymus, or lymph nodes of rats exposed to aspartame at 40 mg/kg

Table 4.2 End-points relevant to genotoxicity in non-human mammals in vivo exposed to aspartame or its impurity, diketopiperazine

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA strand breaks (alkaline comet assay)	Mouse, Swiss albino (M)	Bone marrow	+	35 mg/kg bw	Single oral gavage (7, 14, 28, 35 mg/kg bw), sampling at 18 h after treatment		Bandyopadhyay et al. (2008)
DNA strand breaks (alkaline comet assay)	Rat, Wistar albino (M)	Heart (cardiac myocytes)	(+)	75 mg/kg bw per day	Oral gavage for 4 wk (75, 150 mg/kg bw per day)	Difficult to interpret the results based on the pictures provided.	Al-Eisa et al. (2018)
DNA strand breaks (alkaline comet assay)	Rat (M)	Liver	(+)	75 mg/kg bw per day	Oral gavage for 30 days (75 and 150 mg/kg bw per day)	Difficult to interpret the results based on the pictures provided; rat strain was not reported.	Hamza et al. (2019)
DNA strand breaks (alkaline comet assay)	Mouse, ddY (M)	Glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, bone marrow	–	2000 mg/kg bw	Single oral gavage, sampling at 3 h and 24 h after the treatment	Too few cells scored.	Sasaki et al. (2002)
DNA damage (alkaline comet assay)	Mouse, NMRI (M)	Sperm	+	80 mg/kg bw per day	Oral gavage for 90 days (40, 80, 160 mg/kg bw per day)	DNA damage was due to apoptosis.	Anbara et al. (2020, 2021)
DNA fragmentation	Rat, Wistar albino (M)	Spleen, thymus, lymph node	–	40 mg/kg bw per day	Oral gavage for 90 days	The study was looking for evidence of apoptosis. Single dose tested only.	Choudhary and Sheela Devi (2016)
DNA fragmentation	Rat, albino (F; pregnant and after delivery)	Liver	+	50.4 mg/animal per day [about 265 mg/kg bw per day]	Gavage for GP, or for GP + 3 wk or 9 wk after delivery	Use of a commercial tablet.	Abd Elfatah et al. (2012)
DNA fragmentation	Rat, albino, offspring at birth or at 3 wk or 9 wk after birth	Liver	+	50.4 mg/animal per day to mother [about 265 mg/kg bw per day]	Gavage to mother for GP + 3 wk or 9 wk after delivery	Use of a commercial tablet.	Abd Elfatah et al. (2012)
Dominant lethal mutation	Rat, CD, albino (M)	Offspring	–	2000 mg/kg bw	Oral gavage (1000 mg/kg bw × 2, 2 h interval)	Purity, 99.8% (contained 0.2% DKP).	EFSA E40 (2011)

Table 4.2 (continued)

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Dominant lethal mutation	Rat, CD, albino (M)	Offspring	–	2000 mg/kg bw	Oral gavage (1000 mg/kg bw × 2, 2 h interval)	Purity, 99.25% (contained 0.75% DKP).	EFSA E41 (2011)
Reverse mutation (host-mediated)	Rat, Purina Caesarean-derived, albino (M)	i.p. injection of *Salmonella typhimurium* G46	–	4000 mg/kg bw per day	Oral gavage for 5 days, three equally divided doses every 3 h (500, 1000, 2000, 4000 mg/kg bw per day)	Purity, 99.3% (contained 0.7% DKP).	EFSA E44 (2011)
Reverse mutation (host-mediated)	Mouse, Ha/ICR random-bred Swiss (M)	i.p. injection of *Salmonella typhimurium* G46	–	8000 mg/kg bw per day	Oral gavage for 5 days, three equally divided doses every 3 h (1000, 3000, 4000, 8000 mg/kg bw per day)	Purity unknown.	EFSA E81 (2011)
Chromosomal aberrations	Rat, albino (F, pregnant and after delivery)	Bone marrow	(+)	50.4 mg/animal per day [about 265 mg/kg bw per day]	Gavage for GP, or for GP + 3 wk or 9 wk after delivery	Used a commercial tablet. Included gaps and scored total aberrations.	Abd Elfatah et al. (2012)
Chromosomal aberrations	Rat, albino, offspring at birth or at 3 wk or 9 wk after birth	Liver	(+)	50.4 mg/animal per day to mother [about 265 mg/kg bw per day]	Gavage for gestation period to mother + 3 wk or 9 wk after delivery to mother	Used a commercial tablet. Included gaps and scored total aberrations.	Abd Elfatah et al. (2012)
Chromosomal aberrations	Mouse, Swiss (M)	Bone marrow	(+)	35 mg/kg bw	Single oral gavage (3.5, 35, 350 mg/kg bw), sampling at 24 h after treatment	Questions regarding the validity of the results.	Alsuhaibani (2010)
Chromosomal aberrations	Mouse, C57BL/6	Bone marrow	–	400 mg/kg bw per day	Oral gavage for 5 days (40, 400 mg/kg bw per day), sampling at 6 h after the final treatment		Durnev et al. (1995)

Table 4.2 (continued)

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Chromosomal aberrations	Rat, Holtzman, albino (M)	Bone marrow, spermatogonia	(–)	8000 mg/kg bw per day	Oral gavage for 5 days (2000, 4000, 6000, 8000 mg/kg bw per day), sampling at 29 h after the final treatment	Delayed sampling time; only 50 cells/animal were analysed.	EFSA E12 (2011)
Chromosomal aberrations	Rat, Purina Caesarean-derived, albino (M)	Bone marrow	(–)	4000 mg/kg bw per day	Oral gavage for 5 days, three equally divided doses every 3 h (500, 1000, 2000, 4000 mg/kg bw per day), sampling at 29 h after the final treatment	Purity, 99.3% (contained 0.7% DKP); only 50 cells/animal were analysed.	EFSA E43 (2011)
Chromosomal aberrations	Mouse, Swiss albino (M, F)	Bone marrow	(+)	250 mg/kg bw at 72 h sampling	Single oral gavage (250, 455, 500, 1000 mg/kg bw), sampling at 24, 48, and 72 h after treatment	Difficult to interpret the results based on the pictures provided; no criteria on "aberrations" were provided; inappropriately included gaps.	Kamath et al. (2010)
Chromosomal aberrations	Rat, albino (M)	Bone marrow	(+)	250 mg/kg bw per day	Oral gavage for 90 days	Used a commercial tablet; difficult to interpret the results based on the pictures provided. Single dose tested only.	Elballat and Abas (2020)
Micronucleus formation	Mouse, Swiss albino (M, F)	Bone marrow	(+)	455 mg/kg bw at 24 h sampling, 455 mg/kg bw at 48 h sampling, 250 mg/kg bw at 72 h sampling	Single oral gavage (250, 455, 500, 1000 mg/kg bw), sampling at 24, 48, and 72 h after treatment	Difficult to interpret the results based on the pictures provided; signs of toxicity; altered NCE/PCE ratio.	Kamath et al. (2010)
		Peripheral blood	(+)	500 mg/kg bw at 24 h sampling, 455 mg/kg bw at 48 h sampling, 250 mg/kg bw at 72 h sampling			

Table 4.2 (continued)

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Micronucleus formation	Rat, F344/N (M)	Bone marrow	–	2000 mg/kg bw per day	Oral gavage, 3 × at 24 h intervals (500, 1000, 2000 mg/kg bw per day), sampling at 24 h after the final treatment		NTP (2005)
Micronucleus formation	Mouse, *Tg-AC* hemizygous (M, F), *Cdkn2a*-deficient (M, F), *p53*-haploinsufficient (M)	Peripheral blood	–	50 000 ppm [7280–9560 mg/kg bw per day, depending on the strain or sex]	Feeding for 9 mo (3125, 6250, 12 500, 25 000, 50 000 ppm)		NTP (2005)
Micronucleus formation	Mouse, *p53*-haploinsufficient (F)	Peripheral blood	(+)	50 000 ppm, [9620 mg/kg bw per day]	Oral administration (feed) for 9 mo (3125, 6250, 12 500, 25 000, 50 000 ppm)	A single animal at the highest dose was responsible for the increase.	NTP (2005)
Micronucleus formation	Mouse, CD-1 (M)	Bone marrow	–	2000 mg/kg bw	Single oral gavage (500, 1000, 2000 mg/kg bw), sampling at 24 h and 48 h after treatment		Otabe et al. (2019)
Sister-chromatid exchange	Mouse, Swiss albino (M)	Bone marrow	–	350 mg/kg bw	Single oral gavage (3.5, 35, 350 mg/kg bw), sampling at 24 h after treatment		Alsuhaibani (2010)
Results for the impurity, diketopiperazine							
Dominant lethal mutation	Rat, CD, albino (M)	Offspring	–	1000 mg/kg bw	Oral gavage (500 mg/kg bw × 2, 2 h interval)		EFSA E42 (2011)
Reverse mutation (host-mediated)	Rat, Purina Caesarean-derived, albino (M)	i.p injection of *Salmonella typhimurium* G46	–	2000 mg/kg bw per day	Oral gavage for 5 days, three equally divided doses every 3 h (250, 500, 1000, 2000 mg/kg bw per day)		EFSA E31 (2011)

Table 4.2 (continued)

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Reverse mutation (host-mediated)	Mouse, Ha/ICR random-bred Swiss (M)	i.p. injection of *Salmonella typhimurium* G46	–	8000 mg/kg bw per day	Oral gavage for 5 days, three equally divided doses every 3 h (1000, 3000, 4000, 8000 mg/kg bw per day)		EFSA E82 (2011)
Chromosomal aberrations	Rat, Purina Caesarean-derived, albino (M)	Bone marrow	–	2000 mg/kg bw per day	Oral gavage for 5 days (250, 500, 1000, 2000 mg/kg bw per day), sampling at 29 h after the final treatment		EFSA E30 (2011)

bw, body weight; F, female; GLP, Good Laboratory Practice; GP, gestation period; i.p., intraperitoneal; h, hour(s); HID, highest ineffective dose; LED, lowest effective dose; M, male; mo, month(s); NCE/PCE, normochromatic erythrocytes/polychromatic erythrocytes; NT, not tested; ppm, parts per million; wk, week(s).

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+) or (–), positive or negative result in a study of limited quality.

bw per day by oral gavage for 90 days (Choudhary and Sheela Devi, 2016).

Aspartame did not induce dominant lethal mutation in male CD albino rats exposed, in two separate studies, to aspartame of two different purities (purity, 99.8% or 99.25%) at 2000 mg/kg bw by oral gavage, given twice at 1000 mg/kg bw with a 2-hour interval. Each male was mated with two females weekly for 8 weeks. On day 14 of gestation, the mated females were killed in order to analyse fetal deaths. Paternal body-weight changes and maternal pregnancy rate were assessed, and the uterus and ovaries were examined. No effects were observed (EFSA_E40, 2011; EFSA_E41, 2011).

Aspartame gave negative results in the host-mediated assay in male Purina Caesarean-derived albino rats, Ha/ICR random-bred Swiss mice, and *Salmonella typhimurium* strain G46 (EFSA_E44, 2011; EFSA_E81, 2011). Aspartame was administered by oral gavage for 5 days at a dose of 1000, 2000, and 4000 mg/kg bw per day (rats) or 1000, 3000, 4000, and 8000 mg/kg bw per day (mice) in three equally divided doses every 3 hours. After the final dose, the rodents were inoculated with bacteria by intraperitoneal injection. No increase in mutants was observed among bacteria recovered after 3 hours.

Many cytogenetics studies (chromosomal aberrations and micronucleus formation) have been conducted with aspartame administered orally. Induction of chromosomal aberrations was observed in the bone marrow of albino rat dams and the liver of their offspring at birth or after 3 or 9 weeks. Aspartame was administered to the mother by oral gavage at a dose of 50.4 mg/animal per day [about 265 mg/kg bw per day] during gestation or during gestation plus 3 or 9 weeks after birth (Abd Elfatah et al., 2012). [The Working Group noted that the day of gestation on which exposure began was not clearly mentioned and that the study was of limited quality because of inappropriately included gaps and the scoring of total aberrations, not cells with aberrations.]

Aspartame was administered to male and female Swiss albino mice as a single oral dose at 250–1000 mg/kg bw by gavage, and bone marrow samples were obtained at 24, 48, or 72 hours after dosing. Chromosomal aberrations were observed in mice at 250 mg/kg bw at 72 hours (Kamath et al., 2010). [The Working Group noted that the study limitations included difficulties in the interpretation of the results on the basis of the pictures provided, lack of criteria on "aberration", or inappropriately included gaps.]

Chromosomal aberrations were induced in the bone marrow of male albino rats exposed to aspartame at 250 mg/kg bw per day by oral gavage for 90 days (Elballat and Abas, 2020). [The Working Group noted that the interpretation of results was limited by the quality of the pictures provided; however, data were provided with statistical analysis. An unusual background of 12% of cells with numerical aberrations was reported; normally, numerical aberrations are few or absent. The background of 3.4% of bone marrow cells with structural aberrations was not consistent with the usual value of 0.2–2.0% (Tice et al., 1994).]

In the bone marrow of male Swiss albino mice exposed to aspartame as a single oral dose at 3.5, 35 or 350 mg/kg bw by oral gavage, a small but statistically significant increase in the frequency of chromosomal aberrations was observed at 35 mg/kg bw or more at 24 hours after dosing (Alsuhaibani, 2010). [The Working Group considered the study to be of low informativeness because of questions regarding the validity of the results.] In contrast, negative results were obtained in the chromosomal aberrations assay in bone marrow cells or spermatogonia of male Holtzman albino rats. Aspartame was administered at a dose of 2000, 4000, 6000, or 8000 mg/kg bw per day by oral gavage for 5 days. No chromosomal aberrations were observed

in the bone marrow cells or spermatogonia at 29 hours after the final dose (EFSA E12, 2011).

In a similar study, no chromosomal aberrations were observed in the bone marrow cells of male Purina Caesarean-derived albino rats exposed to aspartame at 500, 1000, 2000, or 4000 mg/kg bw per day by oral gavage for 5 days (EFSA E43, 2011). Aspartame did not induce chromosomal aberrations in the bone marrow cells of C57BL/6 mice exposed at a dose of 40 or 400 mg/kg bw per day by oral gavage for 5 days (Durnev et al., 1995).

In a micronucleus assay in mice, aspartame was administered as a single oral dose at 250–1000 mg/kg bw by gavage, and bone marrow and peripheral blood samples were obtained at 24, 48, or 72 hours after dosing. The most effective induction of micronucleus formation was observed at 72 hours in both bone marrow cells and peripheral blood (Kamath et al., 2010). [The Working Group noted that the study was of limited informativeness because of difficulties in the interpretation of the microphotographs; however, data results were provided with statistical analysis, and there were signs of toxicity. In addition, the increase in micronucleus frequency occurred earlier (at 24 hours) in the peripheral blood than in the bone marrow, whereas, physiologically, changes in peripheral blood micronuclei ratios would be expected to be detected 12–24 hours later than changes in the bone marrow.]

Several micronucleus assays, performed in accordance with GLP, gave negative results. Aspartame was administered at a dose of 500, 1000, or 2000 mg/kg bw per day by oral gavage for 3 days to male F344/N rats. No induction of micronuclei was observed in the bone marrow at 24 hours after the final dose (NTP, 2005). Three strains of transgenic mouse were fed diets containing aspartame at a concentration of 0, 3125, 6250, 12 500, 25 000, or 50 000 ppm (equivalent to average daily doses of approximately 500–8000 or 9000 mg/kg bw per day, depending on the strain and sex) for 9 months. There was no micronucleus formation in the peripheral blood (normochromatic erythrocytes) in Tg-AC hemizygous (male and female), *Cdkn2a*-deficient (male and female), or *p53*-haploinsufficient (male) mice exposed to aspartame at concentrations up to 50 000 ppm (about 7280–9560 mg/kg bw per day). However, a small but statistically significant increase in micronucleus induction was observed in female *p53*-haploinsufficient mice at 50 000 ppm (about 9620 mg/kg bw per day) (NTP, 2005). [The Working Group noted that the increase in frequency of micronucleated normochromatic erythrocytes in female *p53*-haploinsufficient mice at 50 000 ppm was small (1.80‰ versus concurrent female control 0.79‰, male control 1.18‰), and the biological relevance of this extremely high dose was questionable. The Working Group also noted that the study did not provide historical control data.]

In another micronucleus assay in mice, aspartame was administered as a single oral dose of 500, 1000, or 2000 mg/kg bw by gavage to male CD-1 mice. No micronucleus induction was observed in the bone marrow at 24 and 48 hours after dosing (Otabe et al., 2019).

Negative findings were also reported for SCE investigated in the bone marrow of Swiss albino mice exposed to aspartame as a single oral dose of up to 350 mg/kg bw by gavage (at 24 hours after dosing) (Alsuhaibani, 2010). [The Working Group noted that the SCE assay is now considered of less relevance than other currently available genotoxicity tests.]

The genotoxicity of the aspartame impurity DKP was also investigated for different genotoxicity end-points in vivo. Overall, the findings of these studies were negative. In a test for dominant lethal mutations, DKP was administered at a dose of 2000 mg/kg bw per day (two doses of 1000 mg/kg bw with a 2-hour interval) by oral gavage to male CD albino rats. After mating male and female rats, mated females were killed for the analysis of dominant lethal mutations on day

14 of gestation. No dominant lethal effect was observed (EFSA_E42, 2011). In a host-mediated assay, similar strains of male Purina Caesarean-derived albino rats or Ha/ICR random-bred Swiss mice were exposed to DKP at a dose of 1000, 3000, 4000, and 8000 mg/kg bw per day, or 250, 500, 1000, and 2000 mg/kg bw per day, respectively, in three equally divided doses, every 3 hours, by oral gavage, for 5 days. After the final dose, the rodents were inoculated with *S. typhimurium* G46 by intraperitoneal injection. The bacteria were recovered 3 hours later, and the presence of mutants was investigated. No statistically significant increases in mutants were observed (EFSA_E31, 2011; EFSA_E82, 2011). In a test for chromosomal aberrations, DKP was administered at a dose of 250, 500, 1000, or 2000 mg/kg bw per day by oral gavage for 5 days to male Purina Caesarean-derived albino rats. No chromosomal aberrations were observed in the bone marrow at 29 hours after the final dose (EFSA_E30, 2011).

(ii) Non-human mammalian cells in vitro

See Table 4.3.

An alkaline comet assay in MDCK (Madin–Darby canine kidney) cells gave a positive result for DNA damage with aspartame at 2943 µg/mL (10 mM) (Pandurangan et al., 2017). [The Working Group noted that the study was limited by the lack of quantitative or semiquantitative evaluation and difficulties in the interpretation of the results based on the pictures provided.]

Aspartame did not induce unscheduled DNA synthesis (UDS) in rat primary hepatocytes (Jeffrey and Williams, 2000). Cells prepared from the liver of male Sprague-Dawley rats were exposed to aspartame at a concentration of 1472 or 2943 µg/mL (5 or 10 mM) for 20 hours. Unscheduled DNA synthesis was measured by an autoradiographic method and 150 cells were scored per concentration. [The Working Group noted that the study was limited by the lack of short time-point sampling.]

(iii) Non-mammalian experimental systems

See Table 4.4.

Aspartame induced a concentration-related increase in DNA fragmentation in *Danio rerio* (zebra fish) exposed to aspartame at 62.5–500 mg/mL for 5 days (Reshman et al., 2015). [The Working Group noted that the study reported only a qualitative evaluation of the data.] Aspartame induced concentration-related increases in the percentage of DNA fragmentation in *Drosophila melanogaster* exposed to medium containing aspartame at concentrations of 62.5–500 mg/mL for 72 hours (Reshman et al., 2015). [The Working Group noted that the informativeness of the study was low because of the poor quantitative evaluation and because increases were observed only in one duplicate.]

A study was carried out using SMART (somatic mutation and recombination by wing spot test) in two *D. melanogaster* strains (*flr3* females and *mwh* males) with genetic markers that affect the wing-hair phenotype (Mateo-Fernández et al., 2022). After crossing females with males, trans-heterozygous larvae were obtained. After synchronization, the larvae were fed instant medium containing aspartame at 7.4 or 241.3 µg/mL until pupae hatching (10–12 days), when the emerged flies were collected and mutant wing spots (single, large, or twin) were assessed. At concentrations of up to 241.3 µg/mL, aspartame did not induce mutations in SMART.

Aspartame at concentrations up to 5000 µg/plate did not induce reverse mutation with or without metabolic activation in *S. typhimurium* strains TA1535, TA100, TA1538, TA97, and TA1537 (EFSA_E101, 2011; EFSA_E97, 2011). In a study that complied with GLP, negative results were obtained with aspartame at concentrations up to 10 000 µg/plate in *S. typhimurium* strains TA1535, TA100, TA97, TA98, and TA1537, with or without metabolic activation (NTP, 2005). The most recent study conducted under GLP and compliant with OECD test guideline 471

Table 4.3 End-points relevant to genotoxicity in non-human mammalian cells in vitro exposed to aspartame

End-point (assay)	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA strand breaks (alkaline comet assay)	MDCK cells	(+)	NT	2943 µg/mL [10 mM]	No quantitative/ semiquantitative evaluation; difficult to interpret the results based on the pictures provided.	Pandurangan et al. (2017)
Unscheduled DNA synthesis	Primary hepatocyte (male Sprague-Dawley rat, liver)	(–)	NT	2943 µg/mL [10 mM]	No short time sampling.	Jeffrey and Williams (2000)

HIC, highest ineffective concentration; LEC, lowest effective concentration; MDCK, Madin–Darby canine kidney; NT, not tested.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+) or (–), positive or negative result in a study of limited quality.

Table 4.4 End-points relevant to genotoxicity in non-mammalian experimental systems exposed to aspartame or its impurity, diketopiperazine

Test system (species, strain)	End-point (assay)	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Danio rerio (zebra fish)	DNA fragmentation	(+)	NA	250 mg/mL	Only qualitative evaluation.	Reshman et al. (2015)
Drosophila melanogaster	DNA fragmentation	(+)	NA	500 mg/mL	Quantitative evaluation; increase seen in only one duplicate.	Reshman et al. (2015)
Drosophila melanogaster (*flr3* female, *mwh* male)	SMART (somatic mutation and recombination by wing spot test)	–	NA	0.82 mM, [241.3 µg/mL]		Mateo-Fernández et al. (2022)
Salmonella typhimurium TA1535, TA1537, TA1538, TA98, TA100	Reverse mutation	–	–	5000 µg/plate	Purity unknown.	EFSA E97 (2011); EFSA E101 (2011)
Salmonella typhimurium TA98, TA100	Reverse mutation	(–)	(–)	2000 µg/plate	Top concentration[b] tested was not suitable.	Rencüzoğullari et al. (2004)
Salmonella typhimurium TA1535, TA97, TA98, TA100, TA 1537	Reverse mutation	–	(+)	10 000 µg/plate	Equivocal result in TA97 with 30% rat S9.	NTP (2005)
Salmonella typhimurium TA1535, TA1537, TA98, TA100	Reverse mutation	–	–	5000 µg/plate		Otabe et al. (2019)
Escherichia coli WP2 *uvr*A	Reverse mutation	–	–	5000 µg/plate		Otabe et al. (2019)
Salmonella typhimurium TA97a, TA100	Reverse mutation	(–)	(–)	1000 µg/plate	Top concentration[b] tested was not suitable.	Bandyopadhyay et al. (2008)
Salmonella typhimurium TA98	Reverse mutation	(–)	(–)	2400 µg/plate	Purity unknown; top concentration[b] tested was not suitable.	Najam et al. (2017)
Salmonella typhimurium TA100	Reverse mutation	(–)	(+)	150 µg/plate	Unknown reproducibility at low concentrations; top concentration[b] tested was not suitable.	

Table 4.4 (continued)

Test system (species, strain)	End-point (assay)	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100	Reverse mutation	+	+	8 mM [2355 µg/mL]	A nitrosated form of aspartame was tested.	Shephard et al. (1993)
Salmonella typhimurium TA98	Reverse mutation	+	–	20 mM [5886 µg/mL]	A nitrosated form of aspartame was tested.	
Salmonella typhimurium TA102	Reverse mutation	–	NT	20 mM [5886 µg/mL]	A nitrosated form of aspartame was tested.	
Results for the impurity diketopiperazine						
Salmonella typhimurium TA1535, TA1537, TA1538, TA98, TA100	Reverse mutation	–	–	5000 µg/plate		EFSA E98 (2011)
Salmonella typhimurium TA1535, TA1537, TA1538, TA98, TA100	Reverse mutation	–	–	10 000 µg/plate	Precipitate was observed at 5000 and 10 000 µg/plate.	EFSA E106 (2011)

h, hour(s); HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not available; NT, not tested; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+) or (–), positive or negative result in a study of limited quality.

[b] "Top" concentration indicates the highest recommended concentration when toxicity is not limiting.

gave negative results with aspartame at concentrations up to 5000 µg/plate in *S. typhimurium* strains TA1535, TA100, TA97, and TA1537, and *Escherichia coli* strain WP2 *uvrA*, with or without metabolic activation (Otabe et al., 2019). Negative results were also obtained at concentrations up to 1000–2400 µg/plate in *S. typhimurium* strains TA97a, TA98, and TA100, with or without metabolic activation (Rencüzoğullari et al., 2004; Bandyopadhyay et al., 2008; Najam et al., 2017). [The Working Group noted that the studies with aspartame at concentrations up to 2400 µg/plate had limitations because a non-suitable top concentration (the highest recommended concentration when toxicity is not limiting) was tested.] One study, however, reported positive responses at 150 and 300 µg/plate, but not at 600, 1200, or 2400 µg/plate, in *S. typhimurium* TA100 with metabolic activation (Najam et al., 2017). [The Working Group noted that the study had limitations because of unknown reproducibility at low concentrations and testing of a non-suitable top concentration.]

The mutagenicity of nitrosated aspartame was investigated in a bacterial reverse mutation assay in *S. typhimurium* strains TA100, TA98, TA102, and TA104 (Shephard et al., 1993). Aspartame (420 mM in reaction mixtures) was nitrosated with sodium nitrite (40 mM) at pH 3.0 or pH 3.5 for 30 minutes at 37 °C. Concentration-related increases in the number of revertant colonies were obtained in TA100 without metabolic activation. Nitrosated aspartame (20 mM in reaction mixtures) showed mutagenic activity in strain TA100, TA104, and TA98, but not in TA102, without metabolic activation. The effects were decreased with metabolic activation in TA100 or TA98. The most sensitive strain was TA100.

The aspartame impurity DKP did not induce reverse mutations, with or without metabolic activation, in *S. typhimurium* strains TA1535, TA100, TA1538, TA97, or TA1537, at concentrations up to 5000 or 10 000 µg/plate (EFSA_E98, 2011; EFSA_E106, 2011).

4.2.3 Induces oxidative stress

(a) Humans

(i) Exposed humans

No data on induction of oxidative stress in humans were available to the Working Group.

(ii) Human cells in vitro

See Table 4.5.

Several studies evaluated cells for markers of oxidative stress at cytotoxic concentrations of aspartame. There were no signs of increased cellular reactive oxygen species (ROS) in primary human peripheral blood mononuclear cells using colourimetric analysis of a proprietary reagent based on oxidation of a ferrous compound at aspartame concentrations of up to 100 mg/L (339.8 µM). The higher concentrations reduced metabolic activity – oxidative phosphorylation, measured using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, MTT – and caused significant leakage of lactate dehydrogenase (LDH) enzymatic activity into the tissue culture medium. Total antioxidant capacity (TAC) and total antioxidant status (TOS) assays were also performed to determine the antioxidative/oxidative effects of aspartame; no significant changes were reported (Çadirci et al., 2020) [The Working Group noted that this study was limited by the failure to report the numbers of replicates used in the experiment and the lack of statistical analyses of the data, particularly of what appeared to be a concentration-related increase in ROS.]

In contrast, whereas exposure of a human neuroblastoma cell line to either aspartame at 271.7 µM or a mixture containing phenylalanine, aspartic acid, and methanol each at 271.7 µM, caused no cytotoxicity as measured by LDH activity leakage, both exposures led to mitochondrial damage and increased intracellular concentrations of ROS (measured by aminophenyl fluorescein, APF). Transmission electron microscopy was used to demonstrate

Table 4.5 End-points relevant to oxidative stress in human cells in vitro exposed to aspartame

End-points	Assay	Tissue, cell line	Results[a]	Concentration (LEC or HIC) or range	Comments	Reference
Primary cells						
ROS Antioxidant activity Cytotoxicity	TOS TAC MTT, LDH leakage	Human primary peripheral blood mononuclear cells	No change No change ↑: MTT, LDH	10.6–339.8 µM[b]	Number of replicates was not stated; no statistical analysis of the data; a trend towards an increase in "TOS" was observed.	Çadirci et al. (2020)
Cell lines						
ROS SOD1 SOD2 Cytotoxicity Mito-toxicity: cardiolipin FIS1 PINK1	APF qRT-PCR qRT-PCR LDH leakage qRT-PCR qRT-PCR qRT-PCR	Human neuroblastoma cells	↑ ↑ (↑) No changes ↓ ↑ ↑	271.7 µM (aspartame or an isomolar mix of phenylalanine, aspartic acid, and methanol)	Aspartame and the metabolite mix had similar effects for each end-point measured; while there was no cytotoxicity as measured by LDH leakage into the medium, photomicrographs showed significant mitochondrial toxicity and accumulation of cytoplasmic lipid particles. Only a single concentration was used.	Griebsch et al. (2023)
ROS	DCFDA	Caco-2 cells	↑	100 µM	A single non-cytotoxic concentration was used; 1.5 h treatment; the magnitude of the increase was similar to that in the LPS positive control (ROS was restored to vehicle control levels by co-incubation with NAC, T1R3 knockdown, or CLDN3 overexpression).	Shil et al. (2020)
ROS	DCFDA	HUVEC cells IMR-90 cells	↑ No changes	20–100 µM	The biological significance of the HUVEC cell response was questionable due to the flat response over five concentrations.	Alleva et al. (2011)

Table 4.5 (continued)

End-points	Assay	Tissue, cell line	Results[a]	Concentration (LEC or HIC) or range	Comments	Reference
Lipid peroxidation	TBARS	HTR-8/SV neo cells	No changes	0.5 mM	Single concentration used only; 24 h treatment; no cytotoxicity (MTT or LDH leakage assays).	Rodrigues et al. (2022)
Aspartame:		3A-subE trophoblast cells			Pre-treatment with NAC provided only slight protection against cytotoxicity, suggesting that ROS was a minor or secondary factor in causing cytotoxicity. SOD2 expression was not examined with either lactisole or gynemic acid alone, so the impact of co-exposure with aspartame is uninterpretable.	Huang et al. (2023a)
ROS	DCFDA		↑	34 mM[b]		
SOD2	Western blot		↓	0.34 μM[b]		
Cytotoxicity	MTT		↑	8.5–34 mM[b]		
Phenylalanine:						
ROS	DCDFDA		↑	34 mM[b]		
Cytotoxicity	MTT		↑	8.5–34 mM[b]		
ROS	CellROX	HepG2 cells	(↑)	LEC, 713.1 μM	Significant cytotoxicity (the three concentrations tested were the IC_{10}, IC_{25}, and IC_{50}) and evidence of apoptosis made it impossible to tell whether the ROS increase was caused by cytotoxicity or vice versa.	Qu et al. (2019)
Cytotoxicity	MTT		↑			
ROS	DCFDA	HeLa cells	(↑)	LEC, 10 mM	The ROS increases occurred at concentrations that were cytotoxic both by SRB–protein-binding and PI entry into necrotic cells. Thus, the ROS liberated during cell death may be the cause of or secondary to cytotoxicity.	Pandurangan et al. (2016a)
Cytotoxicity	SRB		↑	LEC, 100 μM		
Necrosis	PI		↑	LEC, 10 mM		

Table 4.5 (continued)

End-points	Assay	Tissue, cell line	Results[a]	Concentration (LEC or HIC) or range	Comments	Reference
Cytotoxicity	CCK-8	GMVEC	No changes	0.1–100 µM		Enuwosa et al. (2021)
Membrane permeability	Fluorescence, EVOM2		No changes	100 µM		
			↓	50 ng/mL VEGF + 10 µM aspartame		
ROS	DCFDA		↑	10 µM		
Oxidative stress	GSH reduced/GSH oxidized		↓	10 µM		

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; APF, aminophenyl fluorescein; bw, body weight; Caco-2, epithelial cell line from a human colon carcinoma; CAT, catalase; CCK, cholecystokinin; CLDN3, claudin-3; DMSO, dimethyl sulfoxide; CellRox, a proprietary intracellular dye that fluoresces when oxidized; DCFDA, 2′7′-dichlorofluorescein diacetate; EVOM2, Epithelial Volt Ohm Meter 2; γ-GC, γ-glutamylcysteine; GMVEC, glomerular microvascular endothelial cells; GPx, glutathione peroxidase; GSH, glutathione; GSH RI, glutathione redox index; GSR, glutathione reductase; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; HepG2, human hepatocellular carcinoma cell line; HeLa, human cervical cancer cell line; 3A-subE human trophoblasts; human placental cells transformed by SV40; HUVEC, human umbilical vein endothelial cells; h, hour(s); HTR-8/SV neo, SV40-transformed extravillous trophoblasts from human placenta explants; HCS, high content screening; HIC, highest ineffective concentration; IC, inhibitory concentration; IMR-90, human fetal lung fibroblasts; LDH, lactate dehydrogenase; LEC, lowest effective concentration; LPO, lipid peroxidation; MDA, malondialdehyde; MPO, myeloperoxidase; MTT, dimethylthiazolyl)-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; NPSH, non-protein thiol; NR, not reported; PI, propidium iodide; qRT-PCR, quantitative gene transcription based on reverse transcriptase polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; SOD1, type 1 SOD, found in cytoplasm in human cells; SOD2, type 2 (or Mn) SOD, associated with mitochondria in humans; SRB, sulforhodamine B protein-binding dye; TBARS, thiobarbituric acid reactive substances; T1R3, taste receptor type 1 member 3; TOS, total oxidant status; VEGF, vascular endothelial growth factor.

[a] ↓, decrease; ↑, increase.

[b] Concentration was converted to micromolar or millimolar (µM or mM) for consistency.

morphological changes in mitochondria. These changes were accompanied by increased transcription of superoxide dismutase isoenzyme 1 (cytoplasmic) and isoenzyme 2 (mitochondrial). Evidence of mitochondrial toxicity also included downregulation of cardiolipin and increased expression of genes associated with mitochondrial fission (*FIS1*) and mitophagy (*PINK1*). There were also signs of disruption of lipid metabolism (see also Section 4.2.8) (Griebsch et al., 2023). [The Working Group noted that the limitations of this study included the lack of concentration–response data, the lack of positive controls or antioxidants to determine whether mitochondrial toxicity led to the increase in ROS or vice versa, and the lack of experiments clarifying the relationships between these end-points and the observed changes in cellular lipids.]

Aspartame exposure was reported to disrupt intestinal cell barrier function, an effect that was dependent on oxidative damage and expression of the T1R3 sweet taste receptor (Shil et al., 2020). Exposure to aspartame at high concentrations (up to 10 mM) reduced metabolic activity (measured by the MTT assay) in Caco-2 cells, a human epithelial cell line derived from a colon carcinoma. Intracellular ROS, measured as fluorescence by 2′,7′-dichlorodifluorescein diacetate (DCFDA), was associated with exposure to aspartame at 0.1 mM (100 µM), a non-cytotoxic concentration. A series of well-controlled experiments linked the increase in ROS to reduced permeability in Caco-2 cell monolayers. The permeability reduction was reversed by pre-treatment of the cells with the antioxidant *N*-acetyl cysteine (NAC), by knocking down expression of claudin-3 (a component of the tight junction in the cell membrane) or the T1R3 taste receptor. [The Working Group noted that the study findings, examined in more detail in Section 4.2.8, presented evidence that generation of ROS is associated with and possibly necessary for disruption of membrane permeability in vitro.]

In contrast, Alleva et al. (2011) reported no increase in intracellular ROS, measured as fluorescence by DCFDA, in human fetal lung fibroblasts (IMR-90) exposed to aspartame at non-cytotoxic concentrations of up to 100 µM, although there was a small increase in fluorescence in primary human umbilical vein endothelial cells (HUVECs). [The Working Group noted that the increase observed in HUVEC cells might not be biologically significant because it was small in magnitude and did not increase with increasing concentration.]

No increase in thiobarbituric acid reactive substances (TBARS), a measurement of lipid peroxidation (LPO), was detected by Rodrigues et al. (2022) when HTR-8/SV neo cells (an SV40-transformed cell line derived from extravillous trophoblasts from human placenta explants) were exposed to aspartame at a non-cytotoxic concentration of 0.5 mM for 24 hours.

Increases in markers associated with ROS at cytotoxic aspartame exposures were featured in several reports. In the 3A-subE human trophoblast cell line, aspartame (10 mg/mL) was cytotoxic (measured by the MTT assay), increased intracellular ROS and decreased superoxide dismutase 2 (SOD2) expression (measured by western blot), increased phosphorylation of AKT serine/threonine kinase (AKT), and also inhibited the ability of cells to migrate into a "wound" scratched into the monolayer on the tissue culture plate. The cytotoxicity of aspartame was partially inhibited by exposure of the cells to NAC, and co-incubation of aspartame and either lactisole or gynemic acid increased SOD2 expression to levels higher than those seen in non-exposed cells. The results for lactisole and gynemic acid were attributed to their ability to inhibit binding of aspartame to the sweet taste receptor (Huang et al., 2023a). [The Working Group noted that the antioxidant NAC reversed only a small fraction of the cytotoxicity, thus a secondary effect of ROS could not be excluded. Also, there was no control experiment in which cells were exposed

to lactisol or gynemic acid without aspartame, nor was any evidence presented that these cells expressed a functional sweet taste receptor, which would support a role for the receptor.]

Qu et al. (2019) found a concentration-related increase in ROS using the proprietary fluorescence dye in the CellRox system in HepG2 cells (a human hepatoblastoma cell line) exposed to aspartame at 0.21, 0.32, or 0.48 g/L (713.1, 1088, and 1630 μM), concentrations that also induced signs of apoptosis and corresponded respectively to the IC_{10}, IC_{25}, and IC_{50} for cytotoxicity, measured as MTT reduction. Similarly, exposing a human cervical carcinoma cell line to aspartame at 10 mM resulted in significant cytotoxicity, measured by binding of sulforhodamine B to cellular proteins, binding of propidium iodide to DNA in necrotic cells, and measurement of intracellular ROS (DCFDA fluorescence) (Pandurangan et al., 2016a). [The Working Group noted that, in the absence of additional experimental results, it was impossible to determine whether ROS directly resulting from aspartame exposure led to cytotoxicity or whether toxicity caused by aspartame led to ROS generation secondary to necrotic and/or apoptotic cell death.]

The impact of aspartame on membrane permeability was studied in primary glomerular microvascular endothelial cells (Enuwosa et al., 2021). Exposure of these cells to aspartame at concentrations of up to 100 μM for 24 hours did not reduce metabolic activity as measured by colourimetric evaluation of WST-8 reagent (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt). In these experiments, the cells were exposed to vascular endothelial growth factor (VEGF) at 50 ng/mL to model renal damage and to assess the potential protective effects of pre- or co-exposure to aspartame. Each experiment consisted of a single trial with six replicates. Membrane permeability was assessed by migration of fluorescently labelled dextran across the monolayer in a double-chambered cell. Exposure to VEGF significantly increased membrane permeability. Exposure of the cells to aspartame at concentrations as high as 100 μM had no impact on membrane permeability. Co-incubation of VEGF with aspartame at 10 or 100 μM blocked the permeability increase observed with VEGF alone. The protective effect of aspartame on VEGF-induced membrane leakage (decreased permeability) was reversed by blocking the T1R3 sweet taste receptor, either by addition of lactisole to inhibit receptor binding or by small interfering RNA (siRNA) knockdown of receptor expression. VEGF stimulation of intracellular cyclic adenosine monophosphate (cAMP) was not altered by co-incubation with aspartame at 10 μM. Exposure to aspartame at 10 μM increased intracellular ROS (measured as DCDFA fluorescence) and also depleted intracellular glutathione (GSH). Exposure to VEGF also increased intracellular ROS and depleted intracellular GSH, but co-incubation of VEGF (50 ng/mL) and aspartame (10 μM) resulted in ROS and GSH levels comparable to those in the non-exposed controls. Surface expression of VE-cadherin (detected by enzyme-linked immunosorbent assay, ELISA, of cells fixed to the plates) decreased after exposure to VEGF. Co-incubation with aspartame blocked the reduction in VE-cadherin surface expression. Enuwosa et al. (2021) concluded that, on the basis of the cAMP and VE-cadherin results, aspartame does not act through the signalling pathways associated with T1R3 signalling or VEGF injury.

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.6.

In rats, administration of aspartame at 40 mg/kg bw per day by gavage for 6 weeks resulted in increased concentrations of GSH, catalase (CAT), or lipid peroxidation by thiobarbituric acid reactive substances (LPO/TBARS)

Table 4.6 End-points relevant to oxidative stress in non-human mammals in vivo exposed to aspartame

End-point	Species, strain (sex)/cell line	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
LPO/TBARS, SOD, CAT, GSH, O_2^-, H_2O_2, NO_2^-, $ONOO^-$	Rat, Wistar albino (M)	Erythrocytes	↑: O_2^-, H_2O_2, $ONOO^-$, LPO/TBARS, GSH, CAT No changes: NO_2^-, SOD	40 mg/kg bw per day	Oral gavage for 6 wk	Single dose.	Prokić et al. (2014)
LPO/MDA/TBARS, NO, GSH, GSSG, GSH RI	Rat, Wistar albino (M)	Blood	↓: GSH, GSH RI ↑: NO, LPO, GSSG	40 mg/kg bw per day	Drinking-water, 40 mg/kg bw per day for 6 wk	Single dose.	Prokić et al. (2015)
CAT, TAC	Rat, Wistar albino (M)	Blood	↓: CAT, TAC	250 mg/kg bw per day	Oral gavage for 8 wk (250, 1000 mg/kg bw per day)		Alkafafy et al. (2015)
LPO/MDA, NO, SOD, GPx, GSH, CAT, vit-C	Rat, Wistar albino (M)	Blood (serum)	↓: GSH, SOD, CAT, GPx, vit-C ↑: LPO/MDA, NO	40 mg/kg bw per day	Oral gavage for 90 days	Single dose.	Choudhary and Sheela Devi (2015)
Hsp70	Rat, Wistar albino (M)	Spleen, thymus, lymph node	↑	40 mg/kg bw per day	Oral gavage for 90 days	Indirect measurement of oxidative stress.	Choudhary and Sheela Devi (2016)
TAC, CAT, SOD, GPx, LPO/MDA, NO	Mouse, NMRI (M)	Blood (serum)	↓: TAC, GPx, SOD, CAT ↑: MDA, NO	80 mg/kg bw per day	Oral gavage for 90 days (40, 80 and 160 mg/kg bw per day)	Age 8–10 wk.	Anbara et al. (2020)
MDA, NO, SOD, TAC	Mouse, NMRI (M)	Blood (serum)	↓: TAC, SOD ↑: NO, MDA	80 mg/kg bw per day (SOD, NO, MDA), 160 mg/kg bw per day (TAC)	Oral gavage for 90 days (40, 80, 160 mg/kg bw per day)	Age 10–12 wk.	Anbara et al. (2022)
GSH, GSSG, γ-GC	Mouse, Swiss (M)	Liver	↓: GSH, GSSG, γ-GC	80 mg/kg bw per day	Oral gavage for 90 days	Single dose.	Finamor et al. (2017)
MDA, SOD, CAT, GPx	Mouse, Swiss (M)	Liver	↓: SOD, CAT, GPx ↑: MDA	80 mg/kg bw per day	Oral gavage for 12 wk	Single dose.	Finamor et al. (2021)
LPO/MDA, SOD, CAT, GPx, GSH	Rat (M)	Liver	↓: SOD, CAT, GPx, GSH ↑: LPO/MDA	75 mg/kg bw per day	Oral gavage for 30 days (75, 150 mg/kg bw per day)		Hamza et al. (2019)

Table 4.6 (continued)

End-point	Species, strain (sex)/cell line	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
LPO/TBARS, SOD, CAT, GPx, GSR, GSH	Rat, Wistar (M)	Liver	↓: GPx, GSR, GSH No changes: LPO/TBARS, SOD, CAT	500 mg/kg bw per day (GSH), 1000 mg/kg bw per day (GPx, GSR)	Oral gavage for 180 days (500, 1000 mg/kg bw per day)		Abhilash et al. (2011)
LPO/MDA, GPx, SOD, GST, GSH, CAT	Rat, Wistar albino (M)	Liver	↓: SOD, CAT, GPx, GSH, GST ↑: LPO/MDA	240 mg/kg bw per day	Oral gavage for 60 days	Single dose.	Lebda et al. (2017b)
LPO/MDA, GPx, SOD, GST, GSH, CAT	Rat, Wistar albino (M)	Brain	↓: SOD, CAT, GPx, GSH ↑: LPO/MDA, GST	240 mg/kg bw per day	Oral gavage for 60 days	Single dose.	Lebda et al. (2017a)
LPO/MDA, GPx, SOD, GST, GSH, CAT	Rat, Wistar (M)	Liver, kidney, brain	↓: SOD, CAT, GPx, GSH, GST ↑: LPO/MDA	35 mg/kg bw per day (SOD, CAT, GPx, GSH, GST), 15 mg/kg bw per day (LPO/MDA)	Oral gavage for 9 wk (15, 35, 70 mg/kg bw per day)		Adaramoye and Akanni (2016)
TBARS, vit-C, NPSH, TAC, GST, CAT	Rat, Wistar (M)	Liver Kidney	↓: vit-C, NPSH, TAC ↑: TBARS, GST, CAT ↑: GST, CAT No changes: TBARS, vit-C, NPSH, TAC	75 mg/kg bw per day	Oral gavage for 14 days	Single dose; vehicle, 2% DMSO; rats aged 21 days.	Souto et al. (2021)
MDA, GSH, GST, CAT	Rat, albino (M)	Liver, kidney, heart	↓: GST, GSH, CAT ↑: MDA	250 mg/kg bw per day	Oral gavage for 90 days	Used a commercial tablet; single dose.	Elballat and Abas (2020)
GSH, MDA, SOD, GPx	Rat, offspring	Kidney	↓: GSH, SOD, GPx ↑: MDA	40 mg/kg bw per day	Oral gavage from the first day of pregnancy until postnatal day 30	Single dose.	Fareed and Mostafa (2021)
LPO/TBARS, SOD, CAT, GPx, GSR, GST, LOOH	Rat, Wistar (M)	Brain	↓: SOD, CAT, GPx, GSR, GST ↑: LPO/TBARS, LOOH	40 mg/kg bw per day	Oral gavage for 6 wk (+ i.p. injection of NaCl from wk 5 for 2 wk)		Finamor et al. (2014)

Table 4.6 (continued)

End-point	Species, strain (sex)/cell line	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
LPO/MDA	Rat, SD (M)	Brain	↑: LPO/MDA	3 mg/kg bw per day	Drinking-water for 6 wk	Single dose.	Erbaş et al. (2018)
SOD, NO	Mouse, Swiss (M)	Brain (cerebral cortex)	↑: SOD, NO	40 mg/kg bw per day	Oral gavage for 28 days (20, 40, 80, 160 mg/kg bw per day)		Onaolapo et al. (2016)
SOD, NO	Mouse, Swiss (M)	Brain (hippocampal)	↑: SOD, NO	40 mg/kg bw per day (NO), 80 mg/kg bw per day (SOD)	Oral gavage for 28 days (20, 40, 80 160 mg/kg bw per day)		Onaolapo et al. (2017c)
LPO/MDA, SOD, GSH, NO	Mouse, Swiss (M)	Brain (cerebellum and pons)	↓: GSH ↑: SOD, NO, MDA	20 mg/kg bw per day (SOD, NO), 40 mg/kg bw per day (GSH, MDA)	Oral gavage for 28 days (20, 40, 80, 160 mg/kg bw per day)		Onaolapo et al. (2017b)
SOD, GPx, NO	Mouse, Swiss (M)	Brain	↓: GPx ↑: SOD, NO	160 mg/kg bw per day	Oral gavage for 21 days (160 and 320 mg/kg bw per day)		Onaolapo et al. (2017a)
LPO/MDA, GSH, NO	Mouse, Swiss albino (M)	Brain	↓: GSH ↑: MDA, NO	1.875 mg/kg bw per day	s.c. injection for 2 wk (0.625, 1.875, 5.625 mg/kg bw per day)		Abdel-Salam et al. (2012a)
LPO/MDA, GSH, NO	Mouse, Swiss albino (M)	Brain Liver	↓: GSH ↑: MDA No changes: NO No changes: MDA, NO, GSH	11.25 mg/kg (GSH),22.5mg/kg bw (TBARS)	Single s.c. injection (11.25, 22.5, 45 mg/kg bw)		Abdel-Salam et al. (2012b)
LPO/TBARS, GSH	Mouse, Swiss-Webster (M)	Brain (forebrain)	No changes: TBRAS, GSH	32 mg/kg bw per day	Drinking-water for 30 days	Single dose.	Abu-Taweel et al. (2014)
LPO/MDA, SOD, CAT	Rat, Wistar (M)	Spleen	↓: SOD, CAT ↑: LPO/MDA	500 mg/kg bw per day	Oral gavage for 1 wk	Single dose.	Ibrahim et al. (2022)
MPO, XO, LPO/MDA, SOD, CAT, GPx, GSH, total thiols	Rat, Wistar albino (M)	Heart	↓: MPO, SOD, CAT, GPx, thiols, GSH ↑: XO, LPO/MDA	75 mg/kg bw per day	Oral gavage for 4 wk (75, 150 mg/kg bw per day)		Al-Eisa et al. (2018)

Table 4.6 (continued)

End-point	Species, strain (sex)/cell line	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
TAC, CAT, SOD, GPx, LPO/MDA, NO	Mouse, NMRI (M)	Sperm, testis	↓: TAC, GPx, SOD, CAT ↑: MDA, NO	80 or 160 mg/kg bw per day	Oral gavage for 90 days (40, 80, and 160 mg/kg bw per day)		Anbara et al. (2021)
nNOS, NO	Rat, Wistar albino (M)	Brain	↑: NO	40 mg/kg bw per day	Oral gavage for 90 days (pre-treatment with MTX by s.c. injection, 0.2 mg/kg per day for 7 days)	Pre-treatment with MTX to mimic folate deficiency.	Iyaswamy et al. (2018)
LPO/MDA, GPx, SOD, GST, GSH, GSR	Rat, Wistar (M)	Sperm	↓: SOD, CAT, GPx, GSH, GSR ↑: LPO/MDA, GST	40 mg/kg bw per day	Oral gavage for 90 days (pre-treatment with MTX by s.c. injection, 0.2 mg/kg per day for 7 days)	Pre-treatment with MTX to mimic folate deficiency.	Ashok et al. (2017)
Hsp70	Rat, Wistar albino (M)	Spleen, thymus, lymph node	↑	40 mg/kg bw per day	Oral gavage for 90 days	Indirect measurement of oxidative stress.	Choudhary and Sheela Devi (2016)
LPO/MDA, SOD, GSH, CAT	Rat, Wistar albino (M)	Liver	↓: GSH ↑: SOD, CAT, LPO/MDA	40 mg/kg bw per day	Oral gavage for 90 days (pre-treatment with MTX by s.c. injection, 0.2 mg/kg per day for 7 days)	Pre-treatment with MTX to mimic folate deficiency.	Ashok et al. (2015)
LPO/MDA, SOD, GPx, GSH, CAT, protein thiols	Rat, Wistar albino (M)	Brain	↓: GSH, protein thiols ↑: SOD, CAT, GPx, LPO/MDA	75 mg/kg bw	Single oral gavage		
LPO/MDA, GSR, SOD, GPx, GSH, CAT, GST	Rat, Wistar albino (M)	Brain	↓: GSH, GSR ↑: LPO/MDA, SOD, CAT, GPx, GST	40 mg/kg bw per day	Oral gavage for 90 days (Pre-treatment with MTX by s.c. injection, 0.2 mg/kg per day for 7 days)	Pre-treatment with MTX to mimic folate deficiency.	Ashok and Sheeladevi (2014)

Table 4.6 (continued)

End-point	Species, strain (sex)/cell line	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
LPO, SOD, GPx, GSH, CAT, protein thiols	Rat, Wistar albino (M)	Brain	↓: GSH, protein thiols ↑: LPO, SOD, CAT, GPx	75 mg/kg bw per day	Oral gavage for 90 days (Pre-treatment with MTX by s.c. injection, 0.2 mg/kg per day for 7 days)	Pre-treatment with MTX to mimic folate deficiency.	Iyyaswamy and Rathinasamy (2012)

bw, body weight; CAT, catalase; DMSO, dimethyl sulfoxide; γ-GC, γ-glutamylcysteine; GPx, glutathione peroxidase; GSH, glutathione; GSH RI, glutathione redox index; GSR, glutathione reductase; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; h, hour(s); HID, highest ineffective dose; H_2O_2, hydrogen peroxide; Hsp70, heat shock protein 70; i.p., intraperitoneal; LED, lowest effective dose; LOOH, lipid hydroperoxides; LPO, lipid peroxidation; M, male; MDA, malondialdehyde; MPO, myeloperoxidase; MTX, methotrexate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NO_2^-, nitrite; NPSH, non-protein thiol; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; s.c., subcutaneous; SOD, superoxide dismutase; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; vit-C, vitamin C; XO, xanthine oxidase.

[a] ↑, increase; ↓, decrease.

in erythrocytes, but SOD enzyme activity was unchanged (Prokić et al., 2014). In a subsequent study, the authors reported an increase in LPO and nitric oxide (NO) levels, together with significant alterations in GSH redox status in the blood of male Wistar albino rats (age 2 months) exposed to drinking-water containing aspartame at a dose of 40 mg/kg bw for 6 weeks (Prokić et al., 2015). In two groups of male Wistar albino rats (age 8 weeks) who were exposed to aspartame at daily doses equivalent to five times the ADI (250 mg/kg bw per day) or 10 times the ADI (1000 mg/kg bw per day) for 8 weeks, a decrease was observed in both CAT activity and TAC in blood plasma, when compared with the control group (Alkafafy et al., 2015). In two additional studies, adult male Wistar albino rats were exposed to aspartame at a dose of 40 mg/kg bw per day for 90 days. A significant increase in LPO and elevated levels of malondialdehyde (MDA) and NO were observed in the blood (Choudhary and Sheela Devi, 2015), as well as an increase in messenger RNA (mRNA) transcript levels of heat shock protein Hsp70 (an indirect marker of oxidative stress) in the spleen, thymus, and lymph nodes (Choudhary and Sheela Devi, 2016). In contrast, the activities of CAT and glutathione peroxidase (GPx) and levels of GSH and vitamin C were found to be reduced in the blood (Choudhary and Sheela Devi, 2015, 2016).

Markers of oxidative stress were also assessed in the blood of NMRI male mice (age 8–10 weeks) exposed to aspartame (40, 80, or 160 mg/kg bw per day) by gavage for 90 days. There was a significant increase in MDA levels at the highest dose and in nitric oxide (NO) levels at the intermediate and highest dose compared with the control group. Additionally, aspartame caused a reduction in TAC and CAT activity at the highest dose, resulting in a significant decrease in GPx and SOD activity at the intermediate and highest dose compared with the control group (Anbara et al., 2020). In a subsequent experiment by the same authors, NMRI male mice (age 10–12 weeks) were exposed to aspartame under the same conditions as in the previous study; the same biomarkers were studied and identical findings were reported (Anbara et al., 2022). In male Swiss mice exposed daily to aspartame at 80 mg/kg bw for 90 days, a 30% decrease in GSH levels in the liver was observed; there was no indication of GSH oxidation in the liver, despite the GSH reduction. Additionally, the administration of aspartame led to decreased levels of γ-glutamylcysteine in the liver and induced simultaneous depletion of glutathione disulfide (GSSG), maintaining the normal GSSG:GSH ratio (Finamor et al., 2017). In a later study, the same authors conducted a similar experiment, with the sole difference being an extended exposure duration of 12 weeks. Aspartame caused an increase in MDA levels, which was accompanied by a reduction in the activity of SOD, CAT, and GPx in the liver of exposed mice compared with the control group. Furthermore, mRNA levels of NAD(P)H quinone oxidoreductase 1 (Nqo1) and haem oxygenase 1 (Hmox1), which are known targets of Nrf2 (Nfe2 like bZIP transcription factor 2), were downregulated in the liver of aspartame-exposed mice compared with the control group (Finamor et al., 2021). Another experiment in male rats exposed to aspartame at low (75 mg/kg bw) or high (150 mg/kg bw) doses showed a reduction in the levels of SOD, CAT, GPx, and GSH in the liver (Hamza et al., 2019). In rats exposed to aspartame at doses of up to 1000 mg/kg bw per day by oral gavage for 180 days, tissue concentrations of GSH, GPx, and glutathione reductase (GSR) in the liver were decreased, but LPO/TBARS levels and SOD and CAT activities were unchanged (Abhilash et al., 2011). In a 2-month experiment, male Wistar strain albino rats (age 6–8 weeks) were exposed to aspartame at 240 mg/kg bw (equivalent to six times the ADI in humans). The aspartame-exposed rats exhibited a significant increase in MDA levels and a decrease in GSH levels, and GPx, CAT, and SOD activities in the liver, compared with the

control group (Lebda et al., 2017b). In another study by the same authors, which focused on the brain, it was reported that aspartame increased MDA levels and glutathione *S*-transferase (GST) enzymatic activity. Conversely, GPx, CAT, and SOD activity, and GSH levels were significantly reduced in the aspartame-exposed group (Lebda et al., 2017a).

In another experiment, adult male Wistar rats were exposed to aspartame at daily doses of 15, 35, or 75 mg/kg bw by oral gavage for 9 weeks. The administration of aspartame significantly increased levels of TBARS in the kidney, liver, and brain, and there was a concurrent decline in GSH levels. Additionally, exposure to aspartame at the intermediate and highest dose resulted in a significant decrease in the activities of SOD and GST in the kidney, liver, and brain. In the liver and brain, the activities of CAT and GPx were also adversely affected by aspartame, whereas no significant change was observed in the kidney (Adaramoye and Akanni, 2016).

Similar effects were observed in the kidney, liver, and brain of male Wistar rats (age 21 weeks) exposed to aspartame at 75 mg/kg bw by gavage for 14 days. The study reported an increase in TBARS in the liver, indicating elevated LPO. Consistent with these findings, non-enzymatic antioxidant defences were disrupted by aspartame exposure. Specifically, in the aspartame-exposed group, levels of vitamin C, non-protein thiol (NPSH), and TAC were reduced in the liver, although no significant changes in these biomarkers were reported in the kidney. There was an increase in the activities of hepatic and renal GST and CAT enzymes in rats exposed to aspartame compared with the control group (Souto et al., 2021).

Effects on oxidative stress markers in the liver, kidneys, and heart were assessed in male albino rats exposed daily to aspartame at 250 mg/kg bw by oral gavage for 90 days. The data consistently indicated that aspartame caused a significant rise in MDA levels and a significant decrease in GSH levels and GST and CAT activities across all tissues examined when compared with the control group (Elballat and Abas, 2020). Similarly, adult albino rats exposed to aspartame at 40 mg/kg bw both in utero and postnatally via oral gavage for 30 days after birth demonstrated a statistically significant rise in renal MDA levels associated with a significant decrease in GSH, SOD, and GPx levels compared with the control group (Fareed and Mostafa, 2021). Oral administration of aspartame at a daily dose of 40 mg/kg bw to adult male Wistar rats for 6 weeks concomitant with sodium chloride (NaCl) solution (154 mmol/L) from week 5 resulted in elevated levels of TBARS, lipid hydroperoxides (LOOH), and protein carbonyls in the brain compared with the control group. Additionally, brain CAT activity was lower in the aspartame-exposed group than in the control group. The same pattern was observed for GPx, glutathione reductase (GSR), and GST activities in the brain in rats exposed to aspartame. Moreover, brain non-protein thiol levels were also lower in the exposed group than in the control group. Conversely, brain levels of thrombin receptor-activating protein (TRAP) were higher in the exposed group than in the control group (Finamor et al., 2014). Male Sprague-Dawley albino mature rats exposed to drinking-water containing aspartame at a daily dose of 3 mg/kg bw displayed elevated levels of MDA in the brain (Erbaş et al., 2018). In a study in male Swiss mice (age 6 months), aspartame was administered orally at four different doses (20, 40, 80, and 160 mg/kg bw per day) for 28 days. In both the cerebral cortex and hippocampus of mice at 40, 80 and 160 mg/kg bw, SOD and NO levels showed a significant increase compared with levels in mice in the control group (Onaolapo et al., 2016, 2017c). In the pons and cerebellum of mice that underwent the same exposure paradigm, the same authors reported similar findings. Higher doses of aspartame led to an increase in the activity of SOD, and an elevation in MDA and NO levels. On the other hand, a decrease in

GSH levels was observed when compared with the control group (Onaolapo et al., 2017b). Lastly, Onaolapo et al. (2017a) conducted another study in male Swiss mice exposed to aspartame at 160 and 320 mg/kg bw by gavage for 21 days. The results were consistent with those of their previous studies with regard to SOD, GPx activity, and NO levels in the brain (Onaolapo et al., 2017a). Similar effects were observed in the brain of mice exposed to aspartame at doses up to 45 mg/kg bw or 5.625 mg/kg bw per day by single or multiple (for 2 weeks) subcutaneous injections, respectively. However, such changes were not observed in the liver (Abdel-Salam et al., 2012a, b). No changes in TBARS or GSH were seen in the brain of mice exposed to drinking-water containing aspartame at a dose of 32 mg/kg bw per day for 30 days (Abu-Taweel et al., 2014). Indications of aspartame-induced oxidative stress were also observed in the spleen of male Wistar albino rats (age 6–8 weeks) exposed to aspartame orally at 500 mg/kg bw per day for 1 week. In these rats, levels of LPO in the spleen tissues were increased, whereas the activities of SOD and CAT were significantly decreased compared with those in the control group (Ibrahim et al., 2022). Cardiac oxidative stress was assessed in adult male Wistar albino rats exposed orally to aspartame at 75 or 150 mg/kg bw per day for 4 weeks. The study revealed a significant increase in LPO levels and in the activities of MPO and xanthine oxidase (XO). Additionally, a substantial decrease in the activities of SOD, CAT, and GPx was detected, together with a reduction in levels of GSH and protein thiols. The effects of aspartame on the various markers were found to be dose-dependent when compared with the control group (Al-Eisa et al., 2018). Anbara et al. (2021) reported decreased TAC levels and SOD, CAT, and GPx activities in the sperm of mice exposed to aspartame at 40, 80, or 160 mg/kg bw per day by oral gavage for 90 days. Increased MDA and NO levels were also reported in the testis and sperm (Anbara et al., 2021).

[The Working Group identified several studies from the group of Ashok et al. (Ashok and Sheeladevi, 2014, 2015; Ashok et al., 2015, 2017). The authors, with the aim of mimicking physiological methanol toxicity in humans, and investigating the role of aspartame-derived methanol, investigated the effects of aspartame in conditions of folate deficiency (by pretreatment with methotrexate) in rodents. Long-term exposure to aspartame (40 mg/kg bw per day for 90 days) or a single dose of 75 mg/kg bw were both shown to induce LPO in the liver or in discrete regions of the brain, respectively. However, the lack of proper grouping, including the lack of a negative control group that was exposed to aspartame, limited the interpretation and use of the results.]

(ii) Non-human mammalian cells in vitro

Aspartame was used to prevent the induction of LPO by ochratoxin A. When Vero (African green monkey kidney) cells were exposed to aspartame (0.5 mM [145 µg/mL]) for 24 hours, MDA production was not different from that in the controls (Baudrimont et al., 1997; Creppy et al., 1998).

4.2.4 Induces chronic inflammation

(a) Humans

(i) Exposed humans

See Table 4.7.

The potential for aspartame to exhibit the key characteristic "induces chronic inflammation", by eliciting changes in the key characteristic-associated end-points, was studied in humans in various settings, including consumption of aspartame-sweetened beverages (see Section 1.4.3).

Inflammatory markers

A large cross-sectional study investigated the associations between self-reported habitual intake of aspartame-sweetened beverages (using a food frequency questionnaire) and markers of inflammation in a cohort of women without

Table 4.7 End-points relevant to chronic inflammation in humans exposed to aspartame

End-point	Assay Biosample type	Location, setting, study design	Exposure level and no. of exposed and controls	Response[a] (significance)	Covariates controlled	Comments	Exposure assessment critique	Reference
Inflammatory markers								
CRP serum levels ICAM-1 serum levels Vascular adhesion protein 1 serum levels	Standard clinical chemistry Blood	USA, cross-sectional analysis on prospective cohort (Nurses' Health Study) Analysis: cycle 1, blood draw: 1989–1990; cycle 2, blood draw: 2000–2001	Aspartame-sweetened beverage intake among women Categories for selection: never or almost never; less than once per week; once to twice per week; three to six times per week; or one or more times per day	Frequency of intake associated with CRP serum levels: ($P < 0.048$) No association ($P > 0.05$) No association ($P > 0.05$)	Age, body weight, smoking status, physical activity, medication use and history of chronic diseases	Sample size for each biomarker varied: (CRP, $n = 5939$; ICAM-1, $n = 2290$; vascular adhesion protein 1, $n = 1205$).	Study population consisted of predominantly white health professionals; study authors noted possibility for residual confounding and reverse causation. There is a potential for measurement errors associated with the use of FFQs (frequency of exposure is self-reported). Furthermore, data on frequency of beverage consumption was used rather than actual intake to assess exposure.	Yu et al. (2018)

Table 4.7 (continued)

End-point	Assay Biosample type	Location, setting, study design	Exposure level and no. of exposed and controls	Response[a] (significance)	Covariates controlled	Comments	Exposure assessment critique	Reference
Coronary plaque characteristics: plaque segments, noncalcified, calcified, mixed Inflammatory markers: hsCRP, oxLDL, Lp-PLA2 Immune activation markers: sCD163, sCD14, MCP-1, CD4+, T-cell counts	CT angiography, physical activity questionnaire, standard blood clinical chemistry, immune markers. Blood	Boston, Massachusetts, USA, Matched control study	Dietary intake assessed using a 4-day food record. Cohorts: HIV-subjects (36) Non-HIV controls who consumed aspartame (15)	HIV cohort: ↑ number of coronary plaque segments ($P = 0.002$), noncalcified plaque segments ($P = 0.007$), and mixed segments ($P = 0.047$). Non-HIV cohort: ↑ number of calcified plaque segments ($P = 0.06$), no relationship with coronary plaque characteristics HIV cohort: ↑ MCP-1 ($P = 0.007$) and Lp-PLA2 ($P = 0.02$). ↓ hsCRP, oxLDL, and scCD163 and sCD14, although not significant ($P > 0.05$) Non-HIV cohort: no changes of any marker. ($P > 0.05$)	NA	Standardized 4-day food record based on self-reported dietary intake may not reflect chronic intake patterns.	No clear description of whether it was an open trial or blinded trial and time of sampling Study subjects consumed aspartame ~48 mg/day in 36 HIV-infected participants and ~24 mg/day in 15 non-infected controls.	Hall et al. (2017)

Table 4.7 (continued)

End-point	Assay Biosample type	Location, setting, study design	Exposure level and no. of exposed and controls	Response[a] (significance)	Covariates controlled	Comments	Exposure assessment critique	Reference
CRP serum levels	Standard clinical chemistry Blood	Mexico, 2007 Cross-sectional analysis of a prospective cohort study	Diet soda intake among women, divided into tertiles based on median intake: T1: 0 mL/day; (n = 540) T2: 11.8 mL/day; (n = 122) T3: 50.7 mL/day; (n = 163)	(–)	Age, biomarker batch, state of residence, socioeconomic status, family history of coronary heart disease, menopause, hormone use, smoking, alcohol use, physical activity, fruits and vegetables, and Western and modern Mexican dietary patterns	Intake of beverages containing sugar or artificial sweeteners served as a proxy for total dietary intakes.	Measurements limited to total beverages; aspartame exposure not determined.	Tamez et al. (2018)
CRP serum levels	Standard clinical chemistry Blood	Denmark, 2000 Two-arm-parallel randomized controlled trial, unblinded 10-week intervention	Sucrose group (n = 21) Sweetener group (n = 20)	No correlation was observed between dietary changes and changes in CRP	NA	Use of artificial sweetener mixture (54% aspartame, 23% cyclamate, 22% acesulfame-K, 1% saccharin).	Secondary analysis of an existing RCT. Not specific for aspartame.	Sørensen et al. (2005)

Table 4.7 (continued)

End-point	Assay Biosample type	Location, setting, study design	Exposure level and no. of exposed and controls	Response[a] (significance)	Covariates controlled	Comments	Exposure assessment critique	Reference
IL-6 TNFα Circulating neutrophils LPS-stimulated neutrophil degranulation	Standard clinical chemistry Blood	United Kingdom Randomized control study (counter balanced)	Fluid intake: 5 or 2 mL/kg body mass Healthy, trained soccer players (n = 6)	↑ Neutrophil count at 0 and 30 min post-exercise on both CHO (P < 0.01) and PLA (P < 0.05) trials ↑ IL-6 levels on CHO and PLA trials 0 and 30 min post-exercise (P < 0.01)	NA	In the PLA drink, ingredients were not defined. In the CHO drink, the presence of aspartame was not defined. Unclear sampling timing.	Artificially sweetened beverage. Type of beverage not reported. No information on type of beverage used for placebo. No information on background diet reported.	Bishop et al. (2002)
Allergic response								
Serum IgG, IgA, IgM, IgD, IgE C1q, C3, C4, factor B, glucose Histamine, epinephrine, and norepinephrine Histamine-induced cutaneous flare responsiveness and sensitivity	Cutaneous histamine reactivity. Blood (PBMCs)	Durham, North Caroline, USA, 1987 Double-blinded placebo-controlled crossover challenge study	30 mg/kg Overweight allergic subjects (n = 40)	Incidence of headache in exposed group was not different from incidence after placebo (P = 0.5)	NA	Abstract, lacked details.		Baraniuk et al. (1988)

Table 4.7 (continued)

End-point	Assay Biosample type	Location, setting, study design	Exposure level and no. of exposed and controls	Response[a] (significance)	Covariates controlled	Comments	Exposure assessment critique	Reference
Allergic response defined as urticaria or angioedema	Observation/ pathology Skin	USA and Canada, 1988–1991 Multicentre, randomized, double-blind crossover trial	50, 300, 600 mg, oral administration, three times daily for two alternate days Subjects (both sexes, children and adults, n = 21; 4 males, 17 females; age, 10–55 yr)	No statistically significant difference in the occurrence of allergic reactions after aspartame compared with placebo (P = 1.00).	Heart rate, respiratory rate, temperature, and blood pressure of individuals who had allergic reactions	Small sample size (n = 21). Findings limited to scoring allergic reactions.	Mixed group of children and adults, males and females. Recruitment: selection for those reporting adverse allergic reaction to aspartame-containing products. This affected sample size despite widespread recruitment over 4 yr.	EFSA_UN07 (2011)

Table 4.7 (continued)

End-point	Assay Biosample type	Location, setting, study design	Exposure level and no. of exposed and controls	Response[a] (significance)	Covariates controlled	Comments	Exposure assessment critique	Reference
Reproduction of historical report of hypersensitivity reaction defined as nausea, difficulty swallowing, vomiting, abdominal pain, diarrhoea, abdominal distention, flatulence, urticaria. angioedema, pruritus, cutaneous flushing, eczema. rhinitis, asthma, hypotension, syncope, and/or headache Allergic response as defined by a positive skin test Decrease in lung function as defined by decrease in forced expiratory volume (FEV) > 20%	Observation/ pathology Skin test (prick skin method to various aeroallergens). Lung function tests, breaths.	Maryland, USA, 1986 Combined single-blind, double-blind, placebo-controlled study. Healthy subjects $n = 5$ (1 male, 4 females), Patients with allergic reactions atopic, $n = 6$ (2 males, 4 females) out of 61 referrals	Aspartame dose: 10, 100, 500, 1000, and 2000 mg oral administration	No subject with a clear and reproducible adverse reaction to aspartame was identified.	NA	Small sample size. Findings limited to scoring allergic reactions. No. of exposed and controls provided).	No demographic information provided on the participants. Recruitment: was selective selection for those reporting adverse allergic reaction to aspartame-containing products, which affected sample size.	Garriga et al. (1991)

acesulfame-K, acesulfame potassium; CHO, carbohydrate; CT, computed tomography; CRP, C-reactive protein; FEV, forced expiratory volume; FFQ, food frequency questionnaire; hsCRP, high-sensitivity C-reactive protein; Ig, immunoglobulin; LPS, lipopolysaccharide; min, minute(s); NA, not available; oxLDL, PLA, placebo; PBMC, peripheral blood mononuclear cells; RCT, randomized controlled trial; yr, year

↓ decrease; ↑ increase; (–), negative result in a study of limited quality.

diabetes or cardiovascular disease (n = 8492) in the USA (Yu et al., 2018). The study reported that more frequent intake of aspartame-sweetened beverages was associated with higher blood concentrations of C-reactive protein (CRP) (P for trend, 0.048) and with higher serum concentrations of adiponectin (P for trend, < 0.01). [The Working Group noted that adiponectin, the most abundant adipokine circulating in human blood, seems to play dual roles in both pro-inflammation and anti-inflammation in various chronic diseases in humans (Choi et al., 2020).] However, intake of aspartame-sweetened beverages did not appear to be associated (P > 0.05) with levels of the other markers of inflammation tested, such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (Yu et al., 2018). [The Working Group noted that the reported effects were adjusted for BMI.]

In another cross-sectional study that aimed to characterize the relationship between dietary sweeteners and metabolic dysregulation and cardiovascular disease in a cohort of people who were or were not infected with human immunodeficiency virus (HIV), aspartame consumption in the HIV-positive group (n = 36) was positively (but non-significantly) associated with the inflammatory markers lipoprotein-associated phospholipase A2 (Lp-PLA2; P = 0.02), high-sensitivity C-reactive protein (hsCRP; P = 0.06), and oxidized low-density lipoprotein (oxLDL; P = 0.07). Aspartame intake was associated with a significantly increased level of the immune activation marker monocyte chemoattractant protein 1 (MCP-1; P = 0.007), but there was no association with soluble cluster of differentiation 163 (sCD163) or 14 (sCD14), two other measured markers of monocyte or macrophage activation.

No associations between aspartame intake and markers of inflammation and immune activation were observed among the aspartame consumers in the HIV-negative group (n = 15) (Hall et al., 2017). [The Working Group noted that patterns of consumption of the dietary sweetener in the cohort with 124 HIV-positive and 56 HIV-negative participants were determined on the basis of completion of a 4-day food record. Of these two groups of study participants, 36 HIV-positive and 15 HIV-negative participants were identified as aspartame consumers. The Working Group noted that aspartame intake in the HIV-negative group was much lower than in the HIV-positive group (89 ± 80 versus 164 ± 163 mg/day, P = 0.03) and the sample size was smaller (15 versus 36).]

In the HIV-positive participants in the same study, aspartame consumption was also positively associated with number of coronary plaque segments (P = 0.002), number of noncalcified plaque segments (P = 0.007), and number of mixed plaque segments (P = 0.047). Also, in the HIV-negative participants, aspartame intake appeared to be positively associated with number of calcified plaque segments (P = 0.06), although the findings were not statistically significant (Hall et al., 2017). [The Working Group noted that within this study, the aspartame consumption level was approximately twice as high in the HIV-positive group as in the HIV-negative group. Aspartame consumption was estimated to be 48 mg/day and 24 mg/day among HIV-positive participants and HIV-negative participants, respectively (P = 0.07). The 4-day dietary records provided limited insights into past dietary patterns and might not be reliable indicators of aspartame exposure. Also, the Working Group considered that this study was informative because chronic inflammation is a component of cardiovascular diseases often characterized by coronary plaques (Sorriento and Iaccarino, 2019), although some limitations of the study were identified. For example, no criteria for BMI, a major risk factor for cardiovascular events, were used in patient selection. Recruitment of HIV-positive and control study participants without regard to changes in body composition, weight, or metabolic criteria has been described by Lo et al. (2010); however,

they were considered to be a limitation of that study according to Hall et al. (2017). Exposure to aspartame was quantified from 4-day food records using a US database, although no details on the relevancy and history of the database were provided. The Working Group noted that information on food sources of the sweeteners and duration of aspartame intake was not provided.]

Several other studies investigated the effects of consumption of artificially sweetened food and/or artificially sweetened beverages (ASBs) on markers of inflammation in exposed humans; however, these studies were not particularly informative because the exposure was not clearly identified (Tamez et al., 2018), or because a mixture of artificial sweeteners was investigated (Bishop et al., 2002; Sørensen et al., 2005). For example, two studies in exposed humans reported no association between artificial sweetener consumption and increased levels of CRP. In a cross-sectional study of women from the Mexican Teachers' cohort, no association was reported between CRP and diet soda consumption (Tamez et al., 2018). [The Working Group noted that the focus of the exposure was total beverages, and thus exposure to aspartame was unclear.] Similarly, no correlation was observed between artificial sweetener intake and serum CRP in a study on a randomized controlled trial with a two-arm parallel, unblinded, design in Denmark (Sørensen et al., 2005). [The Working Group noted that an artificial sweetener mixture containing 54% aspartame (the other components being 23% cyclamate, 22% acesulfame-K, and 1% saccharin) was administered to the intervention diet group.]

Bishop et al. (2002) examined the effect of ingesting a carbohydrate beverage ("CHO" solution composed of 6.4% w/v glucose and maltodextrin) or an artificially sweetened placebo drink on plasma interleukin 6 (IL-6), tumour necrosis factor alpha (TNFα), and lipopolysaccharide (LPS)-stimulated neutrophil degranulation responses to high-intensity intermittent running. There was a significant increase in neutrophil count, IL-6, and TNFα levels at both 0 and 30 minutes post-exercise in people who had consumed the CHO drink and in people who consumed the placebo, although changes in TNFα were non-significant. LPS-stimulated elastase release per neutrophil, an indicator of neutrophil degranulation, was lower than it was before exercise in individuals who had been received the CHO drink, although this was also non-significant. [The Working Group noted that this study was deemed less relevant to aspartame since no information on the presence of aspartame in the carbohydrate beverage or in the placebo beverage was provided, and information on the background diet of the study participants was not reported.]

Allergic response

A double-blind, placebo-controlled crossover study reported that a 35% incidence of headache in aspartame consumers (30 mg/kg) was not significantly different from the 45% in the placebo group (P = 0.5). The analysis of the various parameters did not indicate any allergic association with aspartame consumption (Baraniuk et al., 1988). [The Working Group noted that this study summary was provided as an abstract only.]

In a regulatory study carried out in 1993 for marketing authorization, the authors reported that participants (n = 21) who had previously reported experiencing allergy (defined as urticaria or angioedema) to aspartame showed no difference in the occurrence of allergic reaction when challenged with aspartame or placebo in a controlled study. The study reported a non-statistically significant difference in the occurrence of allergic reactions after taking aspartame compared with placebo (EFSA_UN07, 2011). [The Working Group noted that the sample size was small (n = 21) and included male and female children and adults. Recruitment was selective for those reporting adverse allergic reaction to

Table 4.8 End-points relevant to chronic inflammation in human primary cells exposed to aspartame

End-point	Tissue, cell line	Results[a]	Concentration	Comments	Reference
IL-6 sIL-6r VEGF-A sVEGFR-2 secretion	HUVEC IMR-90 fibroblasts	HUVEC: ↑ IL-6 and sIL-6r (after 48 and 72 h) ($P < 0.05$); ↑ VEGF-A (dose-dependent) and sVEGFR-2 after 48 h and 72 h) ($P < 0.05$). IMR-90 fibroblasts: No changes on IL-6, sIL-6r, VEGF-A, or sVEGFR-2 ($P > 0.05$)	20, 40, 60, 80, 100 µM; up to 72 h		Alleva et al. (2011)
IL-6 IL-10 IFNγ	Lymphocytes (primary)	↓ IL-6 secretion ($P < 0.05$). No effect on levels of IL-10 or IFNγ ($P > 0.05$).	10 µg/mL (per well)	Only one dose tested	Rahiman and Pool (2014)

h, hour(s); HUVEC, human umbilical vein endothelial cells; IFNγ, interferon gamma; IL, interleukin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); ↑, increase; ↓, decrease; (+) or (–), positive or negative result in a study of limited quality.

aspartame-containing products, which affected the sample size, despite widespread recruitment over 4 years.]

In a combined single-blind, double-blind, placebo-controlled study ($n = 12$), the capacity of aspartame to reproduce the historically reported hypersensitivity reaction, allergic response (indicated by a positive skin test result), or a decrease in lung function was investigated. Like the above study (EFSA_UN07, 2011), no participant with a clear and reproducible adverse reaction to aspartame was identified (Garriga et al., 1991).

(ii) Human primary cells in vitro

See Table 4.8.

Two studies in primary human cells were relevant to chronic inflammation. In one study, HUVEC cells and IMR-90 fibroblasts were exposed to aspartame at concentrations ranging from 20 to 100 µM. In HUVEC (but not in IMR-90 fibroblasts), aspartame at 20 µM increased the secretion of IL-6 into the growth medium by twofold, with respect to controls, after 48 hours of incubation. IL-6 release was associated with an accompanying release of soluble interleukin-6 receptor (sIL-6r) after 24 hours of incubation, although these effects were not dose-dependent. Dose-dependent increases in cytokines/growth factors VEGF-A and sVEGFR-2 were reported in HUVEC but not in IMR-90 cells (Alleva et al., 2011). [The Working Group noted that Alleva et al. also showed that aspartame induced angiogenesis in HUVEC cells. At low doses, aspartame was a potential angiogenic agent that could induce regenerative cytokine production. It enhanced release of IL-6 and VEGF and their soluble receptors from endothelial cells and lead to the activation of mitogen-activated protein kinases (MAPKs) (extracellular signal-regulated kinase, ERK, and p38 phosphorylation), resulting in the formation of new blood vessels.]

Another study that investigated perturbations in cytokines IL-6, IL-10, and interferon gamma (IFNγ) in whole blood samples ($n = 21$) from human donors exposed to various artificial sweeteners (10 µg/mL), including two brands of commercial artificial sweetener containing aspartame, found no effects in unstimulated cells (Rahiman and Pool, 2014). [The Working Group noted that aspartame was one of the main

ingredients of one of the two aspartame-containing artificial sweeteners, although this branded sweetener also contained maltodextrin and acesulfame-K and may have contained dextrose, depending on the actual formulation applied. Similarly, although aspartame was an important component of the other branded sweetener, the product website indicated that this sweetener contained only 8% aspartame, the primary ingredient being lactose". This information was sought from external sources and was not provided or referenced in either of the primary studies.] After LPS stimulation, the two brands of aspartame-containing artificial sweetener blunted IL-6 secretion relative to the levels in the controls. There were no reported differences in IL-10 synthesis in phytohemagglutinin-stimulated and unstimulated whole blood cultures incubated with either branded aspartame-containing sweetener, relative to untreated controls. Similarly, the comparison of all sweeteners with the control cultures under both stimulated and unstimulated conditions showed that neither branded aspartame-containing sweetener had an effect on IFNγ synthesis, a biomarker of cell-mediated immunity ($P > 0.05$) (Rahiman and Pool, 2014).

(b) Experimental systems

See Table 4.9.

(i) Non-human mammals in vivo

In several studies in vivo, the inflammatory response and chronic inflammation were evaluated by means of measuring alterations in cytokine levels, tissue inflammation, and alterations in inflammation-related gene transcription.

Tissue inflammation

A histopathological analysis of the liver of rats exposed to aspartame at a dose of 240 mg/kg bw by intragastric intubation for 60 days showed severe hydropic degeneration of hepatocytes, which was characterized by swollen cells and clear fluids that replaced the cytoplasm, although nucleus, shape, and location were unaffected. Additionally, intense inflammatory cell aggregation in the portal area, congestion of the portal vein with focal hepatic necrosis, and infiltration of inflammatory cells were observed. Out of the five rats analysed, three showed inflammatory cell infiltration in the portal area (moderate in one rat and mild in two rats). In addition, four rats exhibited hepatic necrosis inflammatory cell infiltration (severe in one rat, moderate in two rats, and mild in one rat) (Lebda et al., 2017b). [The Working Group noted that this was a semi-quantitative histopathological analysis, that the descriptions of inflammation appeared to be inconsistent, and that portal vein congestion may be an artefact since this outcome is often seen in rats that have not been completely exsanguinated. Also, no statistical analysis was conducted for this study, and only five rats were examined microscopically.]

In another study, histopathological analysis of the livers of male Wistar rats exposed to aspartame at 1000 mg/kg bw for 180 days (by oral intubation) indicated a statistically significant increase in leukocyte infiltration in rats compared with the control group, but no pathological changes in the liver of rats exposed at 500 mg/kg bw (Abhilash et al., 2011).

Hamza et al. (2019) demonstrated that, together with altered liver function, alterations in oxidative biomarkers (see also Section 4.2.3), serum hepatic antioxidant enzyme and thiol levels, and lipid profile, and increases in inflammatory cytokines, rats exposed to aspartame at 75 mg/kg bw for 30 days exhibited major alterations in liver histology. Some hepatocytes displayed a large area of haemorrhage surrounded by necrotic and apoptotic hepatocytes. Hepatic lobules of rats exposed to aspartame at 150 mg/kg bw for 30 days exhibited a confused parenchymal architecture, some hepatocytes had infrequent nuclei and a vacuolated cytoplasm, and blood sinusoids were dilated and congested. Kupffer

Table 4.9 End-points relevant to chronic inflammation in non-human mammalian systems in vivo exposed to aspartame

End-point	Species, strain, (sex), tissue	Results	Dose (LED, HID)	Route, duration, dosing regimen	Comments	Reference
Tissue inflammation						
Inflammatory cell infiltration	Rat, Wistar albino (M), liver	Congestion of the portal vein and focal hepatic necrosis with inflammatory cell infiltration Fibrosis: not clearly identified	240 mg/kg bw per day aspartame	Control group: allowed to drink water ad libitum Aspartame group: oral intubation on dose-per-day basis Soft drink group: allowed to freely drink commercial cola beverages for 2 months; three groups (6 mice/group)	Only one dose group in which aspartame was administered.	Lebda et al. (2017b)
Leukocyte infiltration	Rat, adult Wistar, (M), liver	Increased leukocyte infiltration (1000 mg/kg) Fibrosis: not clearly identified	1000 mg/kg bw per day	Oral intubation, 0, 500, 1000 mg/kg bw per day for 180 days (6 rats/group)		Abhilash et al. (2011)
Inflammatory cell infiltration	Rat (M), liver	Accumulation of inflammatory cells in the portal tract (150 mg/kg) Fibrosis: not clearly identified	150 mg/kg bw per day	Oral administration, 0, 75, 150 mg/kg bw per day for 30 days (8 rats/group)	Hepatotoxicity was observed.	Hamza et al. (2019)
Inflammatory cell infiltration	Rat, Wistar albino (M) (age 7 wk), liver	Congestion of the portal vein and focal hepatic necrosis with inflammatory cell infiltration Fibrosis: not clearly identified	250 mg/kg bw per day	Oral administration, 0, 250, 1000 mg/kg bw per day for 8 wk (5 rats/group)		Alkafafy et al. (2015)

Table 4.9 (continued)

End-point	Species, strain, (sex), tissue	Results	Dose (LED, HID)	Route, duration, dosing regimen	Comments	Reference
Inflammatory cell infiltration Fibrosis	Rat, Sprague-Dawley albino (M), liver	Necrosis, disorganization of hepatic tissue, and mononuclear cell infiltration Fibrosis: increased deposition of collagen fibres around portal area in aspartame-only group Collagen fibres in congested blood vessel and bile ducts in diabetes-induced model	200 mg/kg bw per day	Oral administration, 0, 200 mg/kg bw per day for 4 wk (10 rats/group)	Only one dose tested. One additional dose group in which aspartame was administered in a streptozotocin-induced diabetes model.	Khidr et al. (2017)
Leukocyte infiltration	Mouse, Swiss (M), liver	Hepatocellular injury, leukocyte infiltration, reduction in nuclear area, and degeneration of hepatocytes with increased liver sinusoidal diameter in different areas of the liver Fibrosis: not identified	80 mg/kg bw per day	Oral gavage, 0, 80 mg/kg bw per day for 90 days (6 mice/group)	Only one dose tested. One additional dose group in which mice received i.p. NAC at 163 mg/kg on days 60–90 immediately after aspartame exposure.	Finamor et al. (2017)
Leukocyte infiltration	Mouse, Swiss (M), liver	Increased number of leukocytes per mm^2 of liver Fibrosis: increased deposition of collagen fibres	80 mg/kg bw per day	Oral gavage, 0, 80 mg/kg bw per day for 12 wk (6 mice/group)	Only one dose tested.	Finamor et al. (2021)
Fibrosis	Rat, Wistar Albino (M), spleen,	No inflammation recorded Fibrosis: thickening with fibrosis in splenic capsules, lymphoid depletion and necrosis in white pulp	500 mg/kg bw per day	Oral administration, 0, 500 mg/kg bw per day for 1 wk (8 rats/group)	Study assessed the protective effects of seaweed.	Ibrahim et al. (2022)

Table 4.9 (continued)

End-point	Species, strain, (sex), tissue	Results	Dose (LED, HID)	Route, duration, dosing regimen	Comments	Reference
Mechanical foot paw withdrawal thresholds Knee joint inflammation	Rat, Sprague-Dawley (M), foot/paw Knee joint	No effect on attenuating inflammatory response induced by carrageenan injection ↓ Mechanical hyperalgesia at 50 mg/kg Fibrosis: not assessed	50 mg/kg bw per day for hyperalgesia only	s.c. injection, 0, 25, 50, 100 mg/kg bw daily for 6 days; (8–10 rats/group) followed by intra-articular injection of carrageenan.	Aspartame co-administered with carrageenan.	LaBuda and Fuchs (2001)
Foot oedema	Rat, Sprague-Dawley (M), foot/paw	No effect Fibrosis: not assessed		i.g. administration, 0, 300 mg/kg, 1 h before injection of carrageenan) (8 rats/group)	Aspartame administered before carrageenan.	Aspinall et al. (1980)
Altered cytokine levels						
TNFα	Mouse, Swiss albino (M, F), peripheral blood	↑	22.5 mg/kg	i.p. injection 0.625, 1.875, 5.625, 11.25, 22.5 mg/kg (+ 100 µg/kg LPS) or 22.5, 45 mg/kg (no LPS) (single administration before euthanasia) (6 mice/group)		Abdel-Salam et al. (2012a)
IL-6 TNFα Serum CRP	Rat (M), liver	↑ (dose-dependent) ↑ ↑ (dose-dependent)	75 mg/kg bw per day	Oral administration, 0, 75, 150 mg/kg daily for 30 days (8 rats/group)		Hamza et al. (2019)
IL-2 IL-4 TNFα IFNγ Corticosteroid	Rat, adult Wistar (M), peripheral blood	↓ ↑ ↓ ↓ ↑	40 mg/kg bw per day	Oral gavage, 0, 40 mg/kg bw daily for 90 days (6 rats/group)	Only one dose group.	Choudhary and Sheela Devi (2015)
Phosphorylation of p65 subunit of NF-kB and NLRP3 inflammasome activation	Mouse, Swiss (M), liver	↑	80 mg/kg bw per day	Oral gavage, 0, 80 mg/kg bw daily for 12 wk (6 mice/group)	Only one dose group.	Finamor et al. (2021)

Table 4.9 (continued)

End-point	Species, strain, (sex), tissue	Results	Dose (LED, HID)	Route, duration, dosing regimen	Comments	Reference
CD11b+ cells	Mouse, C57BL/6J (age 3 wk) (M), peripheral blood	↑, slight increase	100 µM	Oral administration, 100 µM solution with equivalent sweetness of 30% w/v sucrose solution Fed ad libitum for 8 wk (8–9 mice/group)	Only one dose group.	Choi et al. (2017)
Altered inflammation-related gene transcription						
TNFa NF-κB	Rat, adult Wistar (M), liver	↑ ↑	40 mg/kg bw per day	Oral gavage, 0, 40 mg/kg bw daily for 90 days; folate-deficient diet was provided for 45 days before the experiment and MTX was administered for 1 wk before the experiment; three groups (6 rats/group)	Only one dose group in which MTX was administered; did not investigate effects of MTX isolation.	Ashok and Sheeladevi (2015)
TNFa, *CXCL1*, *Il22ra2*, *Creb3l3*, *Gnb2l1*, *Pex13*, *Ptgs1*	Mouse, C57BL/6J (age 3 wk) (M), brain	Unclear effects	100 µM	Oral administration, 100 µM solution with equivalent sweetness of 30% w/v sucrose solution Fed ad libitum for 8 wk (8–9 mice/group)	Only one dose group.	Choi et al. (2017)

Table 4.9 (continued)

End-point	Species, strain, (sex), tissue	Results	Dose (LED, HID)	Route, duration, dosing regimen	Comments	Reference
Adamts9, *Fga*, *Fgb*, *Fgg*, *Plg*, *Hpx*, *Itih4*, *Ptgs/cox2*, *Tdo2*, *Kynu*, *Adipoq*, *Fabp4*, *Scarb1*, *Cd36* *ALDH1*	Mouse, C57BL/6J (M, F) Adrenal glands Brain Hypothalamus	 ↓ ↑ ↑	45.44 ± 1.16 mg/kg bw (aspartame) 46.59 ± 0.68 mg/kg bw (aspartame + developmental NMDAR antagonist CGP 39551)	Oral administration, mothers treated for 3 wk until breeding, then up to postnatal day 28; F_1 offspring treated up to week 21 Control group: ad libitum drinking-water NMDAR antagonist group: 5 mg/kg bw in drinking-water Aspartame: 0.25 g/L in drinking-water, equivalent to aspartame at 45.44 ± 1.16 mg/kg bw per day Aspartame + NMDAR antagonist: 0.25 g/L in drinking-water, equivalent to aspartame at 46.59 ± 0.68 mg/kg bw per day	Assessed gene transcription only using microarray and RT-PCR.	Collison et al. (2018)
Pro-fibrotic: *TGFβ*, *COL1A1*, *αSMA* Pro-inflammatory: *IL-1b*, *IL-6*, *CXCL1*, *IL-10*, *IL-18*	Mouse, Swiss (M) (n = 12), liver	↑ ↑	80 mg/kg bw per day	Oral gavage, 0, 80 mg/kg bw daily for 12 wk; two groups of mice (6 mice/group)	Only one dose group.	Finamor et al. (2021)

Table 4.9 (continued)

End-point	Species, strain, (sex), tissue	Results	Dose (LED, HID)	Route, duration, dosing regimen	Comments	Reference
TNFa, *IL-6*	Rat, Wistar albino (M) (n = 32), spleen	↑	500 mg/kg bw	Oral administration, 500 mg/kg bw daily for 1 wk; two additional dose groups, one group treated with *S. vulgare*-MeOH extract (150 mg/kg bw per day for 3 wk), the other group was treated with 500 mg/kg bw per day (aspartame) orally for 1 wk before *S. vulgare*-MeOH extract exposure		Ibrahim et al. (2022)
Leptin Adiponectin	Rat, Wistar albino (M) (n = 30), adipose tissue	↑ ↓	240 mg/kg bw per day aspartame	Three groups (5 rats/group) Control group: allowed to drink water ad libitum Aspartame group: i.g. intubation on dose-per-day basis Soft drink group: allowed to freely drink commercial cola beverages for 2 months (Alkhedaide et al., 2016)	Only one dose group in which aspartame was administered.	Lebda et al. (2017b)

CRP, C-reactive protein; F, female; h, hour(s); HID, highest ineffective dose; IFNγ, interferon gamma; i.g., intragastric; IL, interleukin; i.p., intraperitoneal; LED, lowest effective dose; LPS, lipopolysaccharide; M, male; MeOH, methanol; MTX, methotrexate; NA, not available; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; RT-PCR, reverse transcription-polymerase chain reaction; s.c., subcutaneous; TNFα, tumour necrosis factor alpha; wk, week; w/v, weight per volume.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); ↑, increase; ↓, decrease; (+) or (–), positive or negative result in a study of limited quality.

cells became more prominent. The central vein was markedly dilated and congested and there was an accumulation of inflammatory cells in the portal tract. [The Working Group noted that the number of rats exhibiting these microscopic alterations was not reported.]

Similar results were reported with higher doses of aspartame: liver sections from male Wistar albino rats (age 7 weeks) (n = 25) who were treated with aspartame at 250 mg/kg bw or 1000 mg/kg bw showed various histological changes, including disorganized hepatic parenchyma, dilatation and congestion of the central vein and hepatic sinusoids, and proliferation of Kupffer cells. Some hepatocytes showed signs of degenerative changes in the form of cellular swelling and vacuolar cytoplasm, and others were necrotic. In rats at 250 mg/kg bw, liver sections presented focal areas of complete degeneration and diffuse and focal intralobular mononuclear cell infiltration; these changes were more pronounced in liver sections from rats at 1000 mg/kg bw (Alkafafy et al., 2015). [The Working Group noted that pathology descriptions were reported in the text but did not reflect the histopathology figures provided.]

Khidr et al. (2017) reported marked necrosis, disorganization of hepatic tissue, and mononuclear cell infiltration in the livers of adult male Sprague-Dawley rats exposed to aspartame at a dose of 200 mg/kg bw once daily for 4 weeks. In rats in which diabetes had been induced (via a single intraperitoneal dose of streptozotocin at 70 mg/kg bw) before exposure to aspartame, similar findings were reported, namely the presence of large necrotic area, loss of most of the architecture of the liver tissue, loss of cell boundaries, and degeneration in most of the hepatocytes.

Finamor et al. (2017) reported that administration of aspartame at a dose of 80 mg/kg bw by oral gavage to male Swiss mice for 90 days increased hepatocellular injury, triggered leukocyte infiltration, reduced nuclear volume, and contributed to the degeneration of hepatocytes with increased sinusoidal diameter in different areas of the liver. [The Working Group noted that these results were described by the study authors and that the changes described were not discernible in the figures presented in the manuscript.] In a subsequent publication, Finamor et al. (2021) showed that administration of aspartame at a dose of 80 mg/kg bw by oral gavage to male Swiss mice for 12 weeks induced statistically significant inflammatory infiltration, calculated as number of leukocytes per mm^2 of liver tissue. Evidence of fibrosis, as demonstrated by the elevated deposition of collagen fibres in the mouse liver, was also reported in aspartame-exposed mice. [The Working Group noted that the microscopic changes observed appeared to be nonspecific.] These changes were accompanied by the upregulation of several pro-fibrotic markers (e.g. transforming growth factor beta, TGFβ, collagen type 1 alpha 1, and alpha smooth muscle actin), and other pro-inflammatory transcripts (Il6, Cxcl1, Il1b, and Il18) (Finamor et al., 2021). Similarly, rats exposed orally to aspartame (500 mg/kg bw per day for 1 week) showed fibrosis in splenic capsules, lymphoid depletion, and necrosis in the white pulp, accompanied by elevated levels of pro-inflammatory splenic transcripts of TNFα and IL-6 (Ibrahim et al., 2022).

Another study indicated that the subcutaneous administration of aspartame (at concentrations of 2.5, 5, and 10 mg/mL in distilled water, to reach final doses of 25, 50, and 100 mg/kg, delivered in an injection volume of 10 mL/kg) to male Sprague-Dawley rats could attenuate mechanical hyperalgesia (heightened sensitivity to pain) but did not decrease carrageenan-induced inflammation (LaBuda and Fuchs, 2001). Similarly, Aspinall et al. (1980) reported that aspartame (300 mg/kg) administered intragastrically before carrageenan injection did not have any effect on the development of arthritic swelling in male rats with adjuvant arthritis. [The Working Group noted that these studies may be of limited

relevance to the key characteristic of carcinogens "induces chronic inflammation".]

Altered cytokine levels

TNFα levels, measured via ELISA, in the brain tissue of Swiss albino mice exposed subcutaneously to aspartame in saline solution at doses of 22.5 or 45 mg/kg were elevated relative to levels in controls exposed intraperitoneally to saline solution at 0.1 mL/mouse (Abdel-Salam et al., 2012a). Dose-dependent increases in levels of hepatic pro-inflammatory cytokines, TNFα, and IL-6, and serum CRP were observed in male rats exposed orally to aspartame (75 or 150 mg/kg) for 30 days (Hamza et al., 2019). In a 90-day study in which male Wistar albino rats were exposed orally to aspartame at 40 mg/kg bw per day, there was a marked increase in plasma corticosteroid levels, and an increase in circulatory IL-4 levels, whereas a significant decrease in levels of the cytokines IL-2, TNFα, and IFNγ was reported (Choudhary and Sheela Devi, 2015). These effects were reported in aspartame-exposed rats that had or had not been intraperitoneally immunized with sheep erythrocytes, and in groups of rats that had been fed either a normal diet or a folate-deficient diet.

Finamor et al. (2021) reported increased levels of p65 protein and phospho-p65 (Ser536) of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) in the livers of aspartame-exposed male Swiss mice relative to study controls. This study also reported higher levels of NLR family pyrin domain containing 3 (NLRP3) and cleaved caspase-1 protein levels in the livers of exposed rats, suggesting a role for aspartame in inflammasome activation.

Altered inflammation-related gene transcription

As described above, levels of mRNA expression of inflammation-related transcripts were upregulated by aspartame in the liver of male Swiss mice (*Il6*, *Cxcl1*, *Il1b*, and *Il18*) (Finamor et al., 2021) and in the spleen of exposed male Wistar albino rats (*TNFα* and *Il6*) (Ibrahim et al., 2022). Several studies in vivo solely examined the effects of aspartame exposure on mRNA expression of inflammation-related genes. Wistar albino rats (pre-treated with methotrexate to produce folate deficiency similar to in humans exposed to methanol) then exposed to aspartame (40 mg/kg) showed a marked increase in the *TNF* gene expression and a decrease in *NF-κB* gene expression in the liver relative to the liver of unexposed study controls ($P < 0.05$) (Ashok and Sheeladevi, 2015). [The Working Group considered the study to be of low informativeness because of the experimental design in which the pre-treatment with methotrexate was used to induce folate deficiency and lack of proper negative control with aspartame.]

Increases in the expression of neuroinflammatory genes (*Adipoq*, *Fabp4*, *Scarb1*, *Cd36*) have been reported in the brain tissue of exposed C57BL/6J mice (at least fivefold), whereas decreases were shown in *Adamts9*, *Fga*, *Fgb*, *Fgg*, *Plg*, *Hpx*, *Itih4*, *Ptgs2/cox2*, *Tdo2*, and *Kynu* (two- to threefold) (Collison et al., 2018). In a study that assessed the effects of sugar-sweetened beverages in weaned male C57BL/6J mice (age 3 weeks) provided drinks of 30 w/v% sucrose solution (S30), an aspartame solution with sweetness equivalent to the sucrose solution (A30), or plain water (CT) until age 11 weeks, unclear effects were reported on mRNA expression of inflammation-related transcripts (*TNFα*, *Cxcl1*, *Il22ra2*, *Creb3l3*, *Gnb2l1*, *Pex13*, *Ptgs1*) in the brain tissue. The authors reported that the frequency of CD11b+ myeloid lineage cells was slightly higher in the peripheral blood of rats exposed to S30 compared with rats exposed to aspartame or plain water, and a pattern of positive correlation was reported between the frequency of CD11b+ cells and serum levels of corticosterone (Choi et al., 2017).

Increased mRNA expression of leptin (a pro-inflammatory agent and a potential mediator of hepatic fibrosis) and decreased mRNA

Table 4.10 End-points relevant to chronic inflammation in primary non-mammalian cells in vitro exposed to aspartame

End-point	Cells and/or tissue	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Leukotriene C4 Leukotriene B4 15-Hydroxyeicosatetraenoic acid	Macrophages derived from rats	↑ ↑ ↑	NT NT NT	0, 10^{-6} M	Cells pre-treated for 6 h with 0.5 µCi/mL of ^{14}C-arachidonic acid. Only one dose group.	Hardcastle and Bruch (1997)

h, hour(s); HIC, highest ineffective concentration; LEC, lowest effective concentration; NT, not tested.
[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+) or (–), positive or negative result in a study of limited quality.

expression of adiponectin (an anti-inflammatory adipocytokine) were also observed in the adipose tissue of male Wistar albino rats exposed to aspartame by intragastric intubation (Lebda et al., 2017b).

(ii) Non-human mammalian cells in vitro

See Table 4.10.

A large increase in the production of arachidonic acid metabolites and leukotriene C_4 (LTC_4) levels was reported in aspartame-exposed rat macrophage cell cultures compared with control cell cultures (Hardcastle and Bruch, 1997). [The Working Group noted that the cysteinyl leukotrienes and other lipoxygenase products of arachidonic acid are important mediators in both intestinal inflammation and in the inflammatory response in general.]

4.2.5 Is immunosuppressive

(a) Humans

(i) Exposed humans

Bishop et al. (2002) compared the effects of ingesting a carbohydrate beverage ("CHO" solution composed of 6.4% w/v glucose and maltodextrin), or an artificially sweetened placebo drink that may have contained aspartame, on plasma levels of IL-6 and TNFα, and LPS-stimulated neutrophil degranulation responses to high-intensity intermittent running. There was a significant increase in neutrophil count in those who had consumed the placebo drink. In the CHO and the placebo groups, plasma levels of IL-6 ($P < 0.01$) were increased at both right the after the exercise (time 0) and 30 minutes post-exercise compared with pre-exercise levels; however, IL-6 levels were higher in participants ingesting placebo than those ingesting CHO at 30 minutes post-exercise ($P < 0.02$). LPS-stimulated elastase release per neutrophil (an indicator of neutrophil degranulation) was lower after exercise than before exercise in participants who were given the placebo drink ($P < 0.06$). [The Working Group noted that the study was not informative because the type of sweetener in the placebo beverage was not reported. Additionally, no information on the participants' diet was reported.]

(ii) Human primary cells in vitro

Two commercial brands of aspartame-containing artificial sweeteners (diluted to 10 µg/mL), among others, were shown to suppress the secretion of IL-6 in LPS-stimulated (10 ng/mL) whole blood cells from human donors when compared with control cells exposed to cell medium or natural sweeteners, whereas no effect was reported on levels of IL-10. The comparison of all sweeteners with the control cultures under

stimulated and unstimulated conditions showed that none of the sweeteners had an effect on IFNγ synthesis ($P > 0.05$) (Rahiman and Pool, 2014). [The Working Group noted that the sweeteners used in this study contained aspartame, but that the other sweetener constituents were not described. In this study, none of the sweeteners applied to the human lymphocytes had any effect on cell-mediated immunity.]

(b) Experimental systems

Non-human mammals in vivo

In NC/Nga mice in which atopic dermatitis had been induced by dinitrofluorobenzene, aspartame (0.5 µg/kg or 0.5 mg/kg) administered daily for 9 days (from day 9 to day 18) inhibited inflammatory cells (including eosinophils, mast cells, and CD4+ T-cells) and suppressed the expression of cytokines (IL-4 and IFNγ) and total serum IgE levels relative to mice exposed to dinitrofluorobenzene only (Kim et al., 2015a).

4.2.6 Modulates receptor-mediated effects

(a) Humans

(i) Exposed humans

No data on exposed humans were available to the Working Group.

(ii) Human primary cells in vitro

Aspartame (0.4–410 µM) had no effect on the transcriptional activity of the genes encoding the aryl hydrocarbon receptor (AhR) and glucocorticoid receptor (GR), or on AhR-dependent or glucocorticoid receptor-dependent expression of cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) in human primary hepatocytes (Kamenickova et al., 2013).

(iii) Human cell lines

In human granulosa-like tumour (KGN) cells, Chen et al. (2022) assessed the effects of exposure to aspartame (5 and 20 mM) for 48 hours. The higher concentration of aspartame (20 mM) was observed to significantly decrease progesterone secretion, which is normally correlated with adenosine triphosphate (ATP) production. The observed effects were consistent with mitochondrial respiratory dysfunction resulting from oxidative stress caused by aspartame (see Section 4.2.3).

In contrast, Kamenickova et al. (2013) reported that aspartame, and other non-nutritive sweeteners, had no effect on AhR-dependent or glucocorticoid receptor-dependent expression of CYP1A1 in human intestinal cancer cell lines.

(b) Experimental systems

(i) Non-human mammals in vivo

Steroid hormones and receptors

In a study of reproductive toxicity, the effects of aspartame were investigated in mature male NMRI mice exposed to aspartame (40, 80, and 160 mg/kg bw) for 90 days (Anbara et al., 2020). A dose-dependent reduction in serum concentrations of testosterone and pituitary–testicular axis hormones (follicle-stimulating hormone, FSH, and luteinizing hormone, LH) was observed. Aspartame also decreased sperm and testicular functionality indices. [The Working Group noted that the decrease in in serum concentrations of pituitary–testis axis hormones could indicate male reproductive toxicity. The Working Group also noted that the same group had subsequently published a similar study that also reported decreases in testosterone identical to those in the study in 2020 (Anbara et al., 2021).]

In studies by Lebda et al. (2017a, b), rats exposed to drinking-water containing aspartame at a dose of 240 mg/kg for 2 months showed a decrease in triiodothyronine (T3) levels and an increase in thyroxine (T4) and parathyroid hormone levels (Lebda et al., 2017a), as well as upregulation of leptin, adiponectin, and peroxisome proliferator-activated receptor gamma (PPARγ) mRNA gene expression in adipose tissue (Lebda et al., 2017b). In a similar study by

Ibrahim et al. (2022), exposure to aspartame at 500 mg/kg bw per day for 1 week caused increases in markers of apoptosis and inflammation in the spleen of exposed rats, and decreases in thyroid hormones T3 and T4, and antioxidant defence enzymes CAT, and SOD (see also Section 4.2.3).

In male rats, aspartame caused an increase in FSH levels when administered at doses of > 100 mg/kg bw per day but not at doses of > 300 mg/kg bw per day. Levels of LH and prolactin were unaltered (EFSA_E103, 2011).

In a series of regulatory studies carried out in mice, rabbits, and rats in the 1970s (EFSA_E19, 2011), the administration of aspartame was shown not to exert effects on the activities of estrogen, progesterone, or androgen. Estrogenic agonist and antagonist activities were measured by evaluating uterine weight after the administration of aspartame to female albino mice. No significant alterations in uterine weight were observed after the oral administration of 1350 mg of aspartame for three consecutive days. Saunders et al. (1980) showed that aspartame at up to 300 mg/kg bw per day did not have a hormone mimetic response in the endocrine target organs evaluated in mice, rats, and rabbits. [The Working Group noted that the duration of treatment could not be identified.] Progesterone-like activity and progesterone antagonism were measured in female rabbits primed for 6 days with 17β-estradiol after intragastric administration of 300 mg of aspartame. No increase in the concentration of carbonic anhydrase in the uterus (as a measure of the degree of glandular proliferation in the endometrium) was observed compared with controls (EFSA_E19, 2011). Androgenic and myotrophic activity and androgen antagonism were measured in male castrated Sprague-Dawley rats after intragastric administration of 50 or 350 mg of aspartame for 7 days. No significant alterations in the seminal vesicles, ventral prostate gland, or in the elevator ani muscle were observed compared with controls (EFSA_E19, 2011).

[The Working Group noted that these were studies of acute toxicity and would not have identified any effects on receptor systems that may occur after exposure to aspartame for longer periods. In addition, the Working Group noted that the study did not investigate potential interactions of aspartame with hormone receptors and only documented changes in hormone activities.]

Glutamate receptors

The observation of reduced levels of glutamic acid and aspartic acid in the brains of weanling rats exposed perinatally to aspartame led Reilly and Lajtha (1995) to investigate the effects of aspartame on glutamatergic receptor kinetics. Aspartame (500 mg/kg bw per day in drinking-water) was administered to Sprague-Dawley rats throughout gestation and lactation for 30 days. The kinetics of the *N*-methyl-D-aspartate receptor and total glutamatergic binding in the cerebral cortex and hippocampus were found to be unaffected by perinatal exposure to aspartame. However, Pan-Hou et al. (1990) previously showed that aspartame inhibited L-[^{3}H]glutamate binding to its *N*-methyl-D-aspartate (NMDA)-specific receptors in a dose-dependent manner in ex vivo isolated rat synaptic membranes. More recently, Arcego et al. (2020) reported decreased immunocontent of the GluN2A subunit of the *N*-methyl-D-aspartate receptor (NMDAR) in the hippocampus in adolescent male and female rats given unlimited access to aspartame (2 g/L) on postnatal days 21–55. [The Working Group noted that the study (Arcego et al., 2020) was not informative since no evidence linking alterations of the glutamate receptor to carcinogenesis was reported.]

In a binding assay (Monastyrskaia et al., 1999), aspartame (up to 1 mM) did not bind to the rat brain mGlu4 receptor, a G-protein-coupled metabotropic glutamate receptor.

4.2.7 Causes immortalization

Humans

(i) Exposed humans

No relevant data were available in exposed humans.

(ii) Human cells in vitro

Gezginci-Oktayoglu et al. (2021) investigated the effects of aspartame exposure compared with glucose-exposed and non-exposed controls in human pancreatic adenocarcinoma cells (PANC-1). In a first range-finding study, the authors assessed cell viability after 24 hours exposure to aspartame or glucose at increasing concentrations (1 ng/mL to 100 mg/mL and from 5 mM to 25 mM, respectively). Cell viability decreased with aspartame at concentrations > 1 ng/mL and with glucose at > 15 mM. The authors used this information to select high and low doses (aspartame, 1 ng/mL and 100 ng/mL; glucose, 5 mM and 25 mM) for 7 days or 14 days, to verify effects in a cancer stem cell population. Exposure to aspartame at the lower dose (1 ng/mL) for 7 days significantly increased the percentage of CD24+/CD44+ cells and the overall cancer stem cell population, as assessed by flow cytometric analysis, but this effect was not observed for glucose. In contrast, at the higher dose of aspartame (100 ng/mL) there was a reduction in cell viability and in the percentage of CD24+/CD44+ cells at 7 and 14 days. After a 7-day exposure, aspartame (1 ng/L) or glucose (25 mM) did not induce colony formation and did not alter the cell cycle status of the population.

Aspartame at (1 ng/mL) or glucose (25 mM) altered the protein levels of epithelial–mesenchymal transition (EMT) markers of invasion and migration – E-cadherin, vimentin, and N-cadherin. Aspartame induced a significant decrease in levels of the epithelial marker E-cadherin, as did glucose, compared with non-exposed controls, but a significant increase in levels of the mesenchymal marker vimentin and count of cells expressing N-cadherin, compared with both glucose exposure and non-exposed controls.

In addition, aspartame, but not glucose, was shown to increase protein levels of the membrane sweet taste receptor 1 (T1R1) and to decrease protein levels of sweet taste receptor 3 (T1R3), but aspartame did not alter protein levels of sweet taste receptor 2 (T1R2) after exposure at 1 ng/mL for 7 days. Binding to the T1R2/T1R3 complex in PANC-1 cells increased in cells exposed to aspartame or glucose, but more so in aspartame-exposed cells. It is noteworthy that T1R1/T1R3 binding was not present in PANC-1 cells in the absence of a sweet taste stimulus, but it did occur at a low level in PANC-1 cells exposed to glucose or aspartame. The T1R2/T1R3-mediated effects of aspartame were transduced to downstream effectors: levels of Ca^{2+}, ROS, and AKT/GSk3β were observed to increase, leading to activation of Notch intracellular domain (NICD) and GLI family zinc finger 1 (GLI1), nuclear translocation of p21, and expression of octameric transcription factor 3/4 (OCT3/4) and c-Myc transcription factors (Gezginci-Oktayoglu et al., 2021).

4.2.8 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

(i) Exposed humans

A few studies were available that investigated the safety of long-term administration of aspartame in exposed humans, mainly in healthy participants or in participants with diabetes. These studies included biomarker measurements, such as routine haematology analysis, that could be relevant to the key characteristic of carcinogens "alters cell proliferation, cell death, or nutrient supply".

The effects of long-term administration of aspartame at a dose of 75 mg/kg in capsules three times per day with meals for 24 weeks

were investigated in a randomized, double-blind placebo-controlled study in volunteers. Approximately 54 participants per group completed the study (EFSA_UN08, 2011; and reported in Leon et al., 1989). Capsules containing aspartame were administered to 23 men and 30 women, and capsules containing the placebo were administered to 28 men and 27 women. The study incorporated several end-points specific to aspartame metabolism (e.g. serum methanol and amino acids), routine haematology analysis (complete blood count), blood chemistry (collected at baseline and every 3 weeks for the first 12 weeks then at weeks 18 and 24; included alkaline phosphatase, alanine and aspartate aminotransferases, LDH, total bilirubin, creatinine, creatinine phosphokinase, and total blood urea nitrogen, BUN), and urine analysis (collected at baseline and at weeks 6, 12, 18, and 24; included 24-hour creatinine). At each time point, the results for each test with aspartame were compared with those with placebo, using a two-tailed Student *t*-test without correction for multiple comparisons. All tests were done at the 5%, two-sided level of significance. The data did not indicate any toxicologically significant clinical findings. [The Working Group noted that alteration in haematopoietic parameters would be relevant as an indicator of cell proliferation; however, no changes in routine clinical tests were observed during the study.] In two additional placebo-controlled double-blind studies, aspartame was administered in capsule form three times per day with meals (EFSA_E60, 2011; EFSA_E65, 2011). In a long-term tolerability study in healthy adults with normal phenylalanine metabolism, aspartame was initially administered to 11 men and 19 women for 6 weeks, after which the study duration was extended for an additional 21 weeks and the study population was expanded to include 12 additional men and 5 additional women (EFSA_E60, 2011). A placebo was administered to 7 men and 13 women for the entire 27-week duration of the study. Participants took two capsules containing 300 mg of aspartame (or placebo) three times per day with their normal meals (1.8 g/person per day). Blood was drawn and analysed at baseline and at weeks 6, 12, 18 (original participants), 20 (or 26), and 21 (or 27). Haematological parameters were measured: complete blood count, hepatic function (serum glutamic-oxaloacetic transaminase, (SGOT), alkaline phosphatase, direct and indirect bilirubin) or kidney function (creatinine, uric acid, BUN). At 12, 16, 20, and 21 weeks, serum glucose and insulin were measured after a 4-hour fast and again 30 minutes later after oral challenge with 100 g of glucose. The data were evaluated by comparing the mean values for the groups exposed to aspartame versus placebo using the Student *t*-test and by linear regression for each of the parameters as a function of time. No changes were observed in any of the clinical tests in the group receiving aspartame compared with the group receiving the placebo (EFSA_E60, 2011). [The Working Group noted that the small sample size, particularly of the control group, limited the sensitivity of the study to detect changes.]

In a long-term tolerability study in adults with insulin-dependent diabetes, 25 women and 14 men consumed aspartame capsules for 13 weeks (EFSA_E65, 2011). Some study participants had poor insulin control and abnormal kidney function results at baseline but were not excluded from the study. Blood was drawn at baseline and at the conclusion of the study. Haematological parameters included complete blood count; liver function tests included total bilirubin, alkaline phosphatase, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and LDH; and kidney function was monitored via levels of uric acid, creatinine, and BUN. No changes were reported in the parameters of liver or haematological function. The kidney function test results were mixed since eight aspartame consumers had elevated BUN and creatine levels at entry, and these levels did not change significantly at the end of the

study. BUN levels increased in two participants during the study and decreased in three. The kidney function test results did not change in the other participants receiving aspartame, or in the participants receiving the placebo (EFSA E65, 2011). [The Working Group noted that most of the studies in exposed humans had limitations in study design, including sample size.]

In one study that directly evaluated a relevant end-point in humans, aspartame was administered as a single skin prick or intradermal injection of up to 1 mM in three healthy volunteers. The authors concluded that there was no induration or erythema due to histamine release from either route of administration, attributing erythema observed at the highest dose in two volunteers receiving intradermal injection to irritation. Associated experiments using a mouse mast cell line did not elicit histamine release, as noted in Section 4.2.8(b) (Szucs et al., 1986). [The Working Group considered the study to be of low relevance.]

(ii) Human primary cells

See Table 4.11.

In HUVEC cells exposed to aspartame at 20 or 100 µM for 14 days, stimulation of angiogenesis was observed via microscopic examination of the cultures for tubule formation followed by quantification of absorption of a soluble colourimetric marker dye. The time course of growth factor expression was examined on days 0, 4, 7, and 14 using ELISA (also discussed in Section 4.2.4). VEGF levels increased about fourfold on days 7 and 14 at both concentrations; levels of its soluble receptor also increased on day 7 but fell to baseline levels on day 14 (Alleva et al., 2011). [The Working Group noted that the numbers of replicate plates and experiments were not reported.]

Aspartame-induced angiogenesis was also reported in HUVECs co-cultured with primary human adipose stromal cells. The co-cultured cells were exposed to aspartame at about 0.3–100 µM for 6 days, after which angiogenesis was measured by computational evaluation of tubule length in photomicrographs. There was a significant increase ($P < 0.05$, Student *t*-test) at the lowest concentration and at about 3 µM, but not at two intermediate or four higher concentrations. There were no statistically significant changes in metabolic activity measured using a water-soluble tetrazolium salt (Hautanen et al., 2023). [The Working Group estimated the concentrations from a graph and noted that the statistical test did not correct for multiple comparisons, in contrast to the authors' original version of the Angio Kit model assay (Huttala et al., 2015) in which results were analysed using more appropriate methods: a one-way analysis of variance with post-test application of the Dunnett test, where applicable.]

Angiogenesis in HUVECs was also examined by Yesildal et al. (2015). The cells were exposed to aspartame at 20–100 µM. In this study, a shorter time of exposure (16–18 hours) to aspartame at 20 µM did not induce angiogenesis in HUVECs on the basis of evaluation of the ratio of tubule length to area measured in photomicrographs (Yesildal et al., 2015). Cell viability, as measured by a tetrazolium dye (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; XTT), was not reduced after 24 or 48 hours of exposure. [The Working Group noted that the number of replicates for the tubule formation assay was not provided.] However, in the same study, the authors reported angiogenesis in chicken ova and wound healing in rat skin (the results are described in Section 4.2.8(b)). In chicken ova, the stimulation of angiogenesis was observed with aspartame at doses of 15 and 30 mM for 3 days but not at shorter exposures or at lower concentrations (described in more detail in Section 4.2.8(b)).

In HUVEC cells, exposure to aspartame at 1 mM for 48 hours did not affect angiogenesis, as evaluated after exposure of a single plate to a single concentration and by measuring tubule

Table 4.11 End-points relevant to alterations in cell proliferation, cell death, or nutrient supply in human cells in vitro exposed to aspartame

End-point	Assay	Cell or tissue type	Results[a]	Concentration	Comments	Reference
Angiogenesis	Tubule length measurement	HUVEC	No differences	1 mM, 48 h		Schiano et al. (2020)
Angiogenesis	Tubule length measurement	HUVEC	No differences	20 µM, 16–18 h		Yesildal et al. (2015)
Angiogenesis	Tubule length measurement	HUVEC	↑	20 µM, 14 days		Alleva et al. (2011)
Angiogenesis	Membrane ATPase activity	Erythrocytes from healthy donors	↓	0.14 mM methanol 2.8 mM aspartate 0.14 mM phenylalanine		Schulpis et al. (2006)
Cell migration	Wound healing Migration of cells into a "wound" scratched in the monolayer	HT-29	↓	6 mM		Maghiari et al. (2020)
Cell proliferation	^{3}H-thymidine incorporation	HTR8 SV neo	↓	1 µM	Aspartic acid and phenylalanine had the same antiproliferative effect.	Rodrigues et al. (2022)
Apoptosis	Caspase-3 cleavage Phosphorylation of γH2AX	HepG2	↑	680 µM		Qu et al. (2019)
Apoptosis	Downregulation of mRNA expression of tumour suppressor gene *TP53*, and pro-apoptotic gene *BAX*	HeLa	↑	34–102 µM		Pandurangan et al. (2016b)
Cell proliferation	PCNA; Ki-67	HeLa	↑	34–102 µM		Pandurangan et al. (2016b)

BAX, BCL2 associated X, apoptosis regulator; γH2AX, phosphorylated histone H2AX; h, hour(s); HeLa, human cervical adenocarcinoma cell line; HIC, highest ineffective concentration; HT29, human colon carcinoma cell line; HTR-8 SVneo, SV-40-transformed human extra-villous trophoblast cell line; HUVEC, primary human endothelial umbilical vein cells; LEC, lowest effective concentration; PCNA, proliferating cell nuclear antigen; TP53, tumour protein 53.

[a] ↓, decrease; ↑, increase.

length in photomicrographs. Cell viability, measured by the trypan blue exclusion test, was slightly reduced after exposure to aspartame at 1 mM for 96 hours, but not at intermediate time points. Also, exposure to aspartame at 100 μM did not induce cytotoxicity, measured as LDH leakage. Moreover, aspartame did not alter the cell cycle (as measured by cell cycle distribution and blockage in G0/G1 phase) or apoptosis (as measured by annexin-V/propidium iodide staining, compared with controls) (Schiano et al., 2020).

Inhibition of membrane ATPase activity was noted when erythrocytes were isolated, lysed from healthy donors, and the membrane fraction incubated with aspartic acid, phenylalanine, or methanol. An additive effect was observed in mixtures of the three aspartame metabolites at concentrations modelled on literature reports of maximum concentrations (C_{max}) in the plasma of volunteers ingesting aspartame at doses of 34 mg/kg bw and above (Schulpis et al., 2006).

(iii) Human cell lines

See Table 4.11.

Exposure of HT-29 cells (a human colon carcinoma cell line) to aspartame at up to 50 mM had little impact on overall cell viability, measured as oxidation of alamar blue after 72 hours. After creation of a "wound" by scratching a gap in the cell monolayer, a significant reduction in the ability of cells to migrate into the gap was observed with aspartame at non-cytotoxic concentrations of up to 3 mM, but not at 6 mM (Maghiari et al., 2020).

Rodrigues et al. (2022) studied cell proliferative capability in an SV40-transformed human extra-villous trophoblast cell line (HTR-8 SVneo) exposed to aspartame. After exposure to aspartame at concentrations of 1–1000 μM for 24 hours, metabolic activity (as measured by MTT) was reduced to about 85% of the control value at each of the five concentrations tested; however, there was no increase in LDH release into the culture medium or reduction in total cell protein content (as measured by sulforhodamine B staining). Aspartame (500 and 1000 μM) significantly decreased the uptake of glucose ([^{3}H]deoxy-D-glucose, ^{3}H-DG), as well as [^{3}H]thymidine incorporation, as observed after exposure to aspartame at increasing concentrations (1–1000 μM). In addition, aspartame at 500 μM did not affect cell migration (measured by wound-healing assay) or alter the level of lipid peroxidation (LPO/TBARS). Cell cycle analysis indicated an accumulation of cells in S-phase, without any indication of apoptosis, as measured using the TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labelling). The aspartame metabolites aspartic acid and methanol, but not phenylalanine, induced reduction of metabolic activity of about 25–30%. There were smaller or no changes in LDH release and in cell protein levels, and no effects on cell proliferation after exposure to phenylalanine aspartic acid, and methanol; only the latter metabolite induced oxidative stress and reduced the migration rate, and the number of cells in G2/M.

Qu et al. (2019) evaluated the toxicity of aspartame in HepG2 cells (a human liver carcinoma cell line). Concentrations between 0.68 and 3.4 mM caused a steep, concentration-dependent decline in metabolic activity, measured using the MTT assay. This toxicity was accompanied by an increase in the proportion of cells exhibiting apoptosis, indicated by cleavage of caspase-3 and phosphorylation of γH2AX, and by an increase in intracellular ROS (discussed in Section 4.2.5) (Qu et al., 2019).

Exposure of HeLa cells (a human cervical carcinoma cell line) to aspartame at millimolar concentrations of 10–20 mM for 48 hours induced cytotoxicity, generation of intracellular ROS, DNA strand breakage (comet assay), and apoptosis at the highest concentrations (Pandurangan et al., 2016a). In a follow-up study in the same cell line, the same laboratory evaluated exposure to aspartame at low concentrations of 34, 68, and 102 μM (10, 20, and

30 µg/mL) (Pandurangan et al., 2016b). At these concentrations, aspartame did not induce cytotoxicity but suppressed apoptosis, as described by downregulation of mRNA expression of the tumour suppressor gene *TP53* and the pro-apoptotic gene *BAX*, and upregulation of mRNA expression of the anti-apoptotic gene *BCL2*. For cell proliferation marker Ki-67 and proliferating cell nuclear antigen (PCNA), mRNA and protein expression increased in a dose-dependent manner (Pandurangan et al., 2016b). [The Working Group noted that the results from studies in human cell lines did not indicate alteration of cell proliferation, cell death, or nutrient supply. Discrimination between mechanisms of necrosis, apoptosis, and cytotoxicity is a notable data gap for aspartame.]

(b) Experimental systems

(i) Non-human mammals in vivo

Cell proliferation

Repeated-dose toxicity studies (with durations of 14 days, 28 days, 90 days, 562 days, and up to 2 years) evaluating the effects of aspartame administered orally in the feed or drinking-water in several non-human mammalian species were available to the Working Group. The studies reported on end-points related to altered morphology, cell proliferation, and hyperplasia, which are relevant to the key characteristic "alters cell proliferation, cell death, or nutrient supply".

Sun et al. (2019) investigated the gastrointestinal response in pre-weaned Hu sheep (age 14 days) fed with starter diet supplemented with aspartame at a concentration of 200 mg/kg for 35 days. When compared with the control group that was fed with starter diet alone, exposure to aspartame for 35 days did not induce bodyweight gain but significantly increased small intestine weight/live body weight, jejunum weight/live body weight, and jejunum weight. However, aspartame did not affect small intestine weight (P = 0.208), duodenum weight, duodenum weight/live body weight), ileum weight, or ileum weight/live body weight. These effects were accompanied by an increase in crypt depth and villus height in the jejunum, and there was a similar but not significant trend in the ileum but not in the duodenum. Aspartame significantly increased the plasma concentrations of gastrointestinal hormone glucagon-like peptide-2 (GLP-2); however, there were no significant effects on plasma glucose concentrations. After aspartame exposure, differential upregulation of mRNA expression levels of cyclin D1, cyclin A2, cyclin E1, cyclin-dependent kinases CDK2, CDK4, and CDK6, proglucagon (GCG), glucagon-like peptide-2 receptor (GLP-2R), insulin-like growth factor 1 (IGF-1), and insulin-like growth factor 1 receptor (IGF-1R) were observed in the different regions of the intestine. In the duodenum, there was upregulation of cyclin D1 but no changes in the mRNA expression of GCG, GLP-2R, IGF-1, and IGF-1R. In the jejunum, there was upregulation of mRNA expression of cyclin A2, cyclin D1, CDK6, GCG, GLP-2R, and IGF-1. In the ileum, there was upregulation of mRNA expression of cyclin A2, cyclin D1, CDK4, GCG, and IGF-1. [The Working Group noted that the findings did not directly explain the relationship between the changes in villus height and crypt depth in the small intestine and GLP-2 release; however, in experiments in vitro in epithelial cells isolated from the jejunum tissue of four healthy Hu lambs aged 56 days, GLP-2 indirectly promoted cell proliferation mainly through the IGF-1 pathway.]

In Swiss mice exposed to aspartame (20, 40, 80, and 160 mg/kg bw per day) by gavage for 28 days, alterations in cell morphology were observed in various areas of the hippocampus. Lower doses of aspartame induced significant increases in cell density. At the lower doses of aspartame, the number of neurons that were positive for neuron-specific enolase significantly increased within the dentate gyrus, but at the highest dose (160 mg/kg bw per day)

there was marked neuron loss and reduction in neuron-specific enolase-positive neurons. A parallel increase in several oxidative biomarkers was observed, starting at 40 mg/kg bw per day (Onaolapo et al., 2017b). [The Working Group noted that, despite the differential effects on the hippocampal regions and decrease in antioxidant status, it was suggested that aspartame exerts neurogenesis.] In contrast, in an earlier study (see also Section 4.2.3b), Ashok and Sheeladevi (2014) reported neurodegenerative effects mediated by oxidative stress-induced apoptosis in discrete brain regions of Wistar albino male rats exposed to aspartame at 40 mg/kg bw per day for 90 days under conditions of folate deficiency (pre-treatment with methotrexate). [However, as mentioned in Section 4.2.3(b), the Working Group considered this study to be of low relevance because of the lack of proper grouping including negative control group with aspartame exposure that limited the interpretation of the results.]

In Wistar albino rats, aspartame was administered at doses of 250 and 1000 mg/kg bw per day in the drinking-water for 56 days (8-week study) (Alkafafy et al., 2015). Aspartame increased mRNA expression of the oncogene *ha-Ras* [*HRas*] and decreased mRNA expression of the tumour suppressor gene *p27* [*Cdkn1b*]. Liver sections from rats receiving aspartame showed histological changes, including disorganized hepatic parenchyma, dilatation, and congestion of the central vein and hepatic sinusoids with proliferation of Kupffer cells. Some hepatocytes showed signs of degenerative changes as cellular swelling and vacuolar cytoplasm, and others were necrotic. Some sections showed focal areas of complete degeneration and diffuse and focal intralobular mononuclear cell infiltration. Hyperplasia of the bile duct was also observed. Collagen deposition was more pronounced in the liver, indicating periportal fibrosis (Alkafafy et al., 2015). [The Working Group found several limitations in the histopathological part of the study; therefore, some of the findings were considered to be of limited relevance.]

In a subacute toxicity study, groups of 30 male and 30 female young adult albino rats were exposed to feed containing aspartame at a dose of 0, 5, or 125 mg/kg bw per day for 56 days (8-week study). Findings of bile duct hyperplasia were reported at the lower dose of 5 mg/kg bw in both male and female rats; however, these findings were considered incidental. Similarly, an increase in peribronchial lymphoid and reticular hyperplasia was observed in the lung; however, this was diagnosed as chronic murine pneumonia (EFSA_E20, 2011). [The Working Group noted that the purity of the agent was "assumed to be 100% pure".]

Groups of two male and two female young adult purebred Beagle dogs were exposed orally (by capsule) with aspartame at a dose of 0, 5, or 125 mg/kg bw per day for 56 days (8-week study). No cell proliferation or hyperplasia was observed in various tissues. Slight changes in the adrenal gland, spleen, and mesenteric lymph node, including the presence of a moderate to largely moderate amount of reticuloendothelial tissue in males and females, were observed at the lower and higher dose; however, the changes were similar to those in the controls (EFSA_E21, 2011). [The Working Group considered that the study was limited by the small number of animals. In addition, the purity of the agent was "assumed to be 100% active".]

In a 90-day toxicity study in 36 mature male NMRI mice, aspartame was administered at a dose of 0, 40, 80, and 160 mg/kg bw per day by gavage. Aspartame reduced sperm count and spermiogenesis in a dose-dependent manner, and induced reduction of seminiferous tubules and damage to Leydig cells in the testis. These effects were highly significant at the two higher doses, at which cytotoxicity and apoptosis were also observed (Anbara et al., 2021).

In rats, oral administration of aspartame at a dose of 40 mg/kg bw per day for 90 days did not cause alterations in levels of mRNA transcripts or protein for Bcl-2 and Bax in the spleen, thymus, and lymph nodes (Choudhary and Sheela Devi, 2016).

In a 2-year carcinogenicity study in groups of 100–150 male and 100–150 female Sprague-Dawley rats (age 8 weeks), hyperplasia was observed in the renal pelvis and olfactory epithelium in females Sprague-Dawley rats after administration of feed containing aspartame at increasing concentrations up to 100 000 ppm until natural death (Soffritti et al., 2006; reviewed in Section 3.2).

In male and female Slc Wistar rats exposed to feed containing aspartame at a dose of 1000, 2000, or 4000 mg/kg bw per day for 104 weeks, an increased incidence of transitional cell hyperplasia (synonymous with the more recent term, urothelial cell hyperplasia) of the renal pelvis was observed in males at the highest dose (Ishii, 1981). [The Working Group noted that the study was re-analysed by Shibui et al. (2019), who reported that renal pelvis mineralization was increased in males in a dose-dependent manner (with the incidence being significantly increased at 2000 and 4000 mg/kg bw per day), and in females (with the incidence being significantly increased at the highest dose) (see more details in Section 3.1); thus the Working Group considered that the observed transitional cell hyperplasia was likely to be a response to mechanical irritation induced by the mineral deposition with no evidence of cellular atypia.]

In a study in groups of 20–30 male Fischer 344 rats (age 6 weeks), effects on tumour promotion were determined after administration of 0.05% or 0.01% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in the drinking-water for 4 weeks and feed containing 5% aspartame for 32 weeks. Aspartame did not induce promoting effects, measured as preneoplastic lesions (papillary or nodular hyperplasia). At necropsy, there was no evidence of increased proliferative urothelial lesions of the urinary bladder that could be attributed to aspartame exposure (Ito et al., 1984).

In a long-term study (reviewed in detail in Section 3.2) in Charles River albino rats exposed to feed containing aspartame at a dose of 0, 1000, 2000, 4000, or 8000 mg/kg bw per day for 104 weeks, increased nodular hyperplasia in the pancreas (a non-neoplastic proliferative lesion) was reported in female rats – but the increases in male rats were unremarkable. There were increases in the incidence of renal pelvis epithelial hyperplasia in males – but the increases in female rats were unremarkable (EFSA E33, 2011; EFSA E34, 2011). [The Working Group considered that the study was limited by the lack of information on the purity of the agent, the low survival rate, and an infection (murine pneumonia) observed in controls and exposed animals.]

In three different cancer models in genetically modified mice (reviewed in Section 3.2) (NTP, 2005), male and female Tg.AC, *p53*-haploinsufficient, and *Cdkn2a*-deficient mice were exposed to feed containing aspartame (purity, > 98%) at doses ranging from 0 to 50 000 ppm (approximately 0–7660 or to 7400 mg/kg bw in males; 0–8180 or to 9560 mg/kg bw in females, in the respective deficient mouse strains). In the *p53*-haploinsufficient B6.129-*Trp53*^{tm1Brd} (N5) female mice, histopathology reported hyperplasia in the adrenal cortex, endometrium, and mild haematopoietic cell proliferation although not related to aspartame exposure. No other non-neoplastic lesions were observed. In male mice of the same strain, no alteration in any cell growth/proliferation-associated end-points was observed. In B6.129-*Cdkn2a*^{tm1Rdp} (N2) deficient mice, minimal to mild cytoplastic vacuolization of periportal hepatocytes was found in control and exposed male mice, with an increased incidence in groups at 6250 ppm, 25 000 ppm, and 50 000 ppm. In the FVB/N-TgN(v-Ha-*ras*)Led (Tg.AC) hemizygous male and female mice,

histopathology did not show cell proliferation or hyperplasia in any of the tissues evaluated. [The Working Group noted that the authors stated that this study was done in a new test system and there was uncertainty as to whether it possessed sufficient sensitivity; therefore, the Working Group concluded that these findings were of limited informativeness.]

In a long-term study in groups of five male and five female Beagle dogs (age 150–160 days), randomized and housed individually, aspartame was administered at a dose of 0, 1, 2, or 4 g/kg bw per day in the diet for 106 weeks. Microscopic observation of the brain showed ependymal proliferation (focal) and glial cell proliferation (minimal, focal subependymal) (EFSA E28, 2011; EFSA E86, 2011). [The Working Group noted that the study had some limitations since the purity of the aspartame was not clearly reported; it was reported that from week 20 until the end of the 106-week feeding period, the test material contained 0.1–1.0% DKP (by weight). In addition, the stability and homogeneity of aspartame in the diet was not determined.]

Gombos et al. (2007) examined the effects of aspartame consumption on the expression of key oncogenes and a tumour suppressor gene. Female CBA/CA mice were exposed to aspartame at a dose of 40, 200, and 2500 mg/kg by gavage twice daily for 1 week, and the expression of *Tp53*, *Myc*, and *Hras* genes was assessed in the bone marrow, liver, spleen, thymus, lymph nodes, lung, and kidney. An increase in the expression of all the investigated genes was observed, especially in organs with a high proliferation rate, that is, the lymphoreticular organs, including bone marrow, and the kidney. [The Working Group noted that the study was limited by the lack of statistical analysis on pooled tissue samples. No dose–response relationship was observed in the gene comparisons.]

Dooley et al. (2017) investigated the effects of lifelong exposure to aspartame on the development of spontaneous pancreatic acinar carcinoma in C57BL/6 Ela1-Tag mice. These mice express the SV40 large T antigen (TAg) under the control of the elastase-1 acinar cell promoter, driving spontaneous formation of pancreatic cancers of acinar origin. Aspartame was administered at 0.035% w/v in the drinking-water, starting from birth. No change in the growth rate of pancreatic acinar carcinomas, as measured by histopathology, was observed in aspartame-exposed mice compared with the controls.

Angiogenesis

Shalaby et al. (2019) observed histopathological changes in the placenta (excised on day 19 of gestation) of pregnant female albino rats exposed to aspartame at a dose of 14 mg/kg bw by oral gavage for three consecutive days (days 9, 10, and 11) during gestation. Histopathology and morphometric analysis showed a significant decrease in placenta weight and mean thickness of the labyrinth and basal zones, with areas of degeneration and vacuolization in exposed animals compared with non-exposed controls. In addition, placenta sections from aspartame-exposed rats, compared with the non-exposed controls, showed extended areas in which there was a significant increase in the expression of VEGF) (as measured by immunostaining) in trophoblasts in the labyrinth and in the cytoplasm of spongiotrophoblasts and giant cell basal zones, indicating angiogenesis.

(ii) Non-human mammalian cells ex vivo and in vitro

Yesildal et al. (2015) investigated the effects of aspartame on angiogenesis in various test models (see also Section 4.2.8(a)). In a model of skin wound healing in rats, full thickness round portions of skin (diameter, 5 mm) excised from the dorsal area of male Sprague-Dawley rats (age 8 weeks) were exposed to 50 µL of a 50 mM solution of aspartame in phosphate-buffered saline daily for 7 days. Histopathological examination was performed on the treated fixed

specimens. The wound-healing process was scored according to the following parameters: wound surface area, re-epithelization, granulation tissue formation, collagen deposition, presence of inflammatory cells, and angiogenesis. The aspartame-exposed skin showed enhanced cellularity and increased angiogenesis when compared with non-exposed skin. [The Working Group noted that, despite quantification of the wound-healing surface area, the effects on cellularity and angiogenesis were qualitative.] In the same study, angiogenesis was also explored in a chick chorioallantoic membrane (CAM) model using 6-day Atak-S fertilized chicken eggs. A dose-dependent increase in the vascular area of the CAM was observed on day 7 after a single exposure to 50 µL of a solution containing aspartame at concentrations of 6, 15, 30, and 60 mM. The effect, measured using a computer-aided stereomicroscope, was significant at the highest concentration (60 mM).

Szucs et al. (1986) observed a significant increase in cell proliferation rate in the growth factor-dependent mouse PT18 mast cell line after exposure to medium containing aspartame at 2×10^{-3} M for 9 days. Cells were fed by replacing 50% of the supernatant medium with fresh aspartame-containing medium on day 4 or 5. The increase in proliferation rate started as earlier as day 5. To determine whether the increased proliferation rate was related to the additional nutrients provided by the component amino acids of aspartame, the authors performed experiments enriching the controls with free amino acids at concentrations that would be expected after aspartame exposure. The proliferation rate was higher in cells provided with free amino acids than in cells exposed to aspartame. [The Working Group noted that the authors indicated that the observed effect was not related to aspartame itself but to an increase in nutrients in the cell culture.]

Horio et al. (2014) investigated the effects of aspartame in rat adrenal pheochromocytoma (PC12) cells. Cells were incubated with medium containing aspartame at 0–8 µg/L for 72 hours. Cell toxicity was measured by trypan blue exclusion and LDH, and apoptosis was evaluated by DNA fragmentation, changes in apoptotic factors as cytochrome c in the cytosol, and apoptosis-inducing factor (AIF) by western blot. Caspase-8 and caspase-9 mRNA was detected using reverse transcription-polymerase chain reaction (RT-PCR). Cell viability decreased in a concentration-dependent manner, and a significant decrease was observed at concentrations > 1 µg/mL when trypan blue was used, but the relative activity of LDH increased significantly even at the lowest concentration tested (0.001 µg/mL). DNA fragmentation was observed and visualized by electrophoresis as fragments of DNA of 180 to 200 base-pairs that increased in a concentration-dependent manner. Fragmentation was quantified by the TUNEL method and confirmed the results obtained in the electrophoresis, indicating induction of apoptosis. Caspase-8 mRNA increased significantly at aspartame concentrations of > 1 µg/mL, and caspase-9 mRNA started to increase significantly at > 0.01 µg/mL. Cytochrome c and AIF also increased in the cytosol, starting at 0.001 µg/mL, but the increased level of AIF in the exposed cells was not significantly different from that in the controls.

4.2.9 Data relevant to multiple key characteristics

See Table 4.12.

(a) Results from omics

Choi et al. (2017) examined the long-term effects of sugar-sweetened beverages on social aggression in mice. Weaned mice (age 3 weeks) were exposed to plain water (control), a solution of sucrose 30% w/v, or a solution of aspartame

Table 4.12 Microarray and omics results for multiple key characteristics in experimental systems in vivo or in vitro after exposure to aspartame

End-point	Normalization and statistics	Curation	Results	Relevant KCs[a]	Tissue, cell type or cell line	Exposure concentration or range and duration	Comments	Reference
Transcriptomics Illumina MouseWG-6 v2 Expression BeadChip arrays containing > 45 200 transcripts	Background correction was performed, the data were log-transformed to log2 scale and normalized by quantile normalization method implemented in the Genome Studio software (Illumina Inc.). Significant differences among three groups were identified using ANOVA test ($P < 0.05$) on log2-transformed normalized intensities by Partek Genomics Suite software version 6.3 (Partek, Missouri, USA). Transcripts with more than a twofold differential were selected for each specific comparison analysed. Used IPA for pathway analysis.	None noted	The aspartame solution changed the expression of genes in categories related to development and growth, such as cellular function and maintenance, nervous system development and function, cellular development, and cellular growth and proliferation	KC10	Hypothalamus	Male C57BL/6J mice (age 3.5 wk). Treatment groups were composed of 30 w/v% sucrose solution, aspartame solution with equivalent sweetness of 30 w/v% sucrose solution or plain water ad libitum for 8 wk. Mice were killed on day 56.	Aspartame was used as a sweetness control	Choi et al. (2017)

Table 4.12 (continued)

End-point	Normalization and statistics	Curation	Results	Relevant KCs[a]	Tissue, cell type or cell line	Exposure concentration or range and duration	Comments	Reference
Transcriptomics Affymetrix Mouse Gene 1.0 ST arrays containing 28 853 genes	Analysis performed with Partek Genomics Suite software version 6.15. Probe set data summarized and robust multiarray average algorithm used for background correction as implemented in the microarray analysis software. Genes that were regulated in response to the treatments were identified using the false discovery rate method, in which *P* values were adjusted simultaneously across multiple subgroup comparisons. IPA software used for pathway analysis.	GSE100325; GSE100324	Identified 189 aspartame-responsive differentially expressed genes (DEGs) in the adult male hypothalamus and 2188 in the adrenals. In the hypothalamus, alteration in several pathways including mineral corticoid and glucocorticoid biosynthesis, triacylglycerol degradation, retinol biosynthesis, FXR/RXR activation and many others. In the adrenal gland, there were changes in glutamate receptor signalling, FXR/RXR activation, axonal guidance signalling, GABA receptor signalling and many others. Elevated the expression of genes involved in hypothalamic neuro-steroidogenesis, together with cell stress and inflammatory genes	KC8	Hypothalamus and adrenals	Mice were exposed in utero and through weaning to aspartame (0.25 g/L); a group of these mice were administered NMDAR competitive antagonist CGP 39551 in utero only. Control mice were not given aspartame or CGP 39551.	No information about what the control mice were exposed to; unconventional pooling of the samples before microarray analysis which resulted in technical replicates and not biological replicates.	Collison et al. (2018)

Table 4.12 (continued)

End-point	Normalization and statistics	Curation	Results	Relevant KCs[a]	Tissue, cell type or cell line	Exposure concentration or range and duration	Comments	Reference
Transcriptomics Affymetrix Mouse Gene 1.0 ST arrays containing 28 853 genes	Microarray analysis was performed using Partek Genomics Suite software version 6.3 (Partek, Missouri, USA). Probe set data were summarized and background adjusted using the GC-Robust MultiArray (GCRMA) algorithm. All data normalized using nonlinear transformation quantile normalization. Used a one-way ANOVA to test for diet interactions without correction for multiple testing. IPA was used for detection of gene ontology and canonical pathway analysis.	GSE38444; GSE38445	Microarray analysis of liver and adipose tissue of mice treated with aspartame and *trans* fat-enriched diet showed a pattern of gene expression with the significant upregulation of genes involved in the organization of the cytoplasm, morphology of blood vessels, and angiogenesis, and the downregulation of genes with ontologies relating to hepatic protein metabolism, organization of peroxisomes and expression of RNA	KC10	Liver, adipose tissue	The four diet regimens used in this study were: (1) TFA control diet: consisting of 20% (w/w) partially hydrogenated vegetable shortening; (2) MSG + TFA diet: TFA diet with ad libitum drinking-water containing 0.75 g/L of MSG-hydrate; (3) aspartame + TFA diet: TFA diet with ad libutum drinking-water containing 0.25 g/L Asp-Phe methyl ester; (4) aspartame + MSG + TFA diet: TFA diet with ad libitum drinking-water containing 0.25 g/L aspartame and 0.75 g/L MSG. The four groups of breeder dams were maintained on their respective diets throughout gestation and lactation. The offspring used in the experiments were weaned onto the same maternal dietary regimen at age 4 wk and maintained on their respective diets for the duration of the study.	Analysis performed under specific dietary conditions. Gene ontology analysis not focused on canonical pathways or upstream regulators.	Collison et al. (2013)

ANOVA, analysis of variance; FXR/RXR, farnesoid X receptor/retinoid X receptor; GABA, gamma-aminobutyric acid; IPA, Ingenuity Pathway Analysis; KC, key characteristic of carcinogens; MSG, monosodium glutamate; NMDAR, *N*-methyl-D-aspartate receptor; TFA, *trans* fat diet; wk, week(s).

[a] KC10, "alters cell proliferation, cell death, or nutrient supply"; KC8, "modulates receptor-mediated effects".

with sweetness equivalent to the sucrose solution, until they were adults (age 11 weeks). Sucrose solution, but not aspartame solution, promoted social aggression, which was accompanied by increased serum corticosterone levels and reduced body weight. Transcriptome analyses of the hypothalamus indicated that the profiles for mice exposed to sucrose solution were dramatically different from those for mice in the control group or exposed to aspartame. Aspartame dysregulated genes involved in the development and functioning of the nervous system. Aspartame did not mimic the effects of sugar on social aggression and inflammatory responses. [The Working Group noted that aspartame was used as a sweetness control; the dose of aspartame administered was not provided.]

Collison et al. (2018) examined the effects of aspartame on the hypothalamic–pituitary–adrenal axis in the presence or absence of CGP 39551, a competitive antagonist of developmental NMDAR. This receptor was shown previously to modulate the effects of aspartame on several metabolic parameters (Collison et al., 2016). Mice were exposed in utero and through weaning to aspartame (0.25 g/L); a group of these mice were exposed to CGP 39551 in utero only. Control mice were not exposed to aspartame or CGP 39 551. The authors identified 189 aspartame-responsive differentially expressed genes (DEGs) in the adult male hypothalamus and 2188 in the adrenals. Aspartame exposure elevated the expression of a network of genes involved in hypothalamic neurosteroidogenesis, as well as genes involved in cellular stress and inflammation. The changes in these genes were not seen in aspartame-exposed mice that had received the antagonist CGP 39551. In the adrenal glands of aspartame-exposed mice, gamma-aminobutyric acid (GABA) and glutamate receptor subunit genes were among those that were most highly upregulated. In summary, aspartame exposure increased the expression of functional networks of genes involved in hypothalamic neurosteroidogenesis and adrenal catecholamine synthesis; these patterns of expression were not present in aspartame-exposed mice with developmental NMDAR antagonism. [The Working Group could not determine what the control mice were exposed to. Unconventional pooling of samples was carried out. The authors stated that "to minimize the differences of individual variability and increase the statistical power for the identification of potential biomarkers, microarray analysis was performed using equal amounts of purified RNA pooled from all of the study subjects (n = 18 per treatment group) and applied in triplicate". Thus, the authors examined technical replicates, not biological replicates.]

Collison et al. (2013) investigated the effects of changes in hepatic and adipose tissue gene expression induced by the food additives aspartame or monosodium glutamate (MSG), or a combination of both, in C57Bl/6 J mice fed a *trans* fat-enriched diet. Microarray analysis of liver and adipose tissue of mice exposed to aspartame and the *trans* fat-enriched diet showed significant upregulation of genes involved in the organization of the cytoplasm, morphology of blood vessels, and angiogenesis, and downregulation of genes with ontologies relating to hepatic protein metabolism, organization of peroxisomes, and expression of RNA. Aspartame-induced genes relating to cell proliferation and the development of blood vessels were upregulated in the adipose tissue of mice fed a *trans* fat-enriched diet, whereas downregulated ontologies included adipogenesis and the proliferation of immune cells. [The Working Group considered this study less informative, since the effects of aspartame were observed under specific dietary conditions. In addition, the gene ontology (GO) analysis did not focus on canonical pathways of upstream regulators; a global view of the impact of exposure on such pathways was not provided.]

(b) Evaluation of high-throughput in vitro toxicity screening data

The analysis of the in vitro bioactivity of the agents reviewed in *IARC Monographs* Volume 134 was informed by data from high-throughput screening assays generated by the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes of the government of the USA (Thomas et al., 2018). Aspartame was one of thousands of chemicals tested across the large assay battery of the Tox21 and ToxCast research programmes. Detailed information about the chemicals tested, assays used, and associated procedures for data analysis is publicly available (US EPA, 2023).

The ToxCast/Tox21 high-throughput screening results are presented according to the assays that have been mapped to the key characteristics of carcinogens (Reisfeld et al., 2022). The detailed results are available in the supplementary material for this volume (Annex 4, Supplementary material for Section 4, Mechanistic Evidence, online only, available from: https://publications.iarc.who.int/627). Here, for brevity, assays for which there was a positive "hit call" are referred to as "active" assays. A summary of these results is given below as the number of active assays (without any caution flags) divided by the total number of key characteristic-related assays for the chemical.

Among the 192 assays in which aspartame was tested, it was found to be active and without caution flags in only one assay relevant to the key characteristics of carcinogens, that is, one of the 95 assays mapped for key characteristic 8, "modulates receptor-mediated effects" (Reisfeld et al., 2022). This assay, ATG_ERE_CIS_up, is a cell-based, multiplexed-readout assay in HepG2 (a human liver cell line) with measurements taken 24 hours after chemical dosing in a 24-well plate. The assay measures the ability of estrogen receptor to bind to the estrogen receptor binding element (ERE) and activate a reporter gene; the AC_{50} (50% of maximum activity) for aspartame was 39.46 μM. The assay is also one of 17 assays that measure the ability of a chemical to perturb the estrogen receptor pathway (Reisfeld et al., 2022) and have been used to assess estrogen receptor agonism or antagonism described in previous studies (e.g. Judson et al., 2015). [The Working Group noted that the fact that aspartame gave positive results in only one of the 17 assays and the relatively high AC_{50} compared with 17β-estradiol itself makes it unlikely that the compound has any estrogen receptor activity.]

4.3 Other relevant evidence

4.3.1 Alterations in glucose and lipid metabolism

Hyperinsulinaemia (a hallmark of insulin resistance) and dyslipidaemia can play relevant roles in carcinogenesis (Tumminia et al., 2019; Neshat et al., 2022; Rojas et al., 2023). In the past two decades, an increasing number of studies (either cross-sectional or intervention studies) have investigated the effects of various non-nutritive sweeteners, including aspartame (single or repeated dosing), on glucose or lipid metabolism under various conditions, i.e. in participants who were healthy, obese, diabetic, or pregnant, and before, after, or during exercise. Numerous investigations have also been conducted in experimental systems, especially in vivo. The findings are reported in the present section, and several end-points are also relevant to the 10 key characteristics of carcinogens. Although many of the studies in humans reported conflicting results, there was consistent evidence that aspartame increased serum insulin levels in lambs and in several studies in rodents, indicating alterations in insulin sensitivity, including changes to lipid metabolism.

(a) Humans

See Table 4.13.

(i) Cross-sectional studies

Liu et al. (2022b) investigated associations between maternal serum aspartame (and sucralose) levels and metabolic health during pregnancy in a nested population-based case–control study. The study recruited 632 women in early pregnancy (weeks 9–14 of gestation) and undertook detailed biochemical and metabolic assessments at 24–32 weeks (as close to 28 weeks as possible). The 109 women diagnosed with gestational diabetes mellitus at this visit were compared with 109 controls, matched on age and self-reported pre-pregnancy BMI. Serum levels of aspartame and sucralose measured at the same visit were quantified using "ultra-performance liquid chromatography coupled to a tandem mass spectrometer", a method previously used for urine specimens. [The Working Group noted that this was the only study to detect aspartame in the blood. Although advances in the sensitivity of analytical methods may explain the detection of aspartame in this recent study compared with previous studies, concerns about the study methodology limited the informativeness of this study (see Section 1.3).] Other parameters, including those used for the diagnosis of gestational diabetes mellitus, were measured by standard methods. There were no significant differences between cases with gestational diabetes and matched controls with regard to serum levels of aspartame or sucralose. Further analyses of the data from all participants combined examined relations between levels of each of the two non-nutritive sweeteners (both as categorical and continuous variables) and the various metabolic and biochemical markers measured at 28 weeks. The higher serum levels of aspartame levels were positively associated with elevated haemoglobin A1C (HbA1c), insulin resistance, hypercholesterolaemia, and hyper-low-density lipoprotein (LDL) cholesterolaemia. These associations were identified in separate analyses of the cases with gestational diabetes mellitus and the controls.

Hess et al. (2018) reported a cross-sectional study of non-nutritive sweetener consumption and metabolic syndrome in 123 adults. Participants completed three 24-hour dietary recall questionnaires over a 2-week period and had a fasting blood draw for glucose and lipids analyses at the time of the second dietary recall. The dietary data were used to generate estimates of the amount of specific non-nutritive sweeteners consumed and whether specified levels of consumptions had been met. On the basis of the information collected, 18 participants met the criteria for metabolic syndrome, and 63 participants were categorized as consumers of non-nutritive sweeteners, including 33 who were consumers of aspartame. Data were adjusted for age, sex, total caloric intake, dietary quality, physical activity, and non-nutritive sweetener consumption, and a significant positive association was observed between aspartame consumption and levels of blood glucose and triglycerides, but not waist circumference. The latter parameter was, however, statistically significantly associated with consumption of the other non-nutritive sweeteners considered. [The Working Group noted that the exposure assessment had some limitations (see Table 4.13).]

Kuk and Brown (2016) reported analyses of data from a subset of 2856 participants aged 40–74 years in the Third National Health and Nutrition Examination Survey (NHANES III) who had, in addition to the standard survey measures, undergone an oral glucose tolerance test (OGTT) and were not taking medication for diabetes. A 24-dietary recall questionnaire was used to classify participants as either high- or low-level consumers of specific sugars and as consumers versus non-consumers of specific non-nutritive sweeteners (saccharin and aspartame). Consumers of non-nutritive sweeteners had a higher average BMI than did non-consumers (28 versus 27 for both saccharin and

Table 4.13 Effects of aspartame on glucose and lipid metabolism in exposed humans

End-point	Location, setting, study design, study population	Results	Exposure level and no. of exposed and controls	Comments	Exposure assessment critique	Reference
Cross-sectional studies						
Insulin HbA1c Total cholesterol LDL	Shanghai, China, nested case–control study Women with gestational diabetes mellitus	Positive associations of plasma aspartame with cholesterol (total and LDL) levels, and insulin resistance indices	Serum levels of aspartame quantified in 109 women with gestational diabetes mellitus compared with 109 controls	The study measured aspartame in the blood, but the detection method was not standardized for blood samples (see also Section 1.3).		Liu et al. (2022b)
Fasting glucose TG HDL-C	South-western Virginia, USA, cross-sectional study Adults with metabolic syndrome	Fasting glucose and triglyceride values were positively associated with aspartame consumption	24-hour dietary recall questionnaire 33 consumers of aspartame out of 125 study participants	Linear regression models, adjusted for age, sex, caloric intake, dietary quality, and physical activity, examined associations between total and individual types of NNS with metabolic syndrome indices.	Small sample size Limited description on the source of composition data, aspartame amounts consumed, and use of medicines or supplements.	Hess et al. (2018)
Glucose Insulin	USA, cross-sectional study NHANES III cohort	Aspartame intake significantly influenced the association between BMI and glucose tolerance	24-hour dietary recall questionnaire 2856 participants	Single 24-hour dietary recall questionnaire could miss less frequent use	Limited description of how items with non-nutritive sweeteners were classified as containing aspartame or other sweeteners.	Kuk and Brown (2016)
Insulin GLP-1 Leptin	Canada, randomized, controlled, double-blinded, crossover design Healthy subjects, $n = 17$ (10 women and 7 men)	No changes in insulin, glucose, active GLP-1, and leptin levels between the baseline visit and the visit after the 2-wk washout period	Repeated dose, 425 mg/day for 2 wk Measurement at baseline and after 2 wk	No effect of aspartame on insulin sensitivity in normoglycaemic subjects. The dose used was reflective of common consumption. Sucralose was also tested. Well-conducted study	Quantitative aspartame exposure was assessed. Comprehensive measurement of background diet conducted. Compliance with treatment assessed.	Ahmad et al. (2020)

Table 4.13 (continued)

End-point	Location, setting, study design, study population	Results	Exposure level and no. of exposed and controls	Comments	Exposure assessment critique	Reference
Insulin Leptin GLP-1 GIP	USA, randomized, controlled trial Healthy male subjects (n = 100)	No changes in glucose, insulin, resting leptin, GLP-1, or gastric inhibitory peptide	Repeated dose, 0, 350, or 1050 mg of aspartame in a beverage for 12 wk. Measurement at baseline and after 12 wk (about 30 subjects/dose group).	There were no effects of aspartame ingestion on appetite, body weight, or body composition. Well-conducted study.	No record of intakes from other sources. A screening questionnaire was used to select participants who were low or non-consumers of low-calorie sweeteners.	Higgins et al. (2018)
Insulin GLP-1	Türkiye, single-blinded randomized trial Healthy males (n = 8), and participants with diabetes type 2 (n = 8)	No changes of insulin, C-peptide and GLP-1 in both healthy and subjects with diabetes type 2	72 mg of aspartame 15 min before oral glucose tolerance test Control was non-sweetened water.	Also tested was sucralose, which enhances GLP-1 release in healthy subjects. Well-conducted study.		Temizkan et al. (2015)
Intervention studies						
Insulin Glucagon Cholesterol TG HDL	Japan, intervention trial Healthy participants (n = 7) and untreated diabetic patients (n = 22), (gender not reported)	A small but significant decrease in blood glucose. No changes in insulin or glucagon levels.	Single dose, 500 mg of aspartame. Measurements at baseline and after 3 hours.	Well-conducted study.		Okuno et al. (1986)
	Hospitalized diabetic patients (n = 9) (6 women, 3 men)	No changes in glucose, cholesterol, TG, or HDL levels.	Repeated dose, 125 mg of aspartame, 2 wk, to hospitalized diabetics with steady-state glycaemic control. Measurements at the beginning and end of aspartame administration.			Okuno et al. (1986)

Table 4.13 (continued)

End-point	Location, setting, study design, study population	Results	Exposure level and no. of exposed and controls	Comments	Exposure assessment critique	Reference
Glucose Insulin	USA, interventional trial Healthy participants (n = 7) (5 women and 2 men), and PKU patients (n = 7) (4 women and 3 men)	No changes in glucose and insulin levels	(1) 12 oz of unsweetened cherry-flavoured beverage; (2) 12 oz of cherry-flavoured beverage providing 60 g of carbohydrates; (3) 12 oz of cherry-flavoured beverage providing 200 mg of aspartame; (4) 12 oz of cherry-flavoured beverage providing 200 mg of aspartame and 60 g of carbohydrates.	Each participant (healthy and PKU) was studied on four different days after an overnight fast with different treatments. Well-conducted study.		Wolf-Novak et al. (1990)
Prolactin Growth hormone, cortisol Insulin	USA, intervention trial Healthy subjects, (n = 16) (8 women and 8 men)	No changes in prolactin, growth hormone or cortisol levels. Serum insulin decreased slightly, and serum glucose increased slightly over the course of all test procedures.	Single dose of 0.534 g of aspartame, acute administration. Measurements at baseline and after 150 min	Well-conducted study.		Carlson and Shah (1989)
Glucose Insulin CCK GLP-1 GIP	Surrey, United Kingdom, intervention study, triple crossover design Healthy participants (n = 6) (4 women, 2 men)	GLP-1 concentrations decreased after a liquid meal (60–120 min) after aspartame treatment. No changes in CCK, GIP, insulin, and glucose levels.	Single dose, capsules consisting of either aspartame (400 mg), 176 mg aspartic acid + 224 mg phenylalanine, or 400 mg of corn flour (control) in 450 mL of water			Hall et al. (2003)

Table 4.13 (continued)

End-point	Location, setting, study design, study population	Results	Exposure level and no. of exposed and controls	Comments	Exposure assessment critique	Reference
Insulin	Australia, intervention trial Healthy participants (n = 17) in three different studies: study 1 (4 women, 2 men), study 2 and 3 (7 and 5 men, respectively)	No changes in insulin levels, or in cephalic response	Each subject was studied twice (a food stimulus study and a control study) in the morning after an overnight fast. Aspartame dose, 19 mg.	Well-conducted study.		Bruce et al. (1987)
Glucose Insulin	Japan, randomized crossover design Healthy participants (n = 9) (7 women and 2 men)	No changes in glucose or insulin levels.	Participants rinsed their mouths with either 25 mL of water (control) or a 2.5% *Gymnema sylvestre* extract solution, and then ingested 200 g (50 g × 4) of 0.09% aspartame.	*Gymnema sylvestre* is a plant that selectively suppresses sweet taste sensation in humans.	Quantitative aspartame exposure specifically assessed. No measurement of background or usual diet conducted.	Kashima et al. (2019)
Insulin	USA, intervention trial Healthy participants (n = 15) (all men)	No changes.	Participants sipped and spit solutions of water or aspartame. Measurement at baseline and then at 1 min post-stimulus, followed by every 2 min for 15 min and then every 5 min for 15 min.	The study aimed to describe cephalic response. Well-conducted study.		Teff et al. (1995)
Glucose Insulin	France, intervention trial Healthy participants (n = 7) (3 women and 4 men)	No changes in insulin levels.	Single dose, 250 mg of aspartame dissolved in 250 mL of water. Measurements at baseline and 30, 60, 90, 120, 150, and 180 min after the ingestion of aspartame.	The study was designed to measure calcium and oxalate excretion in the urine, which was not affected by aspartame. Well-conducted study.		Nguyen et al. (1998)

Table 4.13 (continued)

End-point	Location, setting, study design, study population	Results	Exposure level and no. of exposed and controls	Comments	Exposure assessment critique	Reference
Glucose Insulin	Denmark, intervention trial Healthy participants (n = 6) (all men)	Decrease in glucose levels compared with control group (water). No changes in insulin levels.	Single dose of 1.0 g of aspartame dissolved in 200 mL of water, or water alone. Measurements at baseline and after 4 h.	The study showed that the intake of aspartame at the high dose produced a marked and persistent increase in the availability of phenylalanine, which was not observed after protein intake.		Møller (1991)
Glucose Glycated haemoglobin (HbA1c) Insulin TG HDL LDL	USA, randomized controlled trial Overweight or obese adult participants (n = 154; total n = 30) (gender not specified), randomly assigned to aspartame consumption.	No changes	Repeated dose of beverages containing 0.73, 0.58, or 0.66 g of aspartame for 12 wk; 123 subjects completed the study. Measurements at baseline and after 12 wk.	No effect on body composition. Glucose and lipid metabolism were included with body weight and related parameters measurements. Aspartame used as positive control in comparison with other sweeteners.	Quantitative aspartame exposure specifically assessed. Measurement of background diet included.	Higgins and Mattes (2019)
Glucose Insulin Glucagon Cholesterol TG	USA, double-blind randomized intervention trial Overweight participants (age 10–21 yr) (n = 59; total, n = 55) completed the study.	No changes	Repeated dose, 2.7 g of aspartame per day in capsules for 7 wk.			Knopp et al. (1976)
Glucose Insulin	Overweight participants (n = 12) (6 men and 6 women), and normal weight participants (n = 12) (6 men and 6 women)	No changes	Single dose, beverage containing 0.25 g of aspartame in 500 mL of deionized distilled water. Blood samples were taken at 10, 18, 25, 33, 40, and 48 mm after the start of ingestion preload			Rodin (1990)

Table 4.13 (continued)

End-point	Location, setting, study design, study population	Results	Exposure level and no. of exposed and controls	Comments	Exposure assessment critique	Reference
Insulin Glucagon	USA, intervention trial Healthy participants (n = 12) (all women), and patients with type 2 diabetes (n = 10) (5 women and 5 men)	No changes in peak insulin and glucagon levels, and glucose in subjects with or without diabetes. AUC for insulin was significantly higher after aspartame ingestion in normal participants, but not in participants with type 2 diabetes.	Single dose of an unsweetened beverage, or a sweetened beverage with 400 mg of aspartame, or a sweetened beverage with 135 mg of saccharin. Measurements at baseline and 15, 30, 45, 60, 75, 90, 120, 150, and 180 min after consuming the beverage.	Glucagon levels showed time-to-time variation but no overall differences, but the magnitude of the difference was small. The amount of sweetener approximated to that in 1 L of sugar-free soft drink. Well-conducted study.		Horwitz et al. (1988)
Insulin Glucagon	USA, open-label crossover study Healthy subjects, n = 12 (all females), and type 2 diabetes patients, n = 10 (5 women and 5 men)	AUC for insulin was significantly higher after aspartame ingestion in normal subjects, but not in subjects with type 2 diabetes.	Single dose 400 mg aspartame. Measurements at baseline and after 3 hours.	Part of the data was published in Horwitz et al. (1988), who prepared a regulatory study report.		EFSA_UN01 (2011)
Glucose	Chicago, USA, randomized controlled trial Diabetic patients, n = 62 (all with both types of diabetes)	No changes	Repeated dose, 2.7 g of aspartame per day for 18 days or placebo.			Nehrling et al. (1985)
Glucose Insulin Cholesterol HDL TG	Australia, double-blind crossover study Type 2 diabetes patients, taking medications (n = 9) (1 woman and 8 men)	No changes	Repeated dose, 162 mg of aspartame for 6 wk.	Well-conducted study. Compared the effects of adding sucrose and aspartame to the usual diet of participants with type 2 diabetes.		Colagiuri et al. (1989)

Table 4.13 (continued)

End-point	Location, setting, study design, study population	Results	Exposure level and no. of exposed and controls	Comments	Exposure assessment critique	Reference
Glucose Insulin	Japan, two groups: (1) type 2 diabetes patients, *n* = 15	No changes	75 g oral glucose tolerance test and 5 days after the test, a 225 mg oral aspartame loading test. Plasma glucose and insulin levels were measured at 0, 30, 60, 90, 120, and 180 min.	Patients were receiving different therapies for diabetes.		Shigeta et al. (1985)
	(2) type 2 diabetes patients, *n* = 20	No changes	Consumed jellies sweetened with 240 mg of aspartame. The fasting plasma glucose was measured in the morning before and after aspartame loading.			
De novo lipogenesis-derived fatty acids Stearoyl-CoA desaturase activity Phospholipids Cholesteryl esters TG	Denmark, Copenhagen, randomized parallel study Healthy subjects, *n* = 41 (11 out of 41 consuming aspartame)	No changes	Consumption of aspartame-sweetened soda for 24 wk.	The study compared the effects of sugar-sweetened soda, semi-skimmed milk, aspartame-sweetened soda, or water.		Bajahzer et al. (2022)
Glucose Insulin	Australia Healthy subjects, *n* = 9 (all men)	No changes	Dosing: (1) carbohydrate 2% maltodextrin and 5% sucrose; (2) 0.04% aspartame, 2% maltodextrin and 5% sucrose; (3) water; and (4) aspartame (0.04% aspartame with 2% maltodextrin).	Well-conducted study. Each participant completed four trials under the same conditions (45 min rest + 60 min self-paced intense exercise) differing only in their fluid intake.		Siegler et al. (2012)

AUC, area under the curve; BMI, body mass index; CCK, cholecystokinin; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; min, minute(s); NHANES, National Health and Nutrition Examination Survey; NNS, non-nutritive sweetener; PKU, phenylketonuria; TG, triglycerides; wk, week(s).

aspartame). There were no significant differences in markers of glucose tolerance and insulin resistance according to level of consumption of either sugars or non-nutritive sweeteners. In participants classified as aspartame consumers, the association between BMI and the 2-hour glucose test was significantly higher than among non-consumers. There was no similar interaction for higher levels of sucrose, fructose, or saccharin. [The Working Group noted that a limitation of this study was that a single 24-dietary recall questionnaire could fail to detect infrequent use. Additionally, it was unclear how non-nutritive sweetener items were classified as containing aspartame or other sweeteners.]

[The Working Group reviewed articles in which aspartame was used as a control to test the effect of other sweeteners and deemed these studies uninformative since the study design precluded any interpretation related to the effects of aspartame (Anton et al., 2010; Maersk et al., 2012; Kimura et al., 2017; Harder et al., 2020; Hieronimus et al., 2020; Sorrentino et al., 2020; Sigala et al., 2021, 2022).]

(ii) Intervention studies

The effects of single or repeated doses of aspartame have been assessed in healthy, obese, and diabetic participants, during pregnancy, and before, after, or during exercise.

No significant effects on glucose metabolism, including insulin levels, were observed in several intervention studies in which aspartame was administered either as a single dose or as repeated doses for up to 12 weeks to healthy participants (Okuno et al., 1986; Bruce et al., 1987; Carlson and Shah, 1989; Wolf-Novak et al., 1990; Møller, 1991; Teff et al., 1995; Nguyen et al., 1998; Hall et al., 2003; Temizkan et al.; 2015; Higgins et al., 2018; Kashima et al., 2019; Ahmad et al., 2020). Similarly, in the few studies conducted in participants who were overweight or obese and were given a single dose of aspartame or repeated doses for up to 12 weeks (Knopp et al., 1976; Rodin, 1990; Higgins and Mattes, 2019), no alterations in blood concentrations of glucose, insulin, leptin, or glucagon were observed. The effect of aspartame in people with type 2 diabetes was assessed in several single-dose studies (Okuno et al., 1986; Horwitz et al., 1988; EFSA_UN01, 2011; Temizkan et al., 2015) and in a few short-term studies (up to 6 weeks) (Nehrling et al., 1985; Shigeta et al., 1985; Colagiuri et al., 1989). In general, no significant changes in plasma glucose, insulin, glucagon, C-peptide, or glucagon-like peptide-1 (GLP-1) were observed. [The Working Group noted that these studies, although well conducted, presented several limitations. The studies were not designed to evaluate the effects of insulin after long-term ingestion of aspartame. Some of these studies measured the cephalic response to food and were therefore not specifically designed to observe effects on insulin. Some studies administered aspartame in capsules that impaired the sweet sensor in the mouth. In addition, the study participants were not clearly screened for past use of artificial sweeteners to determine whether they were non-consumers or light-consumers, or whether they had a history of prolonged use.]

In a randomized parallel study, Bajahzer et al. (2022) did not observe alterations in de novo lipogenesis fatty acids in 11 individuals who consumed aspartame-sweetened soda for 24 weeks. [The Working Group noted that the study aimed to compare the effects of sugar-sweetened soda, semi-skimmed milk, aspartame-sweetened soda, and water, and the change at 24 weeks was assessed and compared across the groups using analysis of variance (ANOVA) and mixed-effects models.]

There were two studies that evaluated glucose metabolism after exposure to aspartame combined with exercise (Ferland et al., 2007; Siegler et al., 2012). Ferland et al. (2007) investigated plasma glucose and insulin levels during an acute bout of exercise in 14 men with type 2 diabetes. In the group that received aspartame, there was a 34%

reduction in glucose and 73% reduction in insulin levels after exercise, in contrast to the group in the fasting state for which there were no changes after exercise. [The Working Group noted that this was a short report and did not report many details on the study design and results.] Siegler et al. (2012) studied the effects of aspartame in nine healthy subjects at rest and during endurance exercise. No differences between pre- and post-exercise levels of insulin and blood glucose were observed after consumption of aspartame. [The Working Group noted that aspartame may affect the release of insulin during exercise, or at least that exercise may interfere with the possible effects of aspartame; however, there are few studies exploring this association.]

In a study by Singleton et al. (1999), 12 male and 10 female healthy adults ingested a milkshake containing 108 g dairy cream alone, or supplemented with 30 g of fructose, 17.5 g of glucose, or 1 g of aspartame. Blood samples were collected at baseline, and 2, 4, 6, and 8 hours after ingestion. All milkshakes resulted in significant elevations of triglycerides at the 2- and 4-hour time points. At 6 hours, only the groups that were given glucose and fructose had significantly higher values of triacylglycerol than at baseline. No significant effects or interactions were noted related to concentrations of insulin, glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, or low-density lipoprotein (LDL) cholesterol.

[The Working Group noted that studies in which aspartame was co-administered with other non-nutritive sweeteners during exercise (Sylvetsky et al., 2016; Kim et al., 2020), or in which the group receiving aspartame was used as a control (Wouassi et al., 1997; Sigala et al., 2020) were reviewed but considered to be uninformative.]

(b) Experimental systems

See Table 4.14.

Several studies that examined the effects of aspartame on glucose metabolism and associated hormones in non-human mammals in vivo (including lambs, rats, and mice) were available to the Working Group.

The studies measured glucose and insulin under several conditions. Glucose levels were measured using standard techniques, such as fasting blood glucose levels, blood glucose levels after a bolus of glucose, by using the glucose tolerance test (GTT), or blood glucose levels after an insulin tolerance test (ITT) (designed to determine whole-body sensitivity of insulin receptors by measuring blood glucose level changes before and after insulin administration). In some studies, the homeostatic assessment insulin resistance (HOMA-IR) computational model was used, measuring glucose and insulin after aspartame exposure. Several observed end-points also linked to the key characteristics of carcinogens "induces oxidative stress", "induces chronic inflammation", and "alters cell proliferation, cell death, or nutrient supply".

In a study in lambs, Sun et al. (2019) observed that, as well as histological alterations in the small intestine, aspartame exposure caused a significant increase in plasma concentrations of gastrointestinal hormone glucagon-like peptide-2 (GLP-2) and induced upregulation of GCG, GLP-2R, IGF-1, and IGF-1R mRNA expression levels in the different intestine regions. No effects on plasma glucose levels were observed (see also Section 4.2.8(b)).

Increases in blood levels of insulin or glucose after the administration of aspartame were observed in several studies in rats (Palmnäs et al., 2014; Janssens et al., 2017, Lebda et al., 2017b; Ryuk et al., 2019; Mbambo et al., 2020). When administered in the drinking-water for approximately 7 weeks, aspartame increased fasting insulin levels without changes in lipid

Table 4.14 Effects of aspartame on glucose and lipid metabolism in experimental systems

End-point	Species, strain (sex)	Tissue	Results	Route, duration, dosing regimen, dose	Comments	Reference
Glucose GLP-2	Sheep, Hu lambs	Blood	No change in glucose Increase in GLP-2	Starter diet by itself or supplemented with aspartame at 200 mg/kg for 35 days ($n = 6$ per group).	Aspartame supplementation in starter diet accelerates small intestinal cell cycle (see Section 4.2.9).	Sun et al. (2019)
Glucose Insulin Hepatic dietary lipid uptake De novo lipogenesis	Rat, Wistar (M)	Blood	Increase in fasting insulin. No changes in glucose or in lipid metabolism	There were four treatment groups: control group: normal drinking-water; glucose group: 13% (w/v) glucose; fructose group: 13% (w/v) fructose; and aspartame group: 0.4% (w/v) aspartame in drinking-water for 7 wk.	Fructose stimulates hepatic de novo lipogenesis.	Janssens et al. (2017)
Glucose	Rat, Sprague-Dawley	Blood	Increase in glucose after 60 and 120 min of glucose ingestion	Rats had access ad libitum to 10% sucrose solution, while rats belonging to the aspartame, sucralose, cyclamate, saccharin, and stevia groups had ad libitum access ad libitum to their respective solutions (dissolved in drinking-water) at concentrations equivalent to the sweetness of 10% sucrose. Treatment continued for a 5-wk period, during which the normal control rats received normal water ad libitum.	Sex not indicated. No information on the concentration of aspartame administered.	Mbambo et al. (2020)
Glucose Cholesterol TG HDL LDL VLDL mRNA leptin Adiponectin PPARγ	Rat, Wistar (M)	Blood	Increase in glucose. Increase in TG, LDL, and VLDL No changes in cholesterol and HDL Upregulation of mRNA leptin, and downregulation of mRNA of adiponectin and PPARγ in adipose tissues	There were three treatment groups: group I was allowed to drink water ad libitum; group II was intragastrically intubated with aspartame at 240 mg/kg bw; and group III was allowed to freely drink commercial cola beverages for 2 mo.		Lebda et al. (2017a)

Table 4.14 (continued)

End-point	Species, strain (sex)	Tissue	Results	Route, duration, dosing regimen, dose	Comments	Reference
Glucose Insulin GLP-1 TG	Rat, ovariectomized Sprague-Dawley (F)	Blood	Increase in HOMA-IR, HOMA-B, serum insulin; increases in IGF-1 mRNA in jejunum. Increase serum GLP-1. Decrease in TG.	Rats were fed five high-fat diets (45% fat) containing: (1) 10% corn starch; (2) 10% sucrose; (3) 10% fructose; (4) 0.05% aspartame + 9.95% starch; or (5) 0.05% sucralose + 9.95% starch (for 8 wk).		Ryuk et al. (2019)
Glucose Insulin	Rat, Wistar (M)	Blood	Increase in glucose at 104 and 197 days. Decrease in glucose at 288 days, and insulin at 9 mo.	Nutritive sweeteners (fructose 7% and sucrose 10%) and non-nutritive or low-calorie sweeteners (acesulfame, 0.015%; aspartame, 0.3%; aspartame/acesulfame mixture, 0.04%; saccharin, 0.3%; and sucralose, 0.19%) in drinking-water, as well as a control group with no sweeteners; treated for up to 288 days.		Mendoza-Pérez et al. (2021)
Glucose Insulin TG	Rat, Sprague-Dawley (M)	Blood	On regular chow diet: increase of fasting blood glucose; increase blood glucose in the insulin tolerance test. On high-fat diet: increase in fasting blood glucose, increase in blood glucose in the insulin tolerance test, decrease in plasma insulin. No changes in TG.	Adult rats were exposed to standard chow diet (12% kcal fat) or high-fat diet (60% kcal fat) and further divided into ad libitum water control or low-dose aspartame (5–7 mg/kg per day in drinking-water) for 8 wk.	Faecal analysis of gut bacterial composition showed that aspartame increased total bacteria, and the abundance of *Enterobacteriaceae* and *Clostridium leptum*. Aspartame increased levels of acetate and butyrate in chow diet groups, while formate and isobutyrate levels remained unchanged.	Palmnäs et al. (2014)
Glucose Insulin Cholesterol LDL HDL	Rat, Sprague-Dawley (M)	Blood	Increase in fasting glucose; increase in HOMA-IR strongly related to the amount of ingested aspartame; no changes in fasting insulin.	Aspartame (0·05%) was added to the diet or drinking-water or both diet and water, and a control group was given a non-sweetened diet with plain water for 7 wk.		Ragi et al. (2021)
Glucose Insulin	Rat, Charles River	Blood	No changes	7 days of exposure to diet containing aspartame (0.2% or 2%).		EFSA E1 (2011)

Table 4.14 (continued)

End-point	Species, strain (sex)	Tissue	Results	Route, duration, dosing regimen, dose	Comments	Reference
Glucose Insulin	Rat, Sprague-Dawley (M)	Blood	No changes	Rats were treated with water as a control or aspartame (6–8 mg/kg bw per day in week 1 to 15–21 mg/kg bw per day in week 6). Rats were evaluated after 6 wk of treatment.		Tovar et al. (2017)
Glucose Insulin Leptin	Rat, Long–Evans	Blood	No changes in glucose or insulin levels Decreased levels of leptin	Tap water controls and aspartame group (1 g/L) made with tap water for 14 wk (starting at weaning).		Beck et al. (2002)
Glucose Insulin	Rat, STZ-induced Sprague-Dawley diabetic (F)	Blood	No changes	After 18 h of fasting, glucose (150 mg per 100 g bw) or aspartame (0.45 mg per 100 g bw) dissolved in 2 mL of saline was administered through a stomach tube to the STZ-induced diabetic and control rats, respectively. Blood samples were obtained through a polyethylene catheter inserted into the femoral vein for the measurement of plasma glucose and insulin levels at appropriate intervals.		Shigeta et al. (1985)
Glucose Insulin	Rat, STZ-induced Wistar (M)	Blood	No changes	Diabetic aspartame group: rats that were given aspartame (50 mg/kg) intragastrically and daily for 6 wk starting from wk 6 after developing diabetes. Diabetic group treated with insulin and aspartame (D-insulin + aspartame): rats that were given NPH insulin (12 UI/kg) daily for 11 wk from the first week of having developed diabetes mellitus. Starting on the fifth week of insulin administration, all the rats in this group were also given aspartame (50 mg/kg bw per day intragastrically) for 6 wk. Control groups were compared to diabetic rats		Nosti-Palacios et al. (2014)

Table 4.14 (continued)

End-point	Species, strain (sex)	Tissue	Results	Route, duration, dosing regimen, dose	Comments	Reference
Glucose Insulin	Mouse, C57BL/6J (M, F)	Blood	Increase of insulin sensitivity in male offspring	Dams were randomly assigned to drinking-water; sucrose (45 g/L, ~7.2 g/kg bw per day anticipating 4 mL water intake, and 25 g bw); aspartame (0.2 g/L, ~32 mg/kg bw per day); or sucralose (0.04 g/L, ~6.4 mg/kg bw per day) throughout pregnancy and lactation. Mice were evaluated at age 4–12 wk.	Maternal aspartame consumption under all conditions increased body weight in male offspring by age 7 wk compared with the male offspring of control dams.	Azad et al. (2020)
Glucose Insulin	Mouse, C57BL/6J mice (M, F)	Blood	Aspartame affects visceral fat deposition and glucose homeostasis particularly in males, and to a lesser extent in females.	Mice were exposed starting in utero up to age 17 wk. Treatment groups were: (1) ad libitum standard chow with ad libitum drinking-water; (2) ad libitum standard chow with ad libitum drinking-water containing aspartame at 0.25 g/L as the only source of drinking-water.		Collison et al. (2012a)
Glucose Insulin	Mouse, C57BL/6J (M, F)	Blood	Increase in fasting glucose in both sexes, and in glucose in insulin tolerance test in males.	Mice were exposed starting in utero up to age 17 wk. Treatment groups were: (1) ad libitum standard chow with ad libitum drinking-water; (2) ad libitum standard chow with ad libitum drinking-water containing MSG at 0.75 g/L; (3) ad libitum standard chow with ad libitum drinking-water containing aspartame at 0.25 g/L; (4) ad libitum standard chow with ad libitum drinking-water containing aspartame at 0.25 g/L and MSG at 0.75 g/L.		Collison et al. (2012b)

Table 4.14 (continued)

End-point	Species, strain (sex)	Tissue	Results	Route, duration, dosing regimen, dose	Comments	Reference
Glucose Insulin	Mouse, C57BL/6J (M)	Blood	Increase in serum glucose and insulin.	Mice were exposed in utero and during weaning to aspartame (0.25 g/L); a group of these mice were treated with the NMDAR antagonist CGP 39551 in utero only. Control mice were not given aspartame or CGP 39551. Dams continued to receive the treatment in the drinking-water until weaning was completed at day 28, whereupon male F_1 offspring (no more than 3 animals per litter) in the control and control + CGP 39551 group were given ad libitum plain drinking-water and standard chow, and the offspring in the aspartame and the aspartame + CGP 39551 group were given ad libitum drinking-water containing aspartame at 46 mg/kg bw together with standard chow for the duration of the study.	Examined effects in males only.	Collison et al. (2016)
Glucose Insulin	Mouse, C57BL/6J (M)	Blood	Increase in fasting glucose levels at 6 wk but decrease at 17 wk. No changes in insulin or HOMA-IR.	The four diet regimens used in this study were: (1) TFA control diet consisting of 20% (w/w) partially hydrogenated vegetable shortening; (2). MSG + TFA diet: TFA diet with ad libitum drinking-water containing 0.75 g/L of L-glutamic acid monosodium salt hydrate; (3) aspartame + TFA diet: TFA diet with ad libitum drinking-water containing 0.25 g/L aspartame; or (4) aspartame + MSG + TFA diet: TFA diet with ad lib drinking-water containing 0.25 g/L aspartame and 0.75 g MSG. After mating, the breeder dams were maintained on their respective diets throughout gestation, birth, and nursing. The offspring used in the following experiments were weaned onto the same maternal dietary regimen at age 4 wk and maintained on their respective diets for the duration of the study. Measurements in males were carried out at age 6 and 17 wk.	The study was performed under specific diet conditions.	Collison et al. (2013)

Table 4.14 (continued)

End-point	Species, strain (sex)	Tissue	Results	Route, duration, dosing regimen, dose	Comments	Reference
Glucose Cholesterol LDL HDL	Mouse, Swiss (M)	Blood	Decrease in fasting glucose levels. Increase in cholesterol, LDL, and TG levels.	Aspartame at 80 mg/kg (2.5 mL/kg, prepared in 0.9% saline solution) or vehicle, administered daily by gavage for 12 wk.		Finamor et al. (2021)
Glucose Insulin	Mouse, C57BL/6	Blood	Increase in fasting glucose.	Four groups: control high-fat diet, aspartame-treated high-fat diet, control normal diet, and aspartame-treated normal diet. Mice received either regular autoclaved drinking-water or an aspartame-containing solution (0.96 mg/mL). Mice were treated for 18 wk.		Gul et al. (2017)
Glucose Insulin	Mouse, C57BL/6 pregnant dams	Blood	Decrease in glucose plasma levels. No change in insulin plasma levels.	Pregnant mice were treated on days 10–17 of gestation in four groups: control (vehicle); low-dose aspartame (3.5 mg/kg); medium-dose aspartame (7 mg/kg); and high-dose aspartame (14 mg/kg).		Huang et al. (2023b)
Glucose Insulin	Mouse, C57BL/6J (M)	Blood	Increase in glucose, insulin, HOMA-IR; decrease in glucose tolerance, and insulin tolerance.	The control group was fed standard chow, and the experimental group was fed standard chow containing 1% aspartame for 12 wk.		Zhou et al. (2022)
Glucose Cholesterol HDL LDL TG	Rat, Sprague-Dawley (M)	Blood	No changes in glucose levels. Increase in total cholesterol, LDL-cholesterol, and TG.	Control (water) or aspartame (4.1 mg/kg bw per day) for 18 wk.		Morales-Ríos et al. (2022)
Cholesterol TG VLDL LDL HDL	Rat, Wistar (M)		Increase in cholesterol, TG, and LDL at the intermediate and highest dose of aspartame. Decrease in HDL at the highest dose.	Control (water); aspartame at a dose of 15 mg/kg; aspartame at a dose of 35 mg/kg; aspartame at a dose of 70 mg/kg for 9 wk		Adaramoye and Akanni (2016)

Table 4.14 (continued)

End-point	Species, strain (sex)	Tissue	Results	Route, duration, dosing regimen, dose	Comments	Reference
Glucose Cholesterol LDL TG	Rat, Wistar (M, F)	Blood	Increase in glucose in male and female offspring. Increase in cholesterol and LDL in males and females. Increase in TG in males.	Female rats were divided into four groups: control (receiving water); sucrose (45 g/L); saccharin (1.35 g/L); and aspartame (2 g/L), and treated for 30 days. The rats were mated and maintained on the diet until birth. The pups were assessed at 21 and 112 days.		von Poser Toigo et al. (2015)

bw, body weight; F, female; GLP-1, glucagon-like peptide-1; HDL, high-density lipoprotein; HOMA-B, homeostatic model assessment for insulin secretion; HOMA-IR, homeostatic model assessment for insulin resistance; IGF-1, insulin-like growth factor-1; LDL, low-density lipoprotein; M, male; mo, month(s); MSG, monosodium glutamate; NMDAR, *N*-methyl-D-aspartic acid receptor; NPH, neutral protamine Hagedorn; PPARγ, peroxisome proliferator-activated receptor gamma; STZ, streptozotocin; TFA, *trans* fat; TG, triglycerides; UI, International Units; VLDL, very low-density lipoprotein; wk, week(s); w/v, weight per volume.

metabolism (Janssens et al., 2017) or it increased glucose levels with alteration in lipid metabolism (Lebda et al., 2017b). In the study by Ryuk et al. (2019), ovariectomized Sprague-Dawley rats fed high-fat diets containing 0.05% aspartame for 8 weeks showed increases in insulin resistance HOMA-IR, HOMA for insulin secretion (HOMA-B), serum insulin, and GLP-1, and decreases in serum triglycerides. In addition, there were increases in IGF-1 mRNA expression levels in the jejunum.

Palmnäs et al. (2014) observed that aspartame alters the gut microbiota and serum metabolome. Groups of adult male Sprague-Dawley rats were fed a standard chow diet or high-fat diet for 2 weeks, then exposed ad libitum to drinking-water with or without aspartame at a low dose (5–7 mg/kg bw per day) for 8 weeks. Aspartame consumption was associated with fasting hyperglycaemia and impaired insulin tolerance in both groups (standard chow diet and high-fat diet). The same study reported distinctive changes in the gut microbiota of the rats consuming aspartame (see Section 4.3.2). In addition, aspartame increased levels of acetate and butyrate in the group receiving the standard diet, whereas levels of formate and isobutyrate remained unchanged. In the study by Ragi et al. (2021), male Sprague-Dawley rats (age 7 weeks) were exposed to aspartame (0.05%) in the diet or the drinking-water, or both. A control group was exposed to a non-sweetened diet and plain drinking-water for 7 weeks. Aspartame ingestion was associated with glucose intolerance. There were no changes in fasting insulin levels; however, there were increases in fasting glucose and HOMA-IR in the aspartame-exposed groups, and these were strongly related to the amount of ingested aspartame ($r = 0.518$, $P = 0.006$).

Mbambo et al. (2020) compared the effects of commonly used commercially available non-nutritive sweeteners, including aspartame, on diabetes-related parameters in non-diabetic rats. Experimental animals exposed to aspartame consistently showed higher blood glucose levels during an OGTT test, particularly at 60 and 120 minutes after glucose ingestion, when compared with other groups [The Working Group noted that the limitations of the study were that there was no indication of the sex used, and no information on the dose administered.]

In contrast, no changes in insulin or glucose metabolism were observed after aspartame administration in some studies in rats. After 7 days of exposure to diet containing aspartame (0.2% or 2%), serum levels of glucose and insulin were not altered in male and female rats (EFSA_E1, 2011). [The Working Group noted that the percentages of aspartame in the diet were estimated to yield an average daily aspartame consumption of 0.2 g and 2 g/kg bw for males and 0.15 g and 1.48 g/kg bw for females.] In the study by Tovar et al. (2017), male Sprague-Dawley rats were exposed to drinking-water containing aspartame at 6–8 mg/kg bw per day in week 1 and 15–21 mg/kg bw per day in week 6. Rats were evaluated after 6 weeks of exposure. No changes in glucose or insulin levels were observed after the OGTT. In the study by Beck et al. (2002), male Long-Evans rats were exposed to tap water (control) or tap water containing aspartame (1 g/L) for 14 weeks, starting at weaning. No changes in glucose or insulin levels were noted after aspartame exposure. There were two studies in streptozotocin-induced diabetic rats. In the study by Shigeta et al. (1985), streptozotocin-induced female Sprague-Dawley diabetic rats were exposed to aspartame (0.45 mg/100 g bw), after 18 hours of fasting, through a stomach tube. The administration of aspartame had no effect on plasma glucose and insulin levels. In the second study carried out by Nosti-Palacios et al. (2014), streptozotocin-induced male Wistar rats were exposed to aspartame (50 mg/kg) intragastrically daily for 6 weeks, starting from the sixth week after developing diabetes. Blood glucose levels did not change in the group exposed to aspartame compared with the diabetic controls.

Some studies examined C57BL/6J mice exposed to aspartame either in utero until lactation or in utero until adulthood. In the study by Azad et al. (2020), dams were randomly assigned to receive drinking-water with or without aspartame (0.2 g/L, ~32 mg/kg bw per day) throughout pregnancy and lactation. Mice were evaluated at age 4–12 weeks. Maternal consumption of aspartame had no effect on glucose intolerance in male and female offspring. In contrast, male but not female offspring exhibited insulin resistance. There were no changes in the area under the curve (AUC) for glucose in the GTT after aspartame exposure in males or females. In addition, there was upregulated expression of the adipocyte differentiation genes *Cebpa* and *Fabp4* in the adipose tissue of male offspring.

Mice were exposed to drinking-water containing aspartame (0.25 g/L, 50 mg/kg bw per day) as the only source of drinking-water, starting in utero until up to age 17 weeks (Collison et al., 2012a, b, 2016). Aspartame-exposed male and female mice had elevated fasting blood glucose levels and aspartame-exposed males presented decreases in insulin sensitivity. Collison et al. (2016) investigated the involvement of the gut–brain axis in regulating glucose homeostasis by administering CGP 39551, an NMDAR-antagonist in utero to one group of mice exposed to aspartame. Alterations in blood glucose and impaired insulin sensitivity caused by aspartame were normalized by NMDAR antagonism. Collison et al. (2013) investigated the effects of changes in hepatic and adipose tissue gene expression induced by aspartame (0.25 g/L) in the offspring of C57Bl/6 J mice fed a *trans*-fat-enriched diet (see Section 4.2.9). The offspring used in the experiments were weaned onto the respective maternal dietary regimens at age 4 weeks and maintained for the duration of the study. Fasting glucose levels were elevated at 6 weeks but decreased at 17 weeks. There were no changes in insulin or HOMA-IR after exposure to aspartame.

There were few studies examining the effects of aspartame in adult mice. Finamor et al. (2021) investigated the effects of aspartame on oxidative stress and inflammatory mechanisms associated with liver fibrosis progression in mice (see also Sections 4.2.3 and 4.2.4). Male Swiss mice were exposed to aspartame at a dose of 80 mg/kg bw (2.5 mL/kg, prepared in 0.9% saline solution) by gavage for 12 weeks. Aspartame decreased fasting induced glucose levels and impaired gluconeogenesis possibly through decreased peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*) mRNA expression levels and PGC-1α protein levels. In addition, aspartame decreased HDL cholesterol and increased total cholesterol, LDL cholesterol, triglycerides, and total lipids. Gul et al. (2017) investigated C57BL/6 mice fed a standard or high-fat diet and drinking-water with or without aspartame (0.96 mg/mL) for 18 weeks. Mice receiving aspartame-infused drinking-water developed glucose intolerance. Fasting blood glucose levels at 16 hours were significantly higher in groups that were exposed to aspartame than in the controls. In pregnant mice exposed to aspartame at a dose of 3.5, 7 or, 14 mg/kg per day by oral gavage for 17 days, there was a significant decrease in fasting plasma glucose levels in aspartame-exposed groups compared with vehicle controls, but no significant differences in plasma insulin levels (Huang et al., 2023b).

More recently, Zhou et al. (2022) found that aspartame, through its metabolite, phenylalanine, modifies insulin receptor β (IRβ) and inactivates insulin signalling and glucose uptake. Male C57BL/6J mice were exposed to diet containing 1% aspartame for 12 weeks. Aspartame increased levels of blood glucose and insulin, and HOMA-IR, and decreased glucose tolerance and insulin tolerance. In addition, C57BL/6J mice fed a diet that was enriched in phenylalanine or phenylalanine-producing aspartame, and C57BL/6J mice overexpressing human phenylalanyl-tRNA synthetase α subunit (hFARSA), which induces

phenylalanylation of lysine, were shown to develop insulin resistance and symptoms of type 2 diabetes. Specifically, FARS catalyses the phenylalanylation of insulin receptor β (IRβ) on lysine residues 1057 and 1079, thus inactivating the receptor. The inactivation of IRβ inhibits insulin signalling and, finally, glucose uptake by cells. The authors showed also that the inhibition of insulin signalling could be abrogated by de-phenylalanylating lysine residues F-K1057/1079 by overexpressing sirtuin 1 (SIRT1) in hepatocytes from C57BL/6J mice. On the other hand, in the hepatocytes of *Sirt1*-knockout C57BL/6J mice, levels of F-K1057/1079 are higher and insulin signalling is inhibited. In another experiment, the authors showed that blocking the activity of F-K1057/1079, using phenylalaninol, induces insulin sensitivity and relieves the symptoms of type 2 diabetes in C57BL/6J mice overexpressing hFARSA or in C57BL/KsJ-db/db diabetic mice (db/db).

Malaisse et al. (1998) observed that a single acute exposure to aspartame (1–10 mM), in the presence or absence of D-glucose, did not elicit insulin secretion from pancreatic islets isolated from female Wistar rats (ex vivo) (Malaisse et al., 1998).

Increases in circulating total cholesterol and LDL cholesterol were observed in many studies (Collison et al., 2012a, b, 2013; von Poser Toigo et al., 2015; Lebda et al., 2017b; Mbambo et al., 2020; Finamor et al., 2021; Mendoza-Pérez et al., 2021; Morales-Ríos et al., 2022). Decreases in HDL cholesterol were observed by Collison et al. (2012a, b) and Mbambo et al. (2020), whereas an HDL increase was observed by Finamor et al. (2021). [The Working Group noted that, overall, exposure to aspartame led to alterations in lipid metabolism in experimental systems.]

Adaramoye and Akanni (2016) investigated the effects of long-term administration of aspartame on redox status, lipid profiles, and biochemical indices in male Wistar rats exposed to distilled water (control) or aspartame at a dose of 15, 35, or 70 mg/kg, respectively, daily by oral gavage for nine consecutive weeks. There were significant increases in concentrations of LPO products in the kidney, liver, and brain at all doses of aspartame in parallel with decreases in antioxidant defences, including GST, GPx, SOD, CAT, and GSH (see also Section 4.2.3). At 35 and 70 mg/kg, aspartame increased the levels of total cholesterol, triglycerides, and LDL cholesterol. Histopathological analysis showed degeneration, monocyte infiltration, and necrotic lesions in the brain, kidney, and liver. [The Working Group noted that aspartame may induce redox and lipid imbalance in rats via a mechanism that involves oxidative stress and depletion of the GSH-dependent system. No statistical analysis was carried out on the histopathological analyses.]

Pandurangan et al. (2014) investigated the effects of aspartame on obesity, focusing on differentiation in the 3T3-L1 preadipocyte cell line. The 3T3-L1 adipocytes were differentiated in culture for 6 days in the absence and presence of aspartame (10 μg/mL). Aspartame reduced lipid accumulation in differentiated adipocytes (as measured by Oil Red O staining) and significantly reduced the gene expression (as measured by quantitative reverse transcription-polymerase chain reaction, qRT-PCR) of PPARγ, FABP4, C/EBPα, and SREBP, which are involved in adipogenesis. In addition, aspartame reduced the protein expression of p-PPARγ, PPARγ, SREBP1, and adiposin, which are normally induced in differentiating cells.

4.3.2 Microbiome

(a) Humans

Exposed humans

In a cross-sectional study, Frankenfeld et al. (2015) investigated bacterial abundance (according to phylogenetical class or order) in consumers and non-consumers of aspartame

(n = 7) or acesulfame-K (n = 7). Thirty-one healthy adults completed a 4-day food record and provided a faecal sample on day 5. No differences in median bacterial abundance were observed between consumers and non-consumers of either sweetener; however, the overall bacterial population composition was different between non-consumers and consumers of aspartame and acesulfame-K, assessed as separate groups.

Suez et al. (2022) assessed the effects of supplementation with aspartame (240 mg/day) for 2 weeks in a randomized controlled trial in 120 healthy adults. Aspartame affected the faecal and oral microbiomes (according to metagenomic analysis) and the plasma metabolome, but had no significant effect on the glycaemic response, (which was the case for other sweeteners). In addition, aspartame was shown to modify oral and faecal microbiome composition and key functions, such as purine or pyrimidine metabolism, glycolysis, and amino acid metabolism. In gnotobiotic mice that were not exposed to aspartame and that were transplanted with the microbiomes from multiple human donors with either a high or a low response to aspartame supplementation, murine glycaemic responses reflected those reported in the respective human donors.

In a study that gave results contrary to those of the above studies, Ahmad et al. (2020) conducted a 12-week crossover clinical trial in 17 healthy participants who were exposed to 425 mg of aspartame or 136 mg of sucralose. Aspartame (n = 9) or sucralose (n = 8) was administered during weeks 5 and 6, after a 4-week period without any artificial sweeteners. In weeks 7 through 10, all participants underwent a washout period during which no artificial sweeteners were consumed. During weeks 11 and 12, all participants who had previously consumed aspartame were exposed to sucralose, and vice versa. No changes in faecal microbiota or faecal short chain fatty acid concentrations were observed before and after exposure.

(b) Experimental systems

Palmnäs et al. (2014) demonstrated that low doses of aspartame (5–7 mg/kg per day) administered for 8 weeks affected gut microbiota in obese rats. Compared with animals receiving water, rats exposed to aspartame consumed fewer calories and gained less weight. However, aspartame elevated fasting glucose levels and impaired insulin-stimulated glucose disposal in rats receiving control and high-fat diets. Faecal analysis of gut bacterial composition showed that aspartame increased total bacteria and the abundance of Enterobacteriaceae and *Clostridium leptum*. Serum metabolomics analysis revealed aspartame to be associated with elevations in butyrate and propionate, a bacterial metabolite and highly gluconeogenic substrate, potentially explaining the negative effects of aspartame on insulin tolerance. [The Working Group noted that modifications of the gut microbiota could be caused by aspartame metabolites (Oppermann et al., 1973a, b; Lipton et al., 1991) undergoing fermentation by the gut microbiota at the colon level, thus inducing the observed modifications in the bacterial population.] Suez et al. (2014) reported that, in mice, aspartame caused glucose intolerance through induction of compositional and functional alterations to the intestinal microbiota and that this derangement was abrogated by antibiotic treatment.

Nettleton et al. (2020) showed that low doses of aspartame (5–7 mg/kg) altered gut microbiota as well as altering the metabolism and mesolimbic reward system, in rat dams (in which obesity had been induced by diet) and their offspring. In line with this evidence, germ-free mice receiving a faecal microbiota transplant from obese aspartame-exposed offspring showed greater gain in body weight and body fat and impaired glucose tolerance than did the obese controls.

In a subsequent study, the same group (Wang et al., 2021) characterized the major alterations in the caecal microbiome of the offspring of obese

dams consuming a high-fat/sucrose diet with or without aspartame (5–7 mg/kg). Maternal consumption of aspartame altered caecal microbial composition and metabolism of propionate/lactate in the offspring. In the offspring, daily body-weight gain, liver weight, and body fat were positively correlated with the relative abundance of key microbes and enzymes involved in succinate/propionate production and negatively correlated to those involved in lactose degradation and lactate production.

Shil and Chichger (2021) investigated the pathogenicity of two model strains of microbiota (*E. coli* NCTC10418 and *Enterococcus faecalis* ATCC19433) exposed to aspartame at different concentrations in vitro and their effects in a co-culture of human colon adenocarcinoma (Caco-2) cells. The ability of bacteria to form a biofilm increased significantly after exposure to aspartame (100 μM) for 24 hours. In the co-culture experiments, aspartame also increased the ability of gut bacteria to adhere to, invade, and kill Caco-2 cells. In addition, the aspartame-mediated increase in the ability of *E. coli* to invade Caco-2 cells was attenuated by the presence of 100 μM zinc sulfate (a potent inhibitor of sweet taste sensing), as was the ability of *E. faecalis* to form biofilm, adhere and invade Caco-2 cells (see also Shil et al. (2020), described in Section 4.2.8(b) and 4.3.3).

In another recent study investigating bacteria quorum sensing (a function that enables microorganisms to communicate within their community and regulate group behaviours) showed that aspartame has inhibitory actions on the Gram-negative bacteria *N*-acyl homoserine lactone-based communication system involved in the quorum sensing process (Markus et al., 2021).

4.3.3 Sweet taste receptors

There is evidence in humans that aspartame binds to the sweet taste receptor, a heterodimeric G protein-coupled receptor consisting of two subunits (T1R2 and T1R3) (Pin et al., 2003). Activation of the lingual sweet taste receptors triggers the release of α-gustducin, which activates phospholipase C, producing inositol phosphate and resulting in intracellular calcium release. In the mouth, this cascade induces neuronal signals to convey the sensation of sweetness to the brain. In addition, sweet taste receptors have also been located outside of the oral cavity, including in the intestine and in pancreatic β cells, where a signalling cascade analogous to that in the oral cavity elicits GLP-1 and insulin secretion, respectively (Jang et al., 2007; Nakagawa et al., 2009).

Some studies in which the interaction of aspartame with the sweet taste receptors can be linked to downstream effects relevant to the key characteristic of carcinogens are reported below.

(a) Human cell lines

Shil et al. (2020) reported that aspartame at high concentrations (1000 μM) induces apoptosis and cell death in intestinal Caco-2 cells, whereas lower concentrations (100 μM) increase epithelial barrier permeability, downregulate claudin-3 expression, and induce ROS production; these effects were attenuated by knockdown of the sweet taste receptor gene *T1R3* (Shil et al., 2020). Gezginci-Oktayoglu et al. (2021) investigated the effects of aspartame in human pancreatic adenocarcinoma cells (PANC-1) (see Section 4.2.8). Aspartame significantly increased the expression of markers of epithelial–mesenchymal transition (EMT) such as vimentin and N-cadherin, activated AKT, and deactivated GSK3β, by increasing levels of ROS and cytoplasmic Ca^{2+}, respectively, through T1R2/T1R3 stimulation.

(b) Experimental systems

In a study on the effects of aspartame exposure on placental growth, Huang et al. (2023b) exposed pregnant mice to aspartame by oral gavage. Aspartame impaired placenta growth, induced ROS levels, hyper-activated Akt, and downregulated manganese superoxide dismutase (MnSOD) expression (see also Section 4.2.5). Importantly, pre-treatment with antioxidants or sweet taste receptor inhibitors reversed the effects of aspartame on trophoblast function. In addition, the aspartame metabolite phenylalanine similarly induced ROS production and affected proliferation of trophoblasts. [The Working Group noted that these data suggested that aspartame consumption during pregnancy may have an impact on the structure, growth, and function of the placenta via sweet taste receptor-mediated stimulation of oxidative stress.]

Several studies evaluated the effects of aspartame on glucose metabolism in healthy, diabetic, or overweight participants. Several intervention trials of up to 12 weeks gave largely negative results. A small number of cross-sectional studies were uninformative with regard to the effects of aspartame on glucose metabolism. These studies presented several limitations associated with difficulty in controlling for confounding variables in a human population, and the complexity of the different study designs and relative protocols. Often unusual regimens or routes of administration (i.e. sweeteners in juice or lemonade) were considered. Additionally, in many studies aspartame was considered as the reference positive control in comparisons with other sweeteners; thus, the lack of an appropriate background control limited informativeness or invalidated the studies (Ahmad et al., 2020). However, there was consistent evidence that exposure to aspartame induced insulin increase in rodents. Recent studies have suggested that aspartame has effects on metabolism and gut microbiota. The interactions of aspartame with sweet taste receptors, which have been implicated in the signalling cascade that activates metabolism in the body, and with the gut microbiota were identified by the Working Group as notable research gaps.

5. Summary of Data Reported

5.1 Exposure characterization

Aspartame is an odourless white crystalline powder that is added as a low-calorie artificial sweetener to foods and beverages. It is also used in cosmetics, medicines, and tobacco products. Aspartame has a sweetness that is 200 times that of sucrose.

Aspartame is measured in consumer products mostly by chromatographic methodologies. It is reported to be present in food in the milligrams-per-kilogram range. Aspartame has been detected in wastewater samples in different regions of the world.

Aspartame was first authorized for food use in most countries in the 1980s. Artificially sweetened beverages are an important source of aspartame exposure, although aspartame is present in a more concentrated form in tabletop sweeteners, chewing gums, and food supplements. In the USA from the mid-1980s until the beginning of the 2000s, artificially sweetened beverages almost exclusively contained aspartame as the sweetener but, more recently, it has been partly or completely replaced by other low-calorie sweeteners. Trends are likely to be similar in Europe and other regions, but data on exact timing are lacking for many countries.

There are no validated biomarkers of dietary intake of aspartame because, upon consumption, aspartame is rapidly metabolized to endogenous human metabolites. Therefore, assessments of human exposure via ingestion are reliant upon self-reported methods for estimating dietary intake, which have limitations relating to accuracy, recall bias, and lack of comprehensive

food composition data. Exposure assessments have typically shown that daily exposure of the general population is in the low milligrams-per-kilogram-body-weight range but can be higher in certain consumer groups.

Occupational exposure via inhalation during the synthesis of aspartame and the production of aspartame-containing products has been reported in the past, but no recent data on occupational exposure are available.

5.2 Cancer in humans

The association between the consumption of aspartame and/or artificially sweetened beverages and cancer was investigated in 12 prospective cohort studies and 13 retrospective case–control studies (two of which were considered to be not informative).

The majority of these studies assessed consumption of artificially sweetened beverages and/or tabletop sweeteners likely or known to contain aspartame as the exposure of interest, and the Working Group decided to use these assessments as a proxy for aspartame exposure. The quality of the proxy varied across studies according to country- and time-period-specific evidence on aspartame use. Only one study, the NutriNet-Santé cohort, provided information on exposure to aspartame across the entire spectrum of dietary sources and reported brand-specific information (the main analysis considered intake over the first 2 years of follow-up and additional analyses using repeated assessments throughout the follow-up period). The aspartame assessment in this study was based on repeated 24-hour dietary records with: (1) brand-specific information (allowing specific assessment of intakes of aspartame and other artificial sweeteners); (2) all dietary sources accounted for (artificially sweetened beverages, tabletop sweeteners, but also dairy and other products); and (3) a dynamic matching of consumption and composition data accounting for the date when the food or beverage was consumed. Several other cohort studies were also considered particularly informative because of the close overlap of the follow-up period with time periods during which aspartame was the prevailing artificial sweetener used in the respective country: the Nurses' Health Study (NHS), the Health Professionals Follow-up Study (HPFS), the European Prospective Investigation on Cancer (EPIC), the Cancer Prevention Study II (CPS-II), the National Institutes of Health-American Association of Retired Persons (NIH-AARP) Diet and Health Study, and the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial. In addition, three of these studies had exposure assessments that were repeated over time and were therefore more comprehensive.

Three informative studies consistently described positive associations for liver cancer overall or in subgroups of the study population (i.e. grouped according to sex, diabetes, and smoking status). No other studies investigated associations with liver cancer. The available studies included one from EPIC (including populations from ten European countries) and two studies from the USA (the pooled NIH-AARP and PLCO cohorts, and the CPS-II cohort). All three cohort studies controlled for potential confounding by diabetes status at baseline through stratification, model adjustment, or restriction. New-onset diabetes after exposure to aspartame could be one among many potential pathways for liver cancer. In the EPIC cohort, an overall positive association was observed for incidence of hepatocellular cancer (HCC) per each increment in 330 mL servings per week of artificially sweetened beverage consumption (hazard ratio, HR, 1.06; 95% confidence interval, CI, 1.03–1.09; 151 cases). In one of the other two studies (the pooled NIH-AARP and PLCO), positive associations between artificially sweetened beverage consumption and incidence of primary cancer of the liver and intrahepatic biliary duct were observed among individuals with diabetes,

in whom consumption of artificially sweetened beverages was higher than in individuals without diabetes. These positive findings were observed in the first 12 years of follow-up, which coincided with the years in which aspartame was used as the predominant artificial sweetener in the USA. Another study in the USA (CPS-II) found a positive association between consumption of artificially sweetened beverages and mortality from cancer of the liver and intrahepatic biliary duct only among men who were never-smokers. This association was attenuated after adjustment for body mass index (BMI); however, this finding was not considered to refute the positive association seen in other studies, which also adjusted for BMI. Other informative studies, including the NutriNet-Santé study, did not investigate liver cancer separately.

The main limitations of these studies related to the potential for non-differential exposure misclassification, lack of repeat exposure assessments in all studies (although in a subgroup of the CPS-II in the USA, it was shown that high consumption of artificially sweetened beverages was stable over 17 years), and lack of detailed information on aspartame consumption from sources other than artificial beverages (although artificially sweetened beverages are estimated to capture most of the aspartame consumption in the USA during the time periods when the studies were conducted). Despite probable bias towards the null, these studies observed positive associations between aspartame consumption and liver cancer; however, the possibility of confounding by exposure to other known liver carcinogens could not be excluded.

For other cancer sites, the restricted epidemiological data available were not sufficiently informative or consistent to permit a conclusion to be drawn about the presence or absence of a causal association. However, some positive findings from single informative studies suggesting a potential link between aspartame exposure and cancer risk were observed. In particular, the NutriNet-Santé cohort observed positive associations between aspartame exposure and increased risk of all cancer sites combined, breast cancer, and obesity-related cancers combined. Other studies on these cancers did not find evidence of positive associations. Additional findings that emerged included positive associations with some cancers of the lymphatic system or tissue (NHS and HPFS studies, assessing artificially sweetened beverages and tabletop sweeteners containing aspartame), as well as pancreatic cancer (CPS-II and EPIC studies, using baseline consumption of artificially sweetened beverages).

5.3 Cancer in experimental animals

Treatment with aspartame caused an increase in the incidence of malignant neoplasms or an appropriate combination of benign and malignant neoplasms in two species (mouse and rat) and both sexes, in three studies that did not comply with Good Laboratory Practice (GLP). During the evaluation of the available studies, the Working Group noted concerns regarding diagnoses for lymphomas predominantly, but not exclusively, located in the lung. Therefore, the synthesis of evidence of carcinogenicity in experimental animals focused on all neoplastic lesions except lymphoid tumours and related combinations.

Aspartame was administered by transplacental and perinatal exposure followed by oral administration (feed) in male and female Swiss mice. In males, aspartame caused hepatocellular carcinoma, hepatocellular adenoma or carcinoma (combined), bronchioloalveolar carcinoma, bronchioloalveolar adenoma or carcinoma (combined), and a significant increase in the incidence of lymphoblastic leukaemia, of monocytic leukaemia, and of total myeloid tumours, was also reported. In females, aspartame caused lymphoblastic leukaemia.

Aspartame administered by oral administration (feed) in Sprague-Dawley rats caused carcinoma and papilloma and carcinoma (combined) of the renal pelvis and ureter in males and females, and mammary gland carcinoma in females. In males, a re-analysis of the above data indicated a significant increase in the incidence of monocytic leukaemia, histiocytic sarcoma, and a positive trend in the incidence of total myeloid tumours at all doses.

Aspartame administered by transplacental and perinatal exposure followed by oral administration (feed) in male Sprague-Dawley rats caused malignant schwannoma and in female rats aspartame caused mammary gland carcinoma, and renal pelvis papilloma. Another re-analysis of these data reported a positive trend for myeloid leukaemia and total myeloid tumours in females.

Although data from the studies on transplacental and perinatal exposure followed by oral administration (feed) in mice and rats and the study on oral administration (feed) in rats indicated that aspartame had carcinogenic activity, overall, the Working Group had serious questions about the adequacy of the design, conduct, interpretation, and reporting of each of the studies. For example, no adjustments were made for litter effects, which can lead to false-positive results regarding increases in incidence and trends. A minority of the Working Group did not consider that these concerns would substantially change the interpretation of the evidence of carcinogenicity in experimental animals.

5.4 Mechanistic evidence

All available studies in humans consistently indicated that ingested aspartame is hydrolysed by esterases and peptidases in the gastrointestinal tract yielding three components – the amino acids aspartic acid and phenylalanine, and methanol. These components undergo absorption from the intestinal lumen, reaching the systemic circulation, and appear to enter normal endogenous metabolic pathways. Alternatively, ester hydrolysis in the lumen may yield methanol and the dipeptide, aspartylphenylalanine, which is absorbed into intestinal mucosal cells via a peptide-transport mechanism and subsequently undergoes efficient hydrolysis to aspartic acid and phenylalanine in the enterocytes. No studies were available that investigated routes of exposure other than ingestion. Upon absorption, phenylalanine enters the plasma pool of free amino acids from the portal blood, after partial conversion to tyrosine in the liver. Aspartic acid undergoes transamination in the enterocytes, producing oxaloacetate, an intermediate in the citric acid cycle. Methanol does not undergo metabolism in the enterocytes, rapidly enters the portal circulation, and is oxidized to formaldehyde. Formaldehyde quickly undergoes further oxidation to formic acid, which is ultimately converted to carbon dioxide (CO_2). Exposure to methanol from aspartame consumption is estimated to account for 1–10% of overall exposure to methanol, whereas endogenous methanol accounts for more than 80%. It is not known whether exposure to formaldehyde from aspartame consumption would significantly affect concentrations of endogenous formaldehyde.

The metabolism of ingested aspartame and the kinetics for each of its components have been studied in multiple settings, including in healthy adults, healthy infants, lactating women, adults who are heterozygous or homozygous for phenylketonuria, and people with diabetes. These studies have included single, repeated, or long-term dosing, and co-administration with meals. Across subpopulations, the concentration of plasma aspartic acid typically remained within the normal range after intake of high doses of aspartame. Plasma phenylalanine concentrations, measured after single or repeated doses of aspartame, were within the normal postprandial levels in normal subjects, and higher in individuals heterozygous for phenylketonuria, but declined in the hours following exposure.

Blood formate concentrations did not differ from baseline levels after high doses of aspartame, and urinary formate excretion was significantly increased.

Similar to the results reported in humans, aspartame appears to be completely hydrolysed in the small intestine of experimental animals, producing methanol, aspartic acid, and phenylalanine. Most of the absorbed methanol and aspartic acid from aspartame are excreted as CO_2 in experimental animals. Phenylalanine is also excreted as CO_2 in experimental animals; however, a greater proportion of phenylalanine is retained. Because of the greater activity of phenylalanine hydroxylase in rats, plasma levels of phenylalanine in rats increase at a much slower rate than in humans, primates, and pigs after aspartame or phenylalanine exposure. Evidence in rats suggests that the effects of aspartame exposure on phenylalanine hydroxylase activity are similar to those of phenylalanine at equimolar doses, and that aspartame exposure has no effects on gastric juice secretion, concentration of gastric acid, acid output, and proteolytic activity. It is reported that aspartame exposure may significantly increase cytochrome P450 (CYP) protein levels (i.e. 1A1, 1A2, 2B, 3A2) and enzymatic activities (i.e. ethoxyresorufin *O*-deethylase, methoxyresorufin *O*-demethylase, pentoxyresorufin *O*-dependentylase, benzyloxyresorufin *O*-debenzylase, 4-nitrophenol hydroxylase, and erythromycin-*N*-demethylase) in the cerebrum and cerebellum of rats after 30 days of aspartame treatment.

Data were available for aspartame for the following key characteristics of carcinogens: "is electrophilic or can be metabolized to an electrophile", "is genotoxic", "induces oxidative stress", "induces chronic inflammation", "is immunosuppressive", "modulates receptor-mediated effects", "causes immortalization", and "alters cell proliferation, cell death, or nutrient supply".

Overall, the mechanistic evidence for aspartame regarding the key characteristics of carcinogens is suggestive. There is consistent and coherent evidence that aspartame induces oxidative stress in experimental systems. There were no studies in exposed humans. Three studies in human primary cells observed alterations in biomarkers of oxidative stress; however, two studies were of limited quality. Aspartame also altered biomarkers of oxidative stress in a variety of human cell lines; however, these biomarker changes were accompanied by significant cytotoxicity or mitochondrial toxicity, and there was no determination of whether those toxicities caused or were caused by oxidative stress. One study in a human intestinal cell line presented clear evidence that, after binding to the sweet taste receptor, aspartame increased reactive oxygen species (ROS) and disrupted cell-membrane barrier function. Consistent and coherent evidence for oxidative stress derives from several studies in rodents. Aspartame induced lipid peroxidation in a wide variety of tissues in rats. There were also many studies in rats, and a few in mice, that showed the increased presence of ROS or activation of biomarkers of cellular responses to ROS, such as increased expression or activity of catalase, superoxide dismutase, or glutathione transferases in several tissues, including the liver, blood, spleen, kidney, and brain.

There is suggestive evidence that aspartame induces chronic inflammation. Several studies in exposed humans were deemed uninformative because it was not possible to determine whether the exposure was to aspartame only. However, aspartame exposure has been confirmed in two cross-sectional studies. One of the studies in exposed humans reported that intake of aspartame-sweetened beverages was associated with a higher concentration of serum C-reactive protein (CRP) but not with two other markers of inflammation, intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1). Another study evaluated the effects

of aspartame consumption in small groups of HIV-positive and HIV-negative individuals; in the HIV-positive group, aspartame consumption was significantly positively associated with monocyte chemoattractant protein 1 (MCP-1), lipoprotein-associated phospholipase A2 (Lp-PLA2), and coronary plaque segments. Levels of two other markers of inflammation, high-sensitivity CRP and oxidized low-density lipoprotein, were increased but not statistically significantly. The informativeness of this study was limited because of the lack of clear group-selection criteria. In addition, information on food sources of the sweeteners and duration of aspartame intake was not provided. In one study in primary human umbilical vein endothelial cells, an increase in the pro-inflammatory cytokine interleukin-6 (IL-6) was observed; however, no alteration in inflammatory cytokines (IL-6, IL-10, and interferon gamma, IFNγ) was reported in human fibroblast cell lines. Dose-dependent increases in levels of pro-inflammatory cytokines – tumour necrosis factor alpha (TNFα) and IL-6 in the liver, and CRP in the serum – were observed in rats treated orally with aspartame for 30 days. An increase in levels of circulatory IL-4 and a decrease in levels of cytokines IL-2, TNFα, and IFNγ were also observed in rats after intraperitoneal injection of aspartame for 90 days. Similarly, TNFα levels were elevated in the brain tissue of mice exposed to aspartame by intraperitoneal injection. Signs of tissue inflammation in various organs were also reported in several repeated-dose toxicity studies of various durations; however, the studies were considered of limited informativeness on the basis of limitations in the design of the study and accuracy of the data analysis.

There is suggestive evidence that aspartame exhibits the key characteristic of carcinogens "alters cell proliferation, cell death, or nutrient supply". Multiple tolerance studies in exposed humans, which reported on standard clinical chemistry and urine analysis were considered not informative to this key characteristic. Regarding cell proliferation, the evidence for this end-point was inconsistent. There were no studies in exposed humans or in human primary cells. In human cell lines, aspartame decreased markers of cell proliferation in one study and increased markers of apoptosis in two studies. In experimental systems in vivo, aspartame promoted cell proliferation via the insulin-like growth factor 1 (IGF-1) pathway in the jejunum (measured as crypt depth and villus height) in lambs fed aspartame for 35 days. In mice, it caused neurogenesis in the dentate gyrus subgranular zone of the hippocampus. In rats, aspartame treatment for 56 days increased expression of HRas and decreased levels of Cdkn1b. In a lifetime study in rats, hyperplasia was observed in the renal pelvis of females and in the olfactory epithelium in both females and males. However, there was no evidence of increased cell proliferation or hyperplasia in the urinary bladders of male rats exposed via drinking-water and food for 4 weeks. In in vitro systems, aspartame increased cell proliferation in a growth-factor dependent mouse mast cell line, but increased gene expression associated with apoptosis induction in rat adrenal pheochromocytoma cells.

With respect to angiogenesis, the evidence was suggestive. There were no studies in exposed humans. In human primary umbilical vein endothelial cells, aspartame increased markers of angiogenesis, such as tubule formation and vascular endothelial growth factor (VEGF), after exposure for 14 days in one study, but not after a shorter exposure in two other studies. Rats treated with aspartame for 3 days showed an increase in VEGF expression in the placenta. In experimental systems in vitro, a qualitative increase in angiogenesis was reported in a rat skin model, and a dose-dependent increase was observed in the vascular area in the chick chorioallantoic membrane. Aspartame inhibited ATPase activity in membranes isolated from human lymphocytes.

For the key characteristic "is genotoxic", the database was extensive; however, the evidence was considered inconsistent, and many studies presented limitations in design and quality. No data were available in exposed humans. Three studies in human primary cells reported induction of chromosomal aberrations and micronucleus formation; however, two had limitations. One chromosomal aberration study employed only a single concentration and no information on aberrant types and mitotic index was given. One study using the micronucleus assay showed a small statistically significant increase in micronuclei frequency by the *t*-test, which was not corrected for multiple comparisons. In studies in human cell lines, both positive and negative results were observed for DNA strand breaks; however, some of the studies that reported positive results had limitations. Aspartame induced chromosomal aberrations or micronucleus formation in several studies that showed limitations; however, the findings were negative for both end-points in two well-designed in vivo studies. Aspartame did not induce dominant lethal mutations or reverse mutations in host-mediated assays. In rodents, mixed results were obtained for DNA strand breaks and DNA fragmentation; some of the studies were associated with apoptosis. In studies of limited quality in non-human mammalian cells in vitro, aspartame induced DNA strand breaks in Madin–Darby canine kidney (MDCK) cells but did not induce unscheduled DNA synthesis in rat primary hepatocytes. Aspartame did not induce SMART (somatic mutation and recombination by wing spot test) in *Drosophila melanogaster*. In two studies of limited quality in *D. melanogaster* or *Danio rerio*, aspartame induced DNA fragmentation. Aspartame did not induce reverse mutation in standard sets of *Salmonella typhimurium* and *Escherichia coli* strains, with or without metabolic activation.

Regarding the key characteristic "modulates receptor-mediated effects", the few available studies gave negative or inconsistent results. Aspartame had no effect on the transcriptional activity of the aryl hydrocarbon receptor and glucocorticoid receptor-dependent expression of CYP1A1 in human primary hepatocytes or in hepatic and intestinal cell lines. In rodents, there were no consistent effects on estrogen, androgen, progesterone, and glucocorticoid receptor function after acute exposure to aspartame. Two studies examining thyroid hormone levels found that aspartame decreased circulating triiodothyronine (T3) with conflicting changes in thyroxine (T4). Aspartame exposure had inconsistent effects on glutamate receptors in rodent brain tissue.

For the other key characteristics, i.e. "is electrophilic or metabolized to an electrophile", "is immunosuppressive", and "causes immortalization", there was either a paucity of data or the results were negative.

Aspartame was essentially without effects in the assay battery of the Toxicity Testing in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes of the government of the USA.

With regard to other relevant evidence, several studies investigated the effects of aspartame exposure on the release of insulin and other hormones such as leptin, C-peptide, glucagon, and glucagon-like peptide-1 (GLP-1), or on glucose metabolism in healthy or diabetic subjects. Several intervention trials of up to 12 weeks in duration gave largely negative results. A small number of cross-sectional studies were uninformative regarding the effects of aspartame on insulin, because of methodological issues.

On the other hand, there was consistent evidence that exposure to aspartame induces an increase in insulin in rodents. One study also reported that aspartame, through its metabolite phenylalanine, induces post-translational inactivation of insulin receptor β (IRβ).

Aspartame was reported to alter the gut (and oral) microbiome in some studies, but not all, in exposed humans. Alteration of the gut microbiome was also identified in studies in experimental systems in vivo and in vitro.

Recent evidence reported that aspartame binds to the heterodimer G-protein-coupled taste receptor type 1 member 2 (T1R2) and taste receptor type 1 member 3 (T1R3) sweet taste receptors, which modulate glucose signalling and metabolism in both humans and rodents. In human colorectal adenocarcinoma cells, aspartame was shown to increase epithelial barrier permeability and induce ROS production, effects that were attenuated by knockdown of the T1R3 receptor. Aspartame was also shown to increase markers of stemness in human pancreatic adenocarcinoma cells, an effect modulated by the T1R1 receptor.

6. Evaluation and Rationale

6.1. Cancer in humans

There is *limited evidence* in humans for the carcinogenicity of aspartame. Positive associations have been observed between aspartame and cancer of the liver (hepatocellular carcinoma).

6.2 Cancer in experimental animals

There is *limited evidence* in experimental animals for the carcinogenicity of aspartame.

6.3. Mechanistic evidence

There is *limited mechanistic evidence*.

6.4 Overall evaluation

Aspartame is *possibly carcinogenic to humans (Group 2B)*.

6.5 Rationale

The Group 2B evaluation for aspartame is based on *limited evidence* for cancer in humans. There is also *limited evidence* for cancer in experimental animals and *limited mechanistic evidence*. There is *limited evidence* that aspartame causes liver cancer (hepatocellular carcinoma) in humans. Among the available studies of cancer in humans, only three studies (including four cohorts) on the consumption of artificially sweetened beverages were available for assessing the association between aspartame and liver cancer. Consumption of artificially sweetened beverages was considered to be a proxy for aspartame exposure, supported by evidence on the country and time period of aspartame use in beverages. A positive association was observed between consumption of artificially sweetened beverages and risk of liver cancer in all three studies, but bias or confounding could not be ruled out as an explanation for the findings. For all other cancer sites, the evidence is *inadequate*, because the studies did not show consistent positive associations, were few in number, or were of low informativeness for aspartame specifically.

The evidence for cancer in experimental animals was *limited* because, although increases in tumour incidence were observed in both species (mice and rats) in three studies, concerns existed regarding the statistical analyses and pathological diagnoses. Thus, the Working Group considered that there are unresolved questions about the adequacy of the design, conduct, or interpretation of the available studies. A minority of the Working Group did not have concerns about the observations of solid and myeloid tumours reported in these studies, and therefore considered that the evidence for cancer in experimental animals was *sufficient* on the basis of an increased incidence of malignant neoplasms or a combination of benign and malignant neoplasms in two species (mouse and rat) of both sexes in three well-conducted studies.

There was also *limited mechanistic evidence*, which was based on consistent and coherent evidence that aspartame induces oxidative stress in experimental systems and suggestive evidence that aspartame induces chronic inflammation and alters cell proliferation, cell death, or nutrient supply in experimental systems.

A minority of the Working Group supported a Group 2A classification for aspartame, based on a combination of *limited evidence* for cancer in humans and *sufficient evidence* for cancer in experimental animals, supported by the *limited mechanistic evidence*, but these conclusions were not supported by the Working Group overall, thus leading to a Group 2B evaluation.

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Zhang T, Gan Z, Gao C, Ma L, Li Y, Li X, et al. (2016). Occurrence of artificial sweeteners in human liver and paired blood and urine samples from adults in Tianjin, China and their implications for human exposure. *Environ Sci Process Impacts*. 18(9):1169–76. doi:10.1039/C6EM00130K PMID:27383923

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Zhou Q, Sun WW, Chen JC, Zhang HL, Liu J, Lin Y, et al. (2022). Phenylalanine impairs insulin signalling and inhibits glucose uptake through modification of IRβ. *Nat Commun*. 13(1):4291. doi:10.1038/s41467-022-32000-0 PMID:35879296

METHYLEUGENOL

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 93-15-2 (Chemical Abstracts Service, 2022a)

EC/List No.: 202-223-0 (ECHA, 2022)

Chem. Abstr. Serv. name: 1,2-dimethoxy-4-(2-propen-1-yl)-benzene (Chemical Abstracts Service, 2022a)

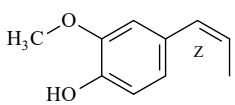
IUPAC systematic name: 1,2-dimethoxy-4-(prop-2-en-1-yl)benzene (ECHA, 2022)

Synonyms: 1-allyl-3,4-dimethoxybenzene; 4-allyl-1,2-dimethyoxybenzene; 4-allyl-veratrole; benzene, 4-allyl-1,2-dimethoxy-; benzene, 1,2-dimethoxy-4-(2-propenyl)-; 1,2-dimethoxy-4-allylbenzene; 3,4-dimethoxyallylbenzene; 1-(3,4-dimethoxyphenyl)-2-propene; 1,3,4-eugenol methyl ether; eugenyl methyl ether; methyl eugenol; *O*-methyleugenol (NCBI, 2022).

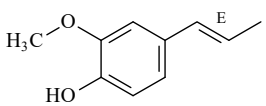
1.1.2 Structural and molecular information

Relative molecular mass: 178.23 (Chemical Abstracts Service, 2022a)

Chemical structure: (Chemical Abstracts Service, 2022a)



Z-(*cis*-) isomer



E-(*trans*-) isomer

Molecular formula: $C_{11}H_{14}O_2$ (Chemical Abstracts Service, 2022a).

1.1.3 Chemical and physical properties

Description: colourless to pale yellow liquid with a clove–carnation odour and a bitter taste (NTP, 2000; Burdock, 2010)

Odour threshold: 820 ppb [µg/L] in water (Ohloff, 1978)

Boiling-point: 254.7 °C (Haynes, 2017)

Melting-point: –2 °C (Haynes, 2017)

Density: 1.0396 g/cm^3 at 20 °C (Haynes, 2017)

Solubility: soluble in ethanol, ethyl ether, chloroform and most other organic solvents; insoluble in water, ethylene glycol, and propylene glycol (NTP, 2000)

Vapour density: > 1.0, relative to air (NCBI, 2022)

Flash-point: 99 °C (NCBI, 2022)

Volatility: evaporates readily at room temperature (NTP, 2000)

Vapour pressure: 0.01 mm Hg (NCBI, 2022)

Auto-ignition temperature: 360 °C at 91 kPa (ECHA, 2022)

Explosion limit: non-explosive (ECHA, 2022)

Octanol/water partition coefficient (P): log K_{ow} = 3.45 (Griffin et al., 1999)

Stability and reactivity: darkens and slowly thickens when exposed to air (NTP, 2000).

1.1.4 Commercial products and impurities

Commercial qualities with purities in the range of 90–94%, 95–98%, and ≥ 99% are available (Chemical Abstracts Service, 2022b). Eugenol is a common impurity with a maximum of 1.0%. (Elan Chemical Co., 2007). β-Caryophyllene (0.68%) and α-humulene (0.08%) were reported as major impurities when eugenol from clove leaf oil was used to produce methyleugenol (Riyanto et al., 2016).

1.2 Production and use

1.2.1 Production process

Methyleugenol is produced by the methylation of eugenol (Burdock, 2010). For example, eugenol may be isolated from leaf oil of *Syzygium aromaticum* (clove), followed by methylation using dimethyl sulfate (Riyanto et al., 2016; Kurniawan et al., 2017). The methylation can be achieved using a one-pot synthesis strategy followed by vacuum fractionation (Agustian et al., 2019). Methyleugenol may also be directly extracted from a variety of plant materials by steam distillation or with organic solvents (Environment Canada, 2010). [The Working Group was unable to find information about which process is currently preferred to produce methyleugenol.]

1.2.2 Production volume

The annual production of methyleugenol in the USA in 1990 was estimated at 11.4 tonnes (NTP, 2000). According to information from 2022, methyleugenol is manufactured in and/or imported to the European Economic Area at a volume of ≥ 1 to < 10 tonnes per annum (ECHA, 2022).

1.2.3 Uses

(a) Direct uses of the isolated compound

Methyleugenol has been used for its delicate clove–carnation odour as a flavouring agent in jellies, baked goods, non-alcoholic beverages, chewing gum, candy, puddings, relishes, and ice cream (Burdock, 2010; IARC, 2013). The presence of methyleugenol in food products may have decreased substantially after the prohibition of its use as such in foods and beverages in the European Union (EU) in 2008 and in the USA in 2018 (see Section 1.5).

Methyleugenol has also been widely used as a fragrance ingredient in various consumer products (NTP, 2000), e.g. in carnation and lilac compositions (Panten and Surburg, 2016). The Consumer Products Information Database (CIPD) lists the use of methyleugenol in six products, including air and fabric fresheners, home pest control products, and automobile cleaners and fresheners (CPID, 2022). According to the Substances in Preparations in Nordic Countries (SPIN) database (SPIN, 2023), four methyleugenol preparations were used in 2000 in the sale, maintenance, and repair of motor vehicles and motorcycles, and in the retail sale of automotive fuel. In 2020, methyleugenol was reported to be present in four cleaning/washing agents (SPIN, 2023).

Methyleugenol is classified as a parapheromone (Australian Government, 2005), and it is used as an insect attractant to male fruit flies in combination with insecticides (NTP, 2000;

NCBI, 2011; Metcalf and Horowitz, 2014a). It is very strongly attractive for the oriental fruit fly, *Bactrocera dorsalis*, and many closely related species (Metcalf and Horowitz, 2014b). In male annihilation programmes for *Bactrocera* species, fibreboard blocks are treated with methyleugenol (the volatile attractant, 15 g/ha) and contact insecticides such as malathion or naled (1 g/ha) (Metcalf and Horowitz, 2014b). Methyleugenol has been used for this purpose in programmes to eradicate the oriental fruit fly in the USA since the 1980s (Turner et al., 1989; US EPA, 2006).

In veterinary medicine, methyleugenol has been used as an anaesthetic agent in rats and mice undergoing surgical procedures (NCBI, 2022).

[The Working Group noted that the literature is often unclear as to whether methyleugenol is used as described above directly or indirectly, due to the use of various plant materials and essential oils that contain it.]

(b) Indirect uses due to natural occurrence in various plant species

Most of the present use of methyleugenol in foods, beverages, or herbal medicinal products may occur inadvertently because of its natural occurrence in essential oils of various herbs and spices (Grosch et al., 2013) (see Section 1.4).

In intensive animal production, laurel leaf oil can be used as a feed additive for fattening piglets, poultry, or fin fish (EFSA, 2023).

1.3 Detection and analysis

Methods for the extraction and quantitation of methyleugenol have been reviewed (Turner and Shuker, 2006; Dang and Quirino, 2021a). Samples are homogenized, and methyleugenol is separated using various extraction techniques, including solvent extraction including liquid–liquid extraction, liquid-phase microextraction, solid-phase extraction, and supercritical fluid extraction. Extraction efficiency can be increased by the use of ultrasound or microwaves. Distillation (steam or hydro-) is commonly used for the extraction of methyleugenol from spices and herbal drugs. Sample preparation techniques, methods of analysis, and the limits of detection (LODs) from a selection of recent studies are presented in Table 1.1.

1.3.1 Air

Gas chromatography-mass spectrometry (GC-MS) has been used to quantify methyleugenol in indoor air (Lamas et al., 2010a) and tobacco smoke (Stanfill and Ashley, 2000) with sample collection using absorbents or filters, respectively.

1.3.2 Water

Methyleugenol in water can be measured using liquid chromatography (LC) (Shaver and Bull, 1980) and in wastewater effluent using liquid chromatography-mass spectrometry (LC-MS) (Clark et al., 1991).

1.3.3 Food

GC-MS (Lopez et al., 2015) or gas chromatography-tandem mass spectrometry (GC-MS/MS) have been used for the quantification of methyleugenol in foods and beverages (Bousova et al., 2011; Ke et al., 2016; Li et al., 2018). A GC-MS method was compared with a nuclear magnetic resonance (NMR)-based method that involves only minimal sample preparation for the measurement of methyleugenol in food. GC-MS proved more sensitive, precise, and suitable for regulatory purposes but was time-consuming and expensive. NMR can be used for a faster screening analysis and appears to be ideal for conducting larger surveys to estimate human exposure (Grosch et al., 2013). [The Working Group noted that NMR would not detect very low levels (LOD, 2.1 mg/L), which in food analysis is

Table 1.1 Analytical methods for the measurement of methyleugenol in various matrices

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Air				
Indoor air	Vacuum pump; activated magnesium silicate (Florisil) adsorbent in glass tube; ultrasound-assisted solvent extraction	GC-MS (0.027 µg/m^3)		Lamas et al. (2010a)
Tobacco smoke particulate	Smoking machine; glass fibre (Cambridge) filter; hexane/MDAP extraction, SPE	SIM-GC-MS (5.1 ng/cigarette)		Stanfill and Ashley (2000)
Water				
Water	Dichloromethane extraction	GC, HPLC (NR)		Shaver and Bull (1980)
Water	No pre-treatment	EFM-based LFIA (16.5 µg/kg)		Lei et al. (2023)
Wastewater effluent	Liquid–liquid (methylene chloride) extraction	LC-MS and GC-MS (NR)		Clark et al. (1991)
Food				
Allspice	Powder hydrodistillated, essential oil diluted in dichloromethane; head-space SPME	GC-MS (NR)		Bajer et al. (2016)
Cinnamon	Ultrasound extraction in methanol	HPLC-UV (0.10 µg/cm^3)		Gursale et al. (2010)
Pepper	Ultrasound extraction with ethyl acetate (SLE)	GC-HRMS-Q-Orbitrap (10 µg/kg)	Validated for black pepper according to SANTE 11813/2017 guidelines.	Rivera-Pérez et al. (2020); European Commission (2017)
Beverages	Head-space SPME	GC-MS/MS (1 µg/kg)		Bousova et al. (2011)
Beverages	Ethyl acetate extraction with $MgSO_4$ and NaCl	GC-MS (62.5 µg/kg)		Lopez et al. (2015)
Beverages	Ultrasound extraction in ethyl acetate with $MgSO_4$ and NaCl, clean-up with PSA	SIDA-GC-MS/MS (0.4 µg/kg)		Li et al. (2018)
Rose water	Liquid–liquid extraction: ultrasound extraction in dichloromethane; SPE: polystyrene-based sorbent and dichloromethane extraction or silica-based sorbent and methanol extraction	GC-MS (0.780 µg/L)		Canbay (2017)
Flavouring ingredients	Ultrasound extraction in methanol	HPLC-UV (0.03 µg/mL)		Dang and Quirino (2021b)
Foods, solid	Homogenization in blender; head-space SPME	GC-MS/MS (10 µg/kg)		Bousova et al. (2011)

Table 1.1 (continued)

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Food	Homogenized in 60% ethanol	^{1}H NMR (2.1 mg/L)		Grosch et al. (2013)
Food	Homogenization using liquid nitrogen; ethyl acetate extraction with $MgSO_4$ and NaCl, clean-up with PSA	GC-MS (500 µg/kg)		Lopez et al. (2015)
Fish fillet	Homogenization, ultrasound extraction in hexane, SPE with ethyl acetate	GC-MS/MS (0.2 µg/kg)		Ke et al. (2016)
Fish and meat	Homogenization, ultrasound extraction in ethyl acetate with $MgSO_4$ and NaCl, clean-up with PSA	SIDA-GC-MS/MS (20 µg/kg)		Li et al. (2018)
Fish	Homogenization, extraction by acetonitrile, SPE, polypropylene sorbent	UPLC-MS/MS (0.03 µg/kg[a])		Liu et al. (2019b)
Fish	Homogenization, ultrasound extraction with acetonitrile, air drying at room temperature, dissolved in methanol solution	EFM-based LFIA (16.7 µg/kg)		Lei et al. (2023)
Fish	Ultrasound extraction in acetonitrile and Na_2SO_4, a second extraction with acetonitrile; degreasing by *n*-hexane; concentration by pressure blowing, dissolved in methanol solution	Electrochemical detection. Pt NPs@RL-SiO_2/GCE (0.16 µmol/L)		Shi et al. (2021)
Fish, shrimp	Ultrasound extraction in acetonitrile; DSPE with polystyrene-glycidylmethacrylate microspheres (PS-GMA), clean-up with PSA and C18; DMSO-assisted concentration	HPLC-UV (43 µg/kg)		Shi et al. (2022)
Fish, shrimp	Fluorinated covalent organic polymer adsorbent, SPME with acetonitrile	HPLC-UV-vis (3.3 µg/kg)		Wang et al. (2021a)
Herbal medicines				
Manchurian wildginger (*Asarum* spp)	Powderization, ultrasound extraction in methanol	HPLC-UV-Vis (2.17 µg/g)		Chen et al. (2009)
Mosla soochowensis	Supercritical fluid extraction with carbon dioxide	GC-MS (NR)		Chen and Wu (2005)
Uncaria hook (*Uncaria rhynchophylla*)	Hydrodistillation	GC-MS (NR)		Iwasa et al. (2015)
Aniba canelilla	Hydrodistillation, head-space SPME	GC-FID/MS (0.16 µg/mL)		Kreutz et al. (2018)
Pimenta pseudocaryophyllus	Hydrodistillation	HPLC UV-Vis (NR)		Niculau et al. (2018)
Ocimum gratissimum, Ocium campechianum	Microwave-assisted hydrodistillation	GC-MS (NR)		Pino Benitez et al. (2009)
Tea tree oil	Mixing with *n*-hexane and *n*-tetradecane	GC-MS (150 µg/L)		Raymond et al. (2017)

Table 1.1 (continued)

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Acori tatarinowii rhizome essential oil	Hydrodistillation	GC-MS (NR)		Yan et al. (2020)
Asarum oils	Distill/steam dried plants to obtain volatile oil; desiccate oil using anhydrous sodium sulfate	GC-MS (NR)		Yang (1986); Xu, (1984, 1986)
Asarum herbs	Hydrodistillation	GC-MS (0.01 µg/mL, LOQ)		Yao et al. (2020)
Kaixin San	Powderization, head-space, no pre-treatment Crushing in grinder, extraction by heating with petroleum ether	GC-IMS (NR) GC-MS (0.02 µg/mL, LOQ)		Yin et al. (2021)
Mahuang Fuzi Xixin	Powderization, microwave extraction in methanol	UPLC-PDA (0.62 µg/mL)		Zhang et al. (2015)
Consumer products				
Cigarette tobacco	SPME	GC-MS (0.0022 µg/g)		Stanfill and Ashley (1999)
Aromatherapy massage oil	Dual dispersive liquid–liquid microextraction	GC-MS (3.0 ng/mL)		Tsai et al. (2015)
Cosmetics				
Creams, lotions	Solid-phase dispersion-pressurized liquid extraction	GC-MS [12 µg/kg]		Lamas et al. (2010b)
Creams	Direct contact sorptive tape extraction	GC-MS (15 µg/kg)		Sgorbini et al. (2010)
Human biological specimens				
Serum	SPE	IDGC-MS (3.1 pg/g)		Barr et al. (2000)
Serum	SPE	GC-HRMS (3.1 pg/g)		Schecter et al. (2004)

DMSO, dimethyl sulfoxide; DSPE, dispersive solid-phase extraction; EFM, europium-fluorescent microspheres; GC-MS, gas chromatography-mass spectrometry; GCE, glassy carbon electrode; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; IDGC, isotope dilution gas chromatography; IMS, ion mobility spectrometry; LFIA, lateral-flow immunoassay; LOD, limit of detection; MDAP, 3′,4′-methylenedioxyacetophenone; MS/MS, tandem mass spectrometry; $MgSO_4$, magnesium sulfate; Na_2SO_4, anhydrous sodium sulfate; ^{1}H NMR, proton nuclear magnetic resonance; NR, not reported; PDA, photometric diode array; PSA, primary secondary amine; PS-GMA, polystyrene-glycidylmethacrylate microspheres; RL-SiO_2, raspberry-like silicon dioxide; SIDA, stable isotope dilution assay; SIM, selected ion monitoring; SLE, solid–liquid extraction; SPE, solid-phase extraction; SPME, solid-phase microextraction; UPLC, ultra-high-performance liquid chromatography; UV, ultraviolet.

[a] The LOD for methyleugenol in fish is 0.10 µg/kg (signal:noise ratio, 10). The authors could detect 0.03 µg/kg (signal:noise ratio, 3) in their samples. This could be interpreted as an LOQ of 0.10 µg/kg and LOD of 0.03 µg/kg.

rarely needed, see Section 1.4.] High-performance liquid chromatography with ultraviolet detection (HPLC-UV) was used to quantify methyleugenol in spices or flavouring ingredients (Gursale et al., 2010; Dang and Quirino, 2021b) and in seafood (Wang et al., 2021a; Shi et al., 2022).

Rivera-Pérez et al. (2020) used ultrasound-assisted solvent extraction (UAE) and GC-MS to measure methyleugenol; the analytical method was validated for black pepper according to SANTE/11813/2017 guidelines (European Commission, 2017).

An immunoassay for the determination of methyleugenol was developed recently. The LOD was 16.5 µg/kg in water and 16.7 µg/kg in fish (Lei et al., 2023).

1.3.4 Medicinal herbs or plants

Both GC-MS and HPLC-photo-diode assay have been used for the quantification of methyleugenol in aromatic plants (e.g. Miele et al., 2001; Kothari et al., 2004; Kreutz et al., 2018; Yao et al., 2020), oils (e.g. Verdian-rizi and Hadjiakhoondi, 2008; Zheljazkov et al., 2008; Pino Benitez et al., 2009; Raymond et al., 2017; Yan et al., 2020) and herbal drugs (e.g. Chen et al., 2009; Zhang et al., 2015; Yin et al., 2021).

1.3.5 Consumer products

GC-MS has been used to quantify methyleugenol in cosmetic creams and lotions (Lamas et al., 2010b; Sgorbini et al., 2010), aromatherapy massage oil (Tsai et al., 2015), and cigarette tobacco (Stanfill and Ashley, 1999).

1.3.6 Biological specimens

Methyleugenol can be measured in human serum by solid-phase extraction followed by isotope dilution gas chromatography-high-resolution mass spectrometry (GC-HRMS), with an LOD of 3.1 pg/g (Barr et al., 2000; Schecter et al., 2004).

1.4 Occurrence and exposure

1.4.1 Environmental occurrence

Methyleugenol is a compound that occurs naturally in the essential oils of more than 450 plant species, including *Artemisia dracunculus* (tarragon), *Syzygium aromaticum* (clove), *Daucus carota* (carrot), *Myristica fragrans* (nutmeg), *Rosmarinus officinalis* (rosemary), *Laurus nobilis* (bay leaf or laurel), *Ocimum basilicum* (basil), and *Thymus serpylloides* (thyme) (De Vincenzi et al., 2000; European Medicines Agency, 2005; Burdock, 2010; Government of Canada, 2010; Tan and Nishida, 2012; Eisenreich et al., 2021). The amount of methyleugenol in the essential oils extracted from these plants is variable and depends on several factors, such as the plant variety, maturity at harvest, harvesting method, storage conditions, and extraction method (Smith et al., 2002; Yahyaa et al., 2019). Several species have essential oils that contain more than 90% methyleugenol, for example, *Croton malambo* (Euphorbiaceae), *Cinnamomum cordatum* (Lauraceae), *Melaleuca bracteata*, *M. ericifolia*, *M. leucadendra*, *M. quinquenervia*, *Pimenta racemosa* (all Myrtaceae), *Piper divaricatum* (Piperaceae), and *Clusena anisata* (Rutaceae). In an additional 68 species, the essential oils of either the whole plant or a part of the plant have a methyleugenol content that ranges from 20% to 90% (Tan and Nishida, 2012). For example, the wood oil of the huon pine (*Lagarostrobos franklinii*) grown in Tasmania, Australia, contains 74% methyleugenol (Brophy et al., 2003).

An overview of concentrations of methyleugenol in the essential oils of common fruits and spices is given in Table 1.2.

Methyleugenol occurs naturally in foods and beverages, can be added to foods as a flavouring agent, and occurs in some herbal medicinal products. Other sources include cosmetics and personal care products, insect repellents, and some tobacco and cannabis products. The

Table 1.2 Relative concentrations of methyleugenol in the essential oils of some common fruits and spices

Common name	Botanical name	Concentration (%)
Allspice berries	*Pimenta dioica*	0.1–68
Allspice leaves	*Pimenta dioica*	2–15.4
Anise seeds	*Pimpinella anisum*	0.1–2
Asafetida	*Ferula assa-fetida*	0.03
Basil leaves	*Ocimum basilicum*	< 0.2–87
Black pepper berries	*Piper nigrum*	0.9
Canadian snake root	*Asarum canadense*	11–45.0
Cardamom	*Elettaria cardamomum*	0.1
Clove	*Syzygium aromaticum*	0.03–0.5
Common sage	*Salvia officinalis*	1.45–19.8
Fennel	*Foeniculum vulgare*	0.18–1
Guava fruit	*Psidium guajava*	0.2
Hyssop	*Hyssopus officinalis*	0.01–43.9
Laurel leaves	*Laurus nobilis*	0.02–8.3
Lemongrass	*Cymbopogon flexuosus*	0.05–82.4
Lovage	*Levisticum officinale*	0.001–1.3
Mugwort	*Artemisia vulgaris*	1–7
Nutmeg kernel	*Myristica fragrans*	0.002–17.9
Parsley	*Petroselinum crispum*	0.01–3.8
Peppermint	*Mentha piperita*	0.1
Rhubarb	*Rheum rhabarbarum*	2–7
Rocket/rucola salad	*Eruca sativa*	0.9
Rose	*Rosa (various species)*	0.04–6.9
Rosemary	*Rosmarinus officinalis*	< 0.01–1.5
Chinese star anise seeds	*Illicium verum*	0.11–0.4
Oregano	*Origanum vulgare*	16.5
Tarragon	*Artemisia dracunculus*	0.03–38
Thyme	*Thymus vulgaris*	0.1–0.2

Compiled by the Working Group using data from Eisenreich et al. (2021); De Vincenzi et al. (2000); Tan and Nishida (2012); Davidsen et al. (2023a); Rosol et al. (2023); Rietjens et al. (2023); Davidsen et al. (2023b); Environment Canada (2010); and Zhao et al. (2021).

production and use of methyleugenol can result in its release to the environment through various waste streams, and its use as an insect attractant is expected to result in its direct release to the environment (NCBI, 2011).

(a) Air

Methyleugenol is expected to exist almost exclusively as a vapour in the ambient atmosphere (NCBI, 2011). Vapour-phase methyleugenol is degraded in the atmosphere by reaction with photochemically generated hydroxyl radicals (Meylan and Howard, 1993). The atmospheric half-life has been estimated at between 5 hours and 1 day (Atkinson and Carter, 1984; Meylan and Howard, 1993). Methyleugenol lacks functional groups susceptible to hydrolysis and is therefore not expected to be hydrolysed in the environment (Lyman, 1990), nor is it expected to be directly photolysed, because of its lack of absorption in the environmental UV spectrum (> 290 nm) (NCBI, 2011).

In the context of the use of methyleugenol in bait stations and lures during invasive pest con-

trol programme activities in California, USA, a screening-level air dispersion model estimated a maximum 1-hour air concentration of 0.052 µg/m³ to which the average resident living near the treated area is probably exposed (OEHHA, 2018). In Los Angeles County, California, USA, the air around insect traps baited with methyleugenol was analysed for the presence of the substance; methyleugenol was found in samples taken from within a distance of 5 m from the traps during the first few days (323–1050 ng/m³ on the day of application) (Turner et al., 1989).

(b) Water

Methyleugenol has been detected at a concentration of 5 ppb [0.005 mg/L] in the effluent of a publicly owned treatment works in New Jersey, USA, located at an industrial site (industrial contribution to the influent was 18%) (Clark et al., 1991), and at concentrations of 0.001–0.002 mg/L in the raw and partially treated effluent of an unbleached kraft paper mill, but not in the final effluent (Keith, 1976). Methyleugenol was found to dissipate rapidly from water. Methyleugenol had a half-life of approximately 6 hours in water at 32 °C and 34 hours in water at 22 °C (Shaver and Bull, 1980). Its potential for bioconcentration in aquatic organisms is low, and biodegradation in water may be an important environmental process (NCBI, 2011).

(c) Soil

On the basis of its physical and chemical properties (see Section 1.1), methyleugenol is not expected to adsorb to suspended solids and sediment and is expected to be highly mobile in soil. However, it was immobile in silty loam, Lufkin fine sandy loam, Houston clay, and Brazos river bottom sand from Texas, USA (Shaver, 1984). Volatilization of methyleugenol from moist soil surfaces is expected to be an important process (NCBI, 2011). Methyleugenol had a half-life of approximately 6 hours in soil at 32 °C and 16 hours in soil at 22 °C (Shaver and Bull, 1980). Methyleugenol is not expected to volatilize from dry soil surfaces on the basis of its vapour pressure (Perry and Green, 1984, cited by NCBI, 2011). Biodegradation may be an important environmental process in soil (NCBI, 2011). [The Working Group noted that limited data were available to support or refute the theoretical assumptions about the fate of the compound in soil. Some of the information available appeared contradictory (e.g. methyleugenol volatilizes from moist but not from dry soil) and in need of experimental validation.]

(d) Consumer products

Some essential oils, including citronella (*Cymbopogon* spp.), basil (*Ocimum* spp.), bay (*Laurus nobilis*), and tea tree (*Melaleuca* spp.), that may contain a high percentage of methyleugenol are used as fragrances in consumer products such as personal care and household cleaning products (Environment Canada, 2010). Citronella oil is an active ingredient in some commercially available personal insect-repellent lotions and sprays that are applied to the skin. It is also used in outdoor candles and torches as an ambient insect repellent (Environment Canada, 2010).

The usual and maximum concentrations of methyleugenol in some cosmetic products were 0.02% and 0.2% in soap, 0.002% and 0.02% in detergents, 0.01% and 0.05% in creams and lotions, and 0.3% and 0.8% in perfumes, respectively (Opdyke, 1975).

In a study of eight commercial brands of cigarettes in the USA, only one brand was found to contain methyleugenol at above the LOD (5.1 ng/cigarette) in the smoke particles of unblocked cigarettes (average of three measurements, 46.5 ng in the particulate matter of one cigarette) (Stanfill and Ashley, 2000). The effect of blocking the ventilation holes in the cigarette filter was investigated in another brand (containing methyleugenol at 81 ng/cigarette). Methyleugenol was not detected in the unblocked

cigarette smoke but was detected in the smoke when the holes were partially or completely blocked (6.4 ng and 10.8 ng in the smoke particulate of one cigarette, respectively).

Bidi cigarettes (small hand-rolled cigarettes produced mainly in India) have been sold in the USA in a wide variety of exotic (e.g. clove and mango) and candy-like flavours (e.g. raspberry, dewberry, and chocolate). Some of these tobacco flavourings contain alkenylbenzenes, including methyleugenol (Stanfill et al., 2003, 2006). Methyleugenol was found in 11 out of 20 bidi cigarette brands purchased in the USA and in Indian bidi cigarettes at levels ranging from 0.49 µg/g to 61 µg/g. Lower levels of methyleugenol, ranging from 0.003 to 0.86 µg/g, were found in US cigarettes (Stanfill et al., 2003).

In a study by Stanfill et al. (2006), compounds were analysed in the combustible parts of the filler and wrapper material consumed during smoking of bidi cigarettes and US cigarettes. Methyleugenol was not detected in the three US cigarettes (< 6.3 µg/cigarette) but was detected in two bidi cigarettes (≤ 36.6 µg/cigarette).

The volatile oil of *Cannabis sativa* may contain approximately 0.1–0.2% methyleugenol (Wanas et al., 2020; Pieracci et al., 2021).

(e) Food

Some of the plant species containing methyleugenol are culinary herbs and spices, e.g. basil, tarragon, lemongrass, bay leaf, nutmeg, allspice, cloves, and mace (Siano et al., 2003; Ávila et al., 2009; WHO, 2009; Environment Canada, 2010; Sharopov et al., 2016; Złotek et al., 2016; Rietjens et al., 2023; Davidsen et al., 2023a). Additionally, some edible fruits, such as grapefruit and bananas also contain methyleugenol (Smith et al., 2002).

Commercially prepared foods could also contain methyleugenol, including ice cream; bakery products such as cookies, pies, pastries, and buns; puddings and other gelatin-based desserts; condiments, soups and sauces, especially pesto; various meat products; candy and chewing gum; and beverages prepared with spices and herbs containing methyleugenol (Environment Canada, 2010). An overview of methyleugenol in foods is given in Table 1.3.

(i) Historical occurrence (before changes in regulations)

Historically, the pure compound has been reported to be added as a flavouring agent to bakery products (27–40 mg/kg), chewing gum (10–45 mg/kg), condiments and relishes (3–7 mg/kg), frozen dairy products (15–17 mg/kg), gelatins and puddings (15–17 mg/kg), hard candy (0.6 mg/kg), non-alcoholic beverages (9–12 mg/kg), and soft candy (19–24 mg/kg) (Burdock, 2010).

Some brands of cookies available in the USA were found to contain methyleugenol at approximately 3.3 mg/kg as an added flavouring, i.e. 18 µg/cookie. Lower concentrations were found (in decreasing order) in other brands of gingersnaps, cinnamon-flavoured oatmeal, vinaigrette salad dressing, cinnamon-flavoured mints, chewing gum, cake doughnuts, and cola beverages. In 20 other brands of gingersnaps and other cookies, doughnuts, colas, and foods flavoured with cinnamon, nutmeg, or ginger, methyleugenol was either not detected or was found at concentrations of < 0.05 mg/kg (Schecter et al., 2004).

[The Working Group noted that these levels are no longer to be expected in foods because of changes in the regulation of use of methyleugenol as a food flavouring (see Section 1.5 for details).]

(ii) Occurrence not affected by regulations and current occurrence (after changes in regulations)

Processed foods can be flavoured with essential oils or extracts of specific plants that contain methyleugenol, such as sassafras (*Sassafras albidum*), tarragon (*Artemisia dracunculus* L.), laurel (*Laurus nobilis* L.), and Ceylon citronella (*Cymbopogon nardus*) (Burdock, 2010).

Table 1.3 Concentrations of methyleugenol in selected food products

Food product	Methyleugenol concentration (mg/kg)
Vienna sausage	0.10–0.14
Tomato sauce with basil	0.01–0.33
Hard candy*	0.6**
Gingersnaps (cookies)*	3.3
Pesto sauce	0.01–5.3
Condiments and relishes*	3–7**
Non-alcoholic beverages*	0.03–12
Frozen dairy products*	15–17**
Gelatins and puddings*	15–17**
Soft candy*	19–24**
Bakery products*	27–40**
Chewing gum*	10–45**
Indonesian instant herbal beverages	3–444***

* Historical use in flavoured products.
** "Usual" and "max." (maximum) values.
*** Range for detected samples. Detection rate was 43%.
Compiled by the Working Group using data from Siano et al. (2003); Schecter et al. (2004); Burdock (2010); Grosch et al. (2013); and Suparmi et al. (2019).

Methyleugenol was measured in food products purchased on the Italian national market and found to be present in tomato sauce with basil (0.01–0.33 mg/kg) and in Vienna sausage (0.10–0.14 mg/kg), probably due to the addition of nutmeg (Siano et al., 2003). In a survey of 120 German food products suspected of containing methyleugenol, the highest levels were found in basil, allspice, and nutmeg (mean, 202 mg/kg for basil, 1351 mg/kg for nutmeg and 4288 mg/kg for allspice), with lower mean levels in tarragon and laurel leaves (56 mg/kg and 26 mg/kg, respectively). Other products such as teas, beverages, and compound foods contained mean levels of around 1 mg/kg and below (Grosch et al., 2013). In a survey of 114 herbal beverages suspected to contain methyleugenol in Indonesia, methyleugenol was detected in 49 samples (2.6–444 mg/kg) (Suparmi et al., 2019).

Whole citrus fruit samples were collected near insect traps baited with methyleugenol in Sacramento County, California, USA, in September 1988. Methyleugenol was detected at concentrations ranging from 70 to 210 ppb [µg/kg] in some fruit from two of the four sites sampled (Turner et al., 1989).

(f) Herbal medicinal products

The WHO monographs on selected medicinal plants describe the use of Folium Ocimi Sancti (fresh or dried leaves of *Ocimum sanctum* L.), which contains methyleugenol as a major constituent (up to 86%) of its essential oil, in traditional systems of medicine and in folk medicine (WHO, 2002). Similarly, Xixin (Asari radix et rhizoma) containing methyleugenol (Asari radix up to 39% in its essential oil), has been used in traditional Chinese medicine as a local anaesthetic and a remedy for toothache, headache, and inflammatory diseases (Wang et al., 1997; Wang et al., 2015). Fennel fruits, possibly containing methyleugenol as a minor constituent (up to 1%) of the essential oil, have been used as a traditional herbal medicine in Europe and China. Fennel tea is administered as a carminative to infants in private homes and in maternity clinics and is highly appreciated for its mild flavour and good tolerance (Iten and Saller, 2004). In several

EU countries, sweet and bitter fennel herbal tea is traditionally used for treating the symptoms of digestive upsets and as a remedy for cough associated with colds (European Medicines Agency, 2008).

Methyleugenol is a component of several essential oils that are sold for use as complementary medicines (Government of Canada, 2010).

1.4.2 Occupational exposure

In the context of use in oriental fruit fly eradication programmes, the United States Environmental Protection Agency (US EPA) states that workers using these insect traps are not expected to have dermal contact with the traps, although dermal contact could occur during preparation of the traps, e.g. preparation of the methyleugenol mixture or filling of the traps with the mixture (US EPA, 2006).

In the USA, the National Institute for Occupational Safety and Health (NIOSH) conducted a National Occupational Exposure Survey (NOES) in 1981–1983. NIOSH randomly selected 4490 fixed-site worksites in 522 different industries employing about 1 800 000 workers in 377 occupational categories. NOES revealed that 12 682 [95% confidence intervals (95% CI), 7402–18 262] employees, including 9413 [95% CI, 4895–13 931] women (74%), were potentially exposed to methyleugenol (NIOSH, 1990a, b, 1994), including 9429 [74% of total] hairdressers or cosmetologists, 967 [8%] machine operators, 827 [7%] packaging and filling machine operators, and 595 [5%] mixing and blending machine operators. [The Working Group noted that these data included all potentially exposed workers and were estimated in the 1980s.] Aromatherapists (professionals who use essential oils for body massages) are liable to be exposed repeatedly to methyleugenol through dermal contact and also through inhalation of vapour (Burfield, 2004).

[The Working Group noted the lack of comprehensive exposure data in an occupational context. Despite lacking exposure data, the Working Group also noted that occupational exposure to methyleugenol by dermal or inhalation routes may occur through the production or use of foods, cosmetics, and insect attractants.]

1.4.3 Exposure of the general population

(a) Exposure data

The average intake of methyleugenol was assessed by the United Kingdom delegation to the Council of Europe and was estimated (for consumers only) to be 13 mg/person per day; the 97.5th percentile was 36 mg/person per day or, expressed on a body-weight basis, 0.19 and 0.53 mg/kg body weight (bw) per day, respectively (Council of Europe, Committee of Experts on Flavouring Substances, 2001; European Commission, 2001). In the USA, Smith et al. (2002) estimated the mean daily per capita intake of methyleugenol from all sources to be approximately 0.8 μg/kg bw per day, with more than 85% resulting from the consumption of basil, allspice, and nutmeg, and their essential oils (Smith et al., 2002). The same authors also highlighted that consumers of some foods containing methyleugenol, such as pesto, could have exposures to methyleugenol that were least 10 times higher, since fresh pesto is prepared from a large quantity of fresh sweet basil (Smith et al., 2002). Burdock (2010) estimated individual exposure to methyleugenol at 0.12 μg/kg bw per day. Miele et al. (2001) estimated intake of methyleugenol from a single serving of pasta with pesto and concluded that it could reach 250 μg/kg [bw] per meal for adults and 500 μg/kg [bw] per meal for children (Miele et al., 2001). Recently, a series of assessments developed under the Flavor and Extract Manufacturers Association of the United States (FEMA) "generally recognized as safe" (GRAS) programme were used to estimate intakes of methyleugenol from the consumption of natural flavouring complexes. Depending on the flavouring ingredients

considered, the intakes were estimated as: for derivatives of basil, nutmeg, parsley, tarragon, and mace, 0.4, 3, 0.01, 0.7, and 0.1 µg/person per day, respectively (Davidsen et al., 2023a); for lemongrass oil, chamomile oils, and citronella oil, 0.0005–0.04 µg/person per day (Rosol et al., 2023); for allspice and anise, fennel-derived, 0.1–0.25 µg/kg bw per day (Rietjens et al., 2023); and for asafetida oil, 0.0007 µg/person per day (Davidsen et al., 2023b).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) also estimated the maximum dietary intake of methyleugenol in the USA, finding an exposure of 424 µg/person per day or about 6–8 µg/kg bw per day for an adult (WHO, 2009).

Levels of exposure to methyleugenol through consumption of instant herbal beverages available on the market in Indonesia were assessed and the estimated daily intake was found to be 0.1–51.2 µg/kg bw per day for adults and 1.1–3.3 µg/kg bw per day for children (Suparmi et al., 2019). Specifically, the consumption of *jamu*, an Indonesian traditional herbal medicine, was estimated to lead to an exposure to methyleugenol equivalents of 0.9–551 µg/kg bw per day (Suparmi et al., 2018). Van den Berg et al. (2011) assessed exposure to methyleugenol from the consumption of different plant food supplements of botanicals and botanical preparations. Possible exposure to methyleugenol was estimated to be in the range of 0.02 to 2960 µg/kg bw per day (Van den Berg et al., 2011). On the basis of an analysis of 120 food products from Germany, exposure was less than 1 µg/kg bw per day for the average population of Germany (Grosch et al., 2013).

Another source of exposure to methyleugenol is the use of personal care products that include essential oils containing methyleugenol. Environment Canada (2010) estimated that daily systemic exposure to methyleugenol in adult women was 1.5 µg/kg bw per day as a result of dermal exposure through the use of body lotion, face moisturizer, skin cleanser, and fragrance including various essential oils containing methyleugenol (Environment Canada, 2010).

On the basis of an assessment by Health Canada (Health Canada, 2004a), exposure to methyleugenol from the use of a personal citronella-based insect repellent has been estimated to be 0.13 µg/kg bw for adults and 0.21 µg/kg bw for children (IARC, 2013).

In conclusion, exposure of the general population to methyleugenol is dominated by the ingestion of food and beverages. In addition to dietary exposure, the use of personal care products containing methyleugenol, as well as insect repellent, represent additional sources of exposure for the general population. [The Working Group noted that the estimations obtained present significant sources of uncertainty, mainly because of the lack of exposure data. Additionally, new regulations concerning the addition of pure methyleugenol to foods might have an impact on the levels reported. For the general population, a daily exposure of low micrograms per kg bw is expected. For some high-level consumers of certain methyleugenol-rich foods, considerably higher levels of exposure of up to milligrams per kg bw are expected. Although exposure through inhalation is expected, no data are presently available.]

(b) Biomonitoring

Few data on biomonitoring in humans are available to determine the exposure of the general population to methyleugenol. The Centers for Disease Control and Prevention in the USA measured methyleugenol levels in serum samples collected during the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994) in a non-representative subset of 206 adults. The results revealed a mean serum methyleugenol concentration of 24 pg/g serum (whole weight), and a range of < 3.1 to 390 pg/g serum (whole weight) (Barr et al., 2000). Methyleugenol was detected in 98% of the samples analysed,

demonstrating that exposure to methyleugenol is ubiquitous in the USA (Barr et al., 2000). Smokers (geometric mean, GM, 17 pg/g for smokers or living with 1 smoker, or 19 pg/g for smokers or living with ≥ 2 smokers) had higher levels of methyleugenol than did non-smokers (GM, 5.6 pg/g for non-smokers with no smokers in the home) (Barr et al., 2000). [The Working Group noted that the report by Barr et al. (2000) did not clearly describe whether the two smoker populations were partly overlapping.] The highest serum levels of methyleugenol were dependent on factors such as diet, genetics, and body weight. It has been noted that because methyleugenol is found in air, water, food and beverages, and oils, day-to-day variations in exposure could have an effect on serum levels of methyleugenol (Albertini et al., 2006). [The Working Group noted that methyleugenol is rapidly metabolized, thus sampling strategy and analytical methods affect the human biomonitoring results for this substance (for more detail about absorption, distribution, metabolism, and excretion, see Section 4.1) (Albertini et al., 2006). For this reason, information on sampling strategies is crucial for the informativeness of biomonitoring studies.]

1.5 Regulations and guidelines

The available regulations for methyleugenol have evolved, mainly due to re-evaluations of its hazard classification (e.g. the previous evaluation by the *IARC Monographs* programme as *possibly carcinogenic to humans*, Group 2B) (IARC, 2013; US FDA, 2004).

In the USA, methyleugenol was classified as GRAS as a food additive by the United States Food and Drug Administration (US FDA) under 21 CFR §172.515 (US FDA, 2004), and its addition as a synthetic flavouring substance was permitted (US FDA, 2010). However, in 2018, the US FDA withdrew its authorization for the use of methyleugenol as a synthetic flavouring substance in foods as a result of a petition submitted by various health, environmental, and consumer rights organizations (US FDA, 2018a). To allow food companies to reformulate their products to remove methyleugenol content, the US FDA set October 2020 as the deadline after which this change in regulations was enforced (US FDA, 2018b).

In the EU, according to the Scientific Committee on Food of the European Commission, and Annex III of Regulation (EC) No. 1334/2008 (European Commission, 2008b), it is not permitted to add methyleugenol as such to foods for flavouring purposes. This regulation entered into force in January 2009 and has applied since January 2011. Furthermore, maximum levels of methyleugenol, (which occurs naturally in certain foods with flavouring properties) were established for specific food groups, i.e. dairy products, 20 mg/kg; meat preparations and meat products, including poultry and game, 15 mg/kg; fish preparations and fish products, 10 mg/kg; soups and sauces, 60 mg/kg; ready-to-eat savouries, 20 mg/kg; and non-alcoholic beverages, 1 mg/kg. It must be noted that these maximum levels do not apply for compound foods that contain no added flavourings and to which the only food ingredients with flavouring properties that have been added are fresh, dried, or frozen herbs and spices. As an example, pesto made with basil is permitted in food preparations, regardless of its methyleugenol content.

The International Organization for Standardization (ISO) has provided international standards for minimum and maximum percentages of methyleugenol in essential oils from various plant species; these oils are widely used in the food and perfumery industries (ISO, 2023; Table 1.4). [The Working Group noted that ISO values that include minimum and maximum requirements for methyleugenol content are used for product standardization and are not health-based values.]

Table 1.4 International standards regarding methyleugenol content in various plant essential oils

Common name	Botanical name	Methyleugenol content (% in essential oil)		ISO Standard No.
		Minimum	Maximum	
Tarragon leaves	*Artemisia dracunculus* L.	ND	< 1.0	10115:2013
Dehydrated tarragon (leaves and flowering tops)	*Artemisia dracunculus* L.	Main constituent[a]		7926:1991
Basil leaves	*Ocimum basilicum* L.	0.3	2.5	11043:1998
Magnolia flower, China type	*Michelia* × *alba* DC	1.2	4.4	17382:2007
Bay leaves	*Pimenta racemosa* (Mill.) JW Moore	0.1	2.0	3045:2004
Rose flowers	*Rosa* × *damascena* Miller	Not reported[a]		9842:2003

ISO, International Organization for Standardization; ND, not determined.
[a] No quantitative range provided in standard.
All ISO standards from ISO (2023).

Regarding the use of methyleugenol in cosmetics, the European Commission Directive 2002/34/EC included methyleugenol in the list of substances that cosmetic products must not contain (Annex II of Council Directive 76/768/EEC), except for normal content in the natural essences used and provided the concentration does not exceed 0.01% in fine fragrance; 0.004% in eau de toilette; 0.002% in fragrance cream; 0.0002% in other leave-on products and in oral hygiene products; and 0.001% in rinse-off products (European Commission, 2002). The same approach has been taken in Canada (Health Canada, 2010).

For insect repellents containing citronella oil, Health Canada's Pest Management Regulatory Agency (PMRA) proposed a phase-out of citronella-based personal insect repellents, based on the re-evaluation of available information on these products, including the fact that these products typically contain methyleugenol (Health Canada, 2004b). Subsequently, the PMRA re-evaluated the associated risks and established guidelines for the registration of nonconventional pest control products, noting that the level of methyleugenol in the final product must be less than 0.0002% (2 ppm) (Health Canada, 2017).

For the use of the technical product of citronella on non-food crops to control for ragwort, the European Commission has specified that the product must contain no more than 0.1% of the manufacturing impurities methyleugenol and (structurally related) methylisoeugenol (European Commission, 2008a). In Australia and the USA, the use of methyleugenol in insect traps and lure products as an insect attractant is permitted as a measure in eradication programmes (Australian Government, 2005; US EPA, 2010).

According to European Standard EN 17648 regarding liquids for electronic cigarettes (e-liquids), the maximum level of methyleugenol in the finished e-liquid is 1 mg/kg (CEN, 2022).

[The Working Group noted that no threshold has been established for occupational exposure to methyleugenol.]

1.6 Quality of exposure assessment in key mechanistic studies in humans

Three studies quantified two DNA adducts of methyleugenol ($[^{15}N_5]N^6$-MIE-dA and $[^{15}N_5]N^2$-MIE-dG) in human tissues by ultra-performance

liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (see discussion on DNA adducts findings in Section 4.2.1). The studies by Herrmann et al. (2013) and Tremmel et al. (2017) were conducted on liver tissue samples collected from individuals undergoing liver surgery in Germany. The study by Monien et al. (2015) was conducted on non-tumour pulmonary tissues from patients undergoing surgery in France. The level of exposure to methyleugenol in people from whom liver and lung tissues were collected was not assessed. [The Working Group noted that the presence of methyleugenol–DNA adducts indicates that exposure has occurred, although the source and amount of exposure could not be determined. As the studies did not determine exposure by any other means, no plausible correlation between exposure levels and amounts of DNA adducts could be ascertained. Because of widespread exposure to food and consumer products containing methyleugenol, background exposure of the general population is plausible.]

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

See Table 3.1.

3.1 Mouse

3.1.1 Oral administration (gavage)

In a well-conducted study of chronic toxicity and carcinogenicity that complied with Good Laboratory Practice (GLP), groups of 50 male and 50 female B6C3F$_1$ mice (age, 5–6 weeks) were treated with methyleugenol (purity, 99%) in 0.5% methylcellulose by gavage at a dose of 0 (vehicle control), 37, 75, or 150 mg/kg body weight (bw) on 5 days per week for 105 weeks (NTP, 2000; also reported by Johnson et al., 2000). At study termination, survival was: 38/49, 36/50, 37/50, and 35/50 in males and 31/50, 18/50, 18/50, and 2/50 in females, for the groups at 0 (control), 37, 75, and 150 mg/kg bw, respectively. In males, the probability of survival to study completion for the treated groups was similar to that for the control group. In females, the probability of survival to study completion for the treated groups was significantly lower than that for the control group ($P \leq 0.013$, life-table test). In males, terminal group mean body-weight values decreased by 10%, 16%, and 13% in the groups at 37, 75, and 150 mg/kg bw per day, respectively. In females, terminal group mean body-weight values decreased by 39%, 44%, and 46% in the groups at 37, 75, and 150 mg/kg bw per day, respectively.

In males, there was a significant positive trend in the incidence of hepatocellular adenoma ($P = 0.006$ poly-3 trend test; [$P = 0.024$, Cochran–Armitage trend test]), and the incidence – 26/49 (53%) 43/50 (86%) 38/50 (76%) 39/50 (78%) – was significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P = 0.0003$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0144$, Fisher exact test]; $P = 0.003$, poly-3 test, [$P = 0.0079$, Fisher exact test], respectively), and exceeding the upper bound of the range observed in historical controls from this laboratory – 201/514 (39.1%); range, 21–58%. The incidence of hepatocellular carcinoma – 10/49 (20%) 20/50 (40%) 19/50 (38%) 9/50 (18%) – was significantly increased at 37 and 75 mg/kg bw ($P = 0.030$, poly-3 test, [$P = 0.0281$, Fisher exact test]; and $P = 0.044$, poly-3 test, [$P = 0.0439$, Fisher exact test], respectively). The incidence of hepatocellular carcinoma exceeded the upper bound of the range observed in historical controls from this laboratory at the lowest dose – 102/514 (19.8%); range, 8–38%. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) ($P = 0.018$, poly-3 trend

Table 3.1 Studies of carcinogenicity in experimental animals exposed to methyleugenol

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 5–6 wk 105 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 37, 75 or 150 mg/kg bw, 5 days/wk for 105 wk 50, 50, 50, 50 38, 36, 37, 35	*Liver* Hepatocellular adenoma 26/49 (53%), 43/50* (86%), 38/50* (76%), 39/50** (78%)	$P = 0.006$ poly-3 trend test; [$P = 0.024$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P = 0.0003$, Fisher exact test] *$P < 0.001$, poly-3 test; [$P = 0.0144$, Fisher exact test] **$P = 0.003$, poly-3 test; [$P = 0.0079$, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals used; randomly allocated in groups; adequate duration; males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 201/514 (39.1%); range, 21–58%; hepatocellular carcinoma, 102/514 (19.8%); range, 8–38%; hepatocellular adenoma or carcinoma, 267/514 (52%); range, 25–72%; hepatoblastoma, 2/514 (0.4%); range, 0–3%; hepatocellular carcinoma or hepatoblastoma, 104/514 (20.2%); range, 8–38%; hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma, 267/514 (52%); range, 25–72%.
		Hepatocellular carcinoma 10/49 (20%), 20/50* (40%), 19/50** (38%), 9/50 (18%)	*$P = 0.030$, poly-3 test; [$P = 0.0281$, Fisher exact test] **$P = 0.044$ poly-3 test; [$P = 0.0439$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma 31/49 (63%), 47/50** (94%), 46/50** (92%), 40/50* (80%)	$P = 0.018$, poly-3 trend test; **$P < 0.001$, poly-3 test, [$P = 0.0002$, Fisher exact test] **$P < 0.001$, poly-3 test, [$P = 0.0005$, Fisher exact test] *$P = 0.016$, poly-3 test, [$P = 0.0517$, Fisher exact test]	
		Hepatoblastoma 0/49, 0/50, 1/50 (2%), 3/50 (6%)	$P = 0.019$, poly-3 trend test; [$P < 0.01$, Cochran–Armitage trend test]	
		Hepatocellular carcinoma or hepatoblastoma (combined) 10/49 (20%), 20/50* (40%), 20/50* (40%), 11/50 (22%)	$P \leq 0.030$, poly-3 trend test; [$P = 0.0281$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 5–6 wk 105 wk NTP (2000) (cont.)		Hepatocellular adenoma, hepatocellular carcinoma, hepatoblastoma (combined) 31/49 (63%), 47/50** (94%), 46/50**(92%), 41/50*(82%)	$P = 0.012$, poly-3 trend test; **$P < 0.001$, poly-3 test; [$P \leq 0.0002$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P \leq 0.0005$, Fisher exact test] *$P = 0.011$ poly-3 test; [$P = 0.0305$, Fisher exact test]	
		Glandular stomach Malignant neuroendocrine tumour 0/49, 0/48, 0/49, 2/50	[NS]	
		Carcinoma 0/49, 0/48, 0/49, 1/50	[NS]	
Full carcinogenicity Mouse, B6C3F$_1$ (F) 5–6 wk 105 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 37, 75 or 150 mg/kg bw, 5 days/wk for 105 wk 50, 50, 50, 50 31, 18, 18, 2	*Liver* Hepatocellular adenoma 20/50 (40%), 48/50* (96%), 46/49* (94%), 41/50* (82%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P \leq 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals used; randomly allocated in groups; adequate duration; males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 108/511 (21.1%); range, 6–40%; hepatocellular carcinoma, 37/511 (7.2%); range, 0–22%; hepatocellular adenoma or carcinoma, 138/511 (27%); range, 8–58%; hepatoblastoma, 0/511; hepatocellular carcinoma or hepatoblastoma, 37/511 (7.2%); range, 0–22%; hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma, 138/511 (27.0%); range, 8–58%; hepatocholangiocarcinoma, 1/511 (0.2%); range, 0–2%.
		Hepatocellular carcinoma 7/50 (14%), 37/50* (74%), 47/49* (96%), 47/50* (94%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 25/50 (50%), 50/50* (100%), 49/49* (100%), 49/50* (98%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (F) 5–6 wk 105 wk NTP (2000) (cont.)		Hepatoblastoma 0/50, 6/50* (12%), 11/49** (22%), 15/50*** (30%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.009$, poly-3 test; [$P = 0.0133$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test] ***$P < 0.001$, poly-3 test; [$P = 0.0001$, Fisher exact test]	
		Carcinoma or hepatoblastoma (combined) 7/50 (14%), 38/50* (76%), 48/49* (98%), 49/50* (98%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma (combined) 25/50 (50%), 50/50* (100%), 49/49* (100%), 49/50* (98%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocholangiocarcinoma 0/50, 0/50, 0/49, 2/50 (4%)	[NS]	
Full carcinogenicity Mouse, B6C3F$_1$ (M) 1 day 18 mo Miller et al. (1983)	Intraperitoneal injection Methyleugenol, > 98% Trioctanoin 0 (control), 4.75 µmol on days 1, 8, 15, and 22 of lactation, weaning at 4 wk then purified diet for 18 mo 60, 59 58, 58	*Liver* Hepatoma Tumour incidence: 24/58, 56/58* Tumour multiplicity: 0.5, 3.2	*$P < 0.001$, Fisher exact test	*Principal strengths*: adequate duration and adequate number of animals per group. *Principal limitations*: one sex; only one dose group; limited number of organs examined.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 1 day 18 mo Miller et al. (1983)	Intraperitoneal injection 1′-hydroxymethyleugenol, > 98% Trioctanoin 0 (control), 2.85 µmol on days 1, 8, 15, and 22 of lactation, weaning at 4 wk then purified diet for 18 mo 60, 44 58, 44	*Liver* Hepatoma Tumour incidence: 24/58, 41/44* Tumour multiplicity: 0.5, 3.5	*$P < 0.001$, Fisher exact test	*Principal strengths*: adequate duration and adequate number of animals per group. *Principal limitations*: only one dose group; one sex; limited number of organs examined.
Full carcinogenicity Rat, F344 (M) 5–6 wk 105 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 37, 75, 150 mg/kg bw 0, 37, 75, 150 mg/kg bw, 5 days/wk for 105 wk 50, 50, 50, 50 20, 16, 15, 0	*Liver* Hepatocellular adenoma 5/50 (10%), 12/50* (24%), 23/50** (46%), 38/50** (76%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.042$, poly-3 test; [$P = 0.0542$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals used; randomly allocated in groups; adequate duration; both males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 12/400 (3.0%); range, 0–8%; hepatocellular carcinoma, 4/400 (1.0%); range, 0–4%; hepatocellular adenoma or carcinoma (combined), 16/400 (4.0%); range, 0–10%; renal tubule adenoma, 3/400 (0.8%); range, 0–2%; renal tubule carcinoma, 2/400 (0.5%); range, 0–4%; renal tubule adenoma or carcinoma (combined), 5/400 (1.3%); range, 0–4%; malignant mesothelioma, 7/402 (1.7%); range, 0–6%; skin fibroma, 17/402 (4.2%); range, 0–12%; skin fibrosarcoma, 3/402 (0.8%); range, 0–2%; skin fibroma or fibrosarcoma (combined), 20/402 (5.0%); range, 0–12%; benign adrenal medulla pheochromocytoma, 94/401 (23.4%); range, 14–34%; malignant adrenal medulla pheochromocytoma, 8/401 (2.0%); range, 0–6%; benign or malignant adrenal medulla pheochromocytoma (combined), gavage, 101/401 (25.20%); range, 16–36%.
		Hepatocellular carcinoma 2/50 (4%), 3/50 (6%), 14/50* (28%), 25/50** (50%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P \leq 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test] **$P \leq 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 7/50 (14%), 14/50 (28%), 28/50* (56%), 43/50** (86%)	$P \leq 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.049$, poly-3 test; [$P < 0.0001$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (M) 5–6 wk 105 wk NTP (2000) (cont.)		Hepatocholangioma 0/50, 0/50, 0/50, 1/50 (2%) Hepatocholangiocarcinoma 0/50, 0/50, 1/50 (2%), 1/50 (2%) Hepatocholangioma or hepatocholangiocarcinoma (combined) 0/50, 0/50, 1/50 (2%), 2/50 (4%) *Glandular stomach* Benign neuroendocrine tumour 0/50, 0/50, 0/50, 3/50 (6%) Malignant neuroendocrine tumour 0/50, 0/50, 0/50, 4/50* (8%) Benign or malignant neuroendocrine tumour (combined) 0/50, 0/50, 0/50, 7/50* (14%) *Kidney* Renal tubule adenoma (single and step sections) 4/50 (8%), 6/50 (12%), 17/50** (34%), 13/50* (26%)	[NS, Fisher exact test] [NS, Fisher exact test] [NS, Fisher exact test] NS, poly-3 test $P = 0.002$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test] *$P = 0.033$, poly-3 test $P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.002$, poly-3 test; [$P = 0.0062$, Fisher exact test] $P < 0.001$, poly-3 trend test; [$P = 0.004$, Cochran–Armitage trend test] **$P < 0.001$, poly-3 test; [$P = 0.0013$, Fisher exact test] *$P = 0.003$, poly-3 test; [$P = 0.0155$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (M) 5–6 wk 105 wk NTP (2000) (cont.)		Renal tubule carcinoma (single and step sections) 1/50, 0/50, 0/50, 0/50 *Mammary gland* Fibroadenoma 5/50 (10%), 5/50 (10%), 15/50** (30%), 13/50* (26%) *All organs* Malignant mesothelioma 1/50 (2%), 3/50 (6%), 5/50 (10%), 12/50* (24%) *Skin (subcutaneous tissue)* Fibroma 1/50 (2%), 9/50** (18%), 8/50* (16%), 5/50 (10%) Fibrosarcoma 0/50, 3/50 (6%), 0/50, 3/50 (6%) Fibroma or fibrosarcoma (combined) 1/50 (2%), 12/50** (24%), 8/50* (16%), 8/50* (16%)	[NS, Fisher exact test] $P < 0.001$, poly-3 trend test; [$P = 0.0061$, Cochran–Armitage trend test] **$P = 0.004$, poly-3 test; [$P = 0.0114$, Fisher exact test] *$P = 0.008$, poly-3 test; [$P = 0.0332$, Fisher exact test] $P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test] **$P = 0.006$, poly-3 test; [$P = 0.0078$, Fisher exact test] *$P = 0.011$, poly-3 test; [$P = 0.0154$, Fisher exact test] NS, poly-3 test; [Fisher exact test] $P = 0.037$, poly-3 trend test; **$P < 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test] *$P \leq 0.011$, poly-3 test; [$P = 0.0154$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (F) 5–6 wk 105 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 37, 75 or 150 mg/kg bw, 5 days/wk for 105 wk 50, 50, 50, 50 22, 25, 22, 11	*Liver* Hepatocellular adenoma 1/50 (2%), 8/50* (16%), 11/49** (22%), 33/49*** (66%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.017$, poly-3 test; [$P = 0.0154$, Fisher exact test] **$P = 0.002$, poly-3 test; [$P = 0.0017$, Fisher exact test] ***$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	*Principal strengths*: well-conducted GLP study, adequate number of animals used; randomly allocated in groups; adequate duration; both males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 1/401 (0.3%); range, 0–2%; hepatocellular carcinoma, 0/401; hepatocellular adenoma or carcinoma (combined), 1/401 (0.3%); range, 0–2%; forestomach squamous cell papilloma, 2/401 (0.5%); range, 0–2%; forestomach squamous cell carcinoma, 0/401; forestomach squamous cell papilloma or carcinoma (combined), 2/401 (0.5%); range, 0–2%; benign adrenal medulla pheochromocytoma, gavage, 18/401 (4.5%); range, 0–10%; malignant adrenal medulla pheochromocytoma, gavage, 3/400 (0.8%); range, 0–4%; benign or malignant adrenal medulla pheochromocytoma (combined), gavage, 22/400 (5.5%); range, 0–14%.
		Hepatocellular carcinoma 0/50, 0/50, 4/49* (8%), 8/49** (16%)	$P < 0.001$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test] *$P = 0.066$, poly-3 test; [$P = 0.056$, Fisher exact test] **$P = 0.002$, poly-3 test; [$P = 0.026$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 1/50 (2%), 8/50* (16%), 14/49** (28%), 34/49*** (68%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.017$, poly-3 test; [$P = 0.0154$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test] ***$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (F) 5–6 wk 105 wk NTP (2000) (cont.)		Hepatocholangiocarcinoma		
		0/50, 0/50, 0/49, 3/49 (6%)	$P = 0.010$, poly-3 trend test; [$P = 0.003$, Cochran–Armitage trend test] NS, poly-3 test; [NS, Fisher exact test]	
		Glandular stomach		
		Benign neuroendocrine tumour		
		0/50, 0/50, 13/50** (26%), 9/50* (18%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] **$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test] *$P = 0.029$, poly-3 test; [$P = 0.0013$, Fisher exact test]	
		Malignant neuroendocrine tumour		
		0/50, 1/50 (2%), 12/50* (24%), 26/50* (52%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P \leq 0.0001$, Fisher exact test]	
		Benign or malignant neuroendocrine tumour		
		0/50, 1/50 (2%), 25/50* (50%), 34/50* (68%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		All organs		
		Mononuclear cell leukaemia		
		17/50 (34%), 20/50 (40%), 19/50 (38%), 28/50* (56%)	$P = 0.007$, poly-3 trend test [$P = 0.012$, Cochran–Armitage trend test] *$P = 0.008$, poly-3 test; [$P = 0.0219$; Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (M) 5–6 wk 52 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 300 mg/kg bw, 5 days/wk for 52 wk followed by vehicle control for 53 wk 50, 50 20, 0	*Liver* Hepatocellular adenoma 5/50 (10%), 32/50* (76%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	*Principal strengths*: well-conducted GLP study, adequate number of animals used; randomly allocated in groups; adequate duration; both males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 12/400 (3.0%); range, 0–8%; hepatocellular carcinoma, 4/400 (1.0%); range, 0–4%; hepatocellular adenoma or carcinoma (combined), 16/400 (4.0%); range, 0–10%; renal tubule adenoma, 3/400 (0.8%); range, 0–2%; renal tubule carcinoma, 2/400 (0.5%); range, 0–4%; renal tubule adenoma or carcinoma (combined), 5/400 (1.3%); range, 0–4%; malignant mesothelioma, 7/402 (1.7%); range, 0–6%; skin fibroma, 17/402 (4.2%); range, 0–12%; skin fibrosarcoma, 3/402 (0.8%); range, 0–2%; skin fibroma or fibrosarcoma (combined), 20/402 (5.0%); range, 0–12%; benign adrenal medulla pheochromocytoma, 94/401 (23.4%); range, 14–34%; malignant adrenal medulla pheochromocytoma, 8/401 (2.0%); range, 0–6%; benign or malignant adrenal medulla pheochromocytoma (combined), gavage, 101/401 (25.20%); range, 16–36%.
		Hepatocellular carcinoma 2/50 (4%), 36/50* (72%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 7/50 (14%), 45/50* (90%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocholangioma 0/50, 6/50* (12%)	$P = 0.004$, poly-3 test; [$P = 0.0133$, Fisher exact test]	
		Hepatocholangiocarcinoma 0/50, 7/50* (14%)	$P < 0.001$, poly-3 test; [$P = 0.0062$, Fisher exact test]	
		Hepatocholangioma or hepatocholangiocarcinoma (combined) 0/50, 13/50* (26%)	$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Glandular stomach Benign and malignant neuroendocrine tumour (combined) 0/50, 4/50* (8%)	*$P = 0.032$, poly-3 test	
		Kidney Renal tubule adenoma (single and step sections) 4/50 (8%), 20/50* (40%)	*$P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test]	
		Renal tubule adenoma or carcinoma (single and step sections) 5/50 (10%), 20/50* (40%)	$P < 0.001$, poly-3 test; [$P = 0.0005$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (M) 5–6 wk 52 wk NTP (2000) (cont.)		*All organs* Malignant mesothelioma 1/50 (2%), 5/50* (10%)	*P = 0.041, poly-3 test	
Full carcinogenicity Rat, F344 (F) 5–6 wk 52 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 300 mg/kg bw, 5 days/wk for 52 wk followed by vehicle control for 53 wk 50, 50 22, 16	*Liver* Hepatocellular adenoma 1/50 (2%), 43/50* (86%) Hepatocellular carcinoma 0/50, 22/50* (44%) Hepatocellular adenoma or carcinoma (combined) 1/50 (2%), 43/50* (86%) Hepatocholangioma 0/50, 8/50* (16%) Hepatocholangiocarcinoma 0/50, 9/50* (18%) Hepatocholangioma or hepatocholangiocarcinoma (combined) 0/50, 17/50* (34%) *Glandular stomach* Benign neuroendocrine tumour 0/50, 5/50* (10%)	**P < 0.001, poly-3 test; [P < 0.0001, Fisher exact test] *P < 0.001, poly-3 test; [P < 0.001, Fisher exact test] *P < 0.001, poly-3 test; [P < 0.0001, Fisher exact test] P = 0.003, poly-3 test; P = 0.0029, Fisher exact test] P < 0.001, poly-3 test; P = 0.0013, Fisher exact test] P < 0.001, poly-3 test; P < 0.0001, Fisher exact test] *P = 0.029, poly-3 test; [P = 0.0287, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals used; randomly allocated in groups; adequate duration; both males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 1/401 (0.3%); range, 0–2%; hepatocellular carcinoma, 0/401; hepatocellular adenoma or carcinoma (combined), 1/401 (0.3%); range, 0–2%; forestomach squamous cell papilloma, 2/401 (0.5%); range, 0–2%; forestomach squamous cell carcinoma, 0/401; forestomach squamous cell papilloma or carcinoma (combined), 2/401 (0.5%); range, 0–2%; benign adrenal medulla pheochromocytoma, gavage, 18/401 (4.5%); range, 0–10%; malignant adrenal medulla pheochromocytoma, gavage, 3/400 (0.8%); range, 0–4%; benign or malignant adrenal medulla pheochromocytoma (combined), gavage, 22/400 (5.5%); range, 0–14%.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (F) 5–6 wk 52 wk NTP (2000) (cont.)		Malignant neuroendocrine tumour 0/50, 36/50* (72%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Benign or malignant neuroendocrine tumour 0/50, 41/50* (82%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
Initiation–promotion (tested as initiator) Rat, F344 (M) 5–6 wk 40 wk Williams et al. (2013)	Gavage Methyleugenol, > 98% Methylcellulose 0, 62, 125, 250 mg/kg bw, by gavage, 3 days/wk, for 16 wk followed by control diet for 24 wk 12, 12, 12, 12 12, 12, 12, 12	*Liver* Hepatocellular adenoma 0/12, 0/12, 1/12, 12/12* Tumour multiplicity: 0, 0.1, 1, 1.1 Hepatocellular carcinoma Tumour incidence: 0/12, 0/12, 0/12, 0/12 Tumour multiplicity: 0, 0, 0, 0	[$P < 0.001$, Cochran–Armitage trend test] *[$P < 0.001$, Fisher exact test]	*Principal strengths*: multiple dose study. *Principal limitations*: limited number of organs examined; limited number of animals; short duration; one sex study.
Initiation–promotion (tested as initiator) Rat, F344 (M) 5–6 wk 40 wk Williams et al. (2013)	Gavage Methyleugenol, > 98% Methylcellulose 0, 62, 125, 250 mg/kg bw gavage, 3 days/wk, for 16 wk followed by 500 ppm phenobarbital in the diet for 24 wk 14, 14, 14, 14 14, 13, 14, 13	*Liver* Hepatocellular adenoma Tumour incidence: 0/14, 0/13, 3/14, 10/13* Tumour multiplicity: 0, 0, < 1, 2.8 Hepatocellular carcinoma Tumour incidence: 0/14, 0/13, 0/14, 0/13 Tumour multiplicity: 0, 0, 0, 0	[$P < 0.001$, Cochran–Armitage trend test] *[$P < 0.001$, Fisher exact test]	*Principal strengths*: multiple dose study. *Principal limitations*: limited number of organs examined; limited number of animals; short duration; one sex.

bw, body weight; F, female; GLP, Good Laboratory Practice; M, male; mo, month(s); NS, not significant; wk, week(s).

test), with the incidence – 31/49 (63%), 47/50 (94%), 46/50 (92%), 40/50 (80%) – being significantly increased in all treated groups ($P < 0.001$, poly-3 test, [$P = 0.0002$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0005$, Fisher exact test]; $P = 0.016$, poly-3 test, [$P = 0.0517$, Fisher exact test], respectively), and exceeding the upper bound of the range observed in historical controls – 267/514 (52%); range, 25–72%. There was a significant positive trend in the incidence of hepatoblastoma ($P = 0.019$, poly-3 trend test; [$P < 0.01$, Cochran–Armitage trend test]), with the incidence at 150 mg/kg bw per day (3/50, 6%) exceeding the range observed in historical controls from this laboratory – 2/514 (0.4%); range, 0–3%. There was a significant positive trend in the incidence of hepatocellular carcinoma or hepatoblastoma (combined) ($P \leq 0.030$, poly-3 trend test), with the incidence – 10/49 (20%), 20/50 (40%), 20/50 (40%), 11/50 (22%) – being significant [$P = 0.0281$; Fisher exact test] at 37 and 75 mg/kg bw per day. The incidence at these two doses exceeded the upper bound of the range observed in historical controls from this laboratory – 104/514 (20.2%); range, 8–38%. There was a significant positive trend in the incidence of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma (combined) ($P = 0.012$, poly-3 trend test), with the incidence – 31/49 (63%), 47/50 (94%), 46/50 (92%), 41/50 (82%) – being significantly increased at all doses ($P < 0.001$, poly-3 test, [$P \leq 0.0002$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0005$, Fisher exact test]; ($P = 0.011$, poly-3 test, [$P = 0.0305$, Fisher exact test], respectively), and exceeding the upper bound of the range observed in historical controls from this laboratory – 267/514 (52%); range, 25–72%.

In females, there was a significant positive trend in the incidence of hepatocellular adenoma ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 20/50 (40%), 48/50 (96%), 46/49 (94%), 41/50 (82%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 108/511 (21.1%); range, 6–40%. There was a significant positive trend in the incidence of hepatocellular carcinoma ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 7/50 (14%), 37/50 (74%), 47/49 (96%), 47/50 (94%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 37/511 (7.2%); range, 0–22%. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 25/50 (50%), 50/50 (100%), 49/49 (100%), 49/50 (98%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 138/511 (27%); range, 8–58%. There was a significant positive trend in the incidence of hepatoblastoma ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 6/50 (12%), 11/49 (22%), 15/50 (30%) – being significantly increased in each treated group ($P = 0.009$, poly-3 test, [$P = 0.0133$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0002$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0001$, Fisher exact test], respectively), and exceeding the historical controls from this laboratory (0/511). There was a significant positive trend in the incidence of hepatocellular carcinoma or hepatoblastoma (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 7/50 (14%), 38/50 (76%), 48/49 (98%), 49/50 (98%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this

laboratory – 37/511 (7.2%); range, 0–22%. There was a significant positive trend in the incidence of hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 25/50 (50%), 50/50 (100%), 49/49 (100%), 49/50 (98%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 138/511 (27%); range, 8–58%. The incidence of hepatocholangiocarcinoma at the highest dose – 2/50 (4%) – exceeded the upper bound of the range observed in historical controls from this laboratory – 1/511 (0.2%); range, 0–2%. [The Working Group noted that hepatoblastoma and hepatocholangiocarcinoma are rare neoplasms of the liver and that neuroendocrine tumours of the glandular stomach are rare neoplasms of the glandular stomach in experimental animals of this strain and age.]

In treated groups, a significantly increased incidence of preneoplastic and non-neoplastic lesions was observed in liver (eosinophilic foci and chronic active inflammation in males; oval cell hyperplasia, hypertrophy, bile duct hyperplasia, haematopoietic cell proliferation, and haemosiderin pigmentation in females), and glandular stomach (atrophy, ectasia, hyperplasia, chronic inflammation in both males and females).

[The Working Group noted that this was a well-conducted study that complied with GLP and used an adequate number of animals (randomly allocated in groups), both males and females, and multiple doses, and with a duration of most of the lifespan.]

3.1.2 Intraperitoneal administration

In a study performed by Miller et al. (1983), groups of 60 male B6C3F$_1$ mice received methyleugenol (purity > 98%; dissolved in trioctanoin) by intraperitoneal injection at a dose of 0 (vehicle controls) or total dose of 4.75 µmol/mouse on days 1, 8, 15, and 22 of lactation. Mice were weaned at 4 weeks and then maintained on a purified diet for 18 months. A group of 60 animals was used as controls.

A statistically significant increase ($P < 0.001$, Fisher exact test) in the incidence of hepatoma was observed in the methyleugenol-treated group (56/58, 96%) compared with controls (24/58, 41%). [The Working Group noted that the distinction between benign and malignant hepatoma had not been clearly defined at the time when the study was conducted.]

Non-neoplastic lesions have not been reported.

[The Working Group noted that this study used an adequate duration of exposure and observation and an adequate number of animals; however, only one sex and one dose group were used, and a limited number of organs was examined (which is appropriate for this particular assay).]

3.1.3 Intraperitoneal administration of the metabolite, 1′-hydroxymethyleugenol

In a study performed by Miller et al. (1983), groups of 44 male B6C3F$_1$ mice received 1′-hydroxymethyleugenol (purity, > 98%; dissolved in trioctanoin) by intraperitoneal injection at a dose of 2.85 µmol/mouse on lactation days 1, 8, 15 and 22. Mice were weaned at 4 weeks and then maintained on a purified diet for 18 months. A group of 60 animals was used as controls.

A statistically significant increase ($P < 0.001$, Fisher exact test) in the incidence of hepatoma was observed in the group treated with 1′-hydroxymethyleugenol (41/44, 93%) compared with the controls (24/58, 41%)). [The Working Group noted that the distinction between benign and malignant hepatoma had not been clearly defined at the time when the study was conducted.]

[The Working Group noted that this study used an adequate duration of exposure and

observation and an adequate number of animals per group; however, it was limited by the use of one sex only, only one dose group, and the limited number of organs examined (which is appropriate for this particular assay).]

3.2 Rat

3.2.1 Oral administration (gavage)

In a well-conducted study of chronic toxicity and carcinogenicity that complied with GLP, groups of 50 male and 50 female Fischer 344 (F344) rats (age, 5–6 weeks) were treated with methyleugenol (purity, 99%; in 0.5% methylcellulose) by gavage at a dose of 0 (vehicle control), 37, 75, or 150 mg/kg bw per day on 5 days per week for 105 weeks. Additional groups of 50 males and 50 females received methyleugenol (in 0.5% methylcellulose) at a dose of 300 mg/kg bw per day by gavage for 52 weeks, followed by the vehicle alone for the remaining 53 weeks of the study (NTP, 2000; also reported by Johnson et al., 2000). At study termination, survival was: 20/50, 16/50, 15/50, 0/50, and 0/50 in males and 22/50, 25/50, 22/50, 11/50, and 16/50 in females, for the groups at 0 (control), 37, 75, 150, and 300 mg/kg bw per day, respectively. In males, the probability of survival to study completion for the groups at 150 and 300 mg/kg bw per day was significantly decreased ($P < 0.001$, life-table test) and all animals from these groups died before the end of the study. In females, the probability of survival to study completion for the group at 150 mg/kg bw per day approached a significant decrease ($P = 0.053$, life-table test). In males, terminal group mean body-weight values decreased by 12%, 23%, and 26% in the groups at 75, 150, and 300 mg/kg bw per day, respectively. In females, terminal group mean body-weight values decreased by 20%, 26%, and 26% in the groups at 75, 150, and 300 mg/kg bw per day, respectively.

In males, there was a significant positive trend in the incidence of hepatocellular adenoma ($P \leq 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 5/50 (10%), 12/50 (24%), 23/50 (46%), and 38/50 (76%), for the groups at 0 (control), 37, 75, or 150 mg/kg bw per day, respectively – being significantly increased at the lowest dose ($P = 0.042$, poly-3 test; [approached significance, $P = 0.0542$, Fisher exact test]), and the two other doses ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 12/400 (3.0%); range, 0–8%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular adenoma in the treated group – 5/50 (10%) and 32/50 (64%) at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of hepatocellular carcinoma ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 2/50 (4%), 3/50 (6%), 14/50 (28%), and 25/50 (50%) in groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 mg/kg bw per day ($P \leq 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test]) and at 150 mg/kg bw ($P \leq 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]), respectively, and exceeding the upper bound of the range observed in historical controls from this laboratory – 4/400 (1.0%); range, 0–4%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular carcinoma in the treated group – 2/50 (4%) and 36/50 (72%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) ($P \leq 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 7/50 (14%), 14/50 (28%), 28/50 (56%), and 43/50 (86%)

for the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 37 mg/kg bw ($P = 0.049$, poly-3 test; [$P < 0.0001$, Fisher exact test]); and at 75 mg/kg bw and 150 mg/kg bw ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test], for each), and exceeding the upper bound of the range observed in historical controls from this laboratory – 16/400 (4.0%); range, 0–10%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased the incidence of hepatocholangioma – 0/50 (control), 6/50 (12%) ($P = 0.004$, poly-3 test; [$P = 0.0133$, Fisher exact test]; $P < 0.001$, poly-3 test; [$P = 0.0062$, Fisher exact test]; $P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) – hepatocholangiocarcinoma – 0/50 (control), 7/50 (14%) – and hepatocholangioma or hepatocholangiocarcinoma (combined) – 0/50 (control), 13/50 (26%) – in the treated groups compared with controls. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular adenoma or carcinoma (combined) in the treated group – 7/50 (14%) and 45/50 (90%), for the groups at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of malignant neuroendocrine tumours of the glandular stomach ($P = 0.002$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 0/50, 0/50, and 4/50 (8%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at the highest dose ($P = 0.033$, poly-3 test). There was a significant positive trend in the incidence of benign or malignant neuroendocrine tumours of the glandular stomach (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 0/50, 0/50, 7/50 (14%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at the highest dose ($P = 0.002$, poly-3 test; [$P = 0.0062$, Fisher exact test]). Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P = 0.032$, poly-3 test) the incidence of benign or malignant neuroendocrine tumours of the glandular stomach (combined) in the treated group – 0/50, 4/50 (8%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of renal tubule adenoma (single and step sections) ($P < 0.001$, poly-3 trend test; [$P = 0.004$, Cochran–Armitage trend test]), with the incidence – 4/50 (8%), 6/50 (12%), 17/50 (34%), and 13/50 (26%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 ($P < 0.001$, poly-3 test; [$P = 0.0013$, Fisher exact test]) and 150 mg/kg bw ($P = 0.003$, poly-3 test; [$P = 0.0155$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 3/400 (0.8%); range, 0–2%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test]; $P < 0.001$, poly-3 test; [$P = 0.0005$, Fisher exact test]) the incidence of renal tubule adenoma (single and step sections) – 4/50 (8%) (control), 20/50 (40%) ($P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test]) – and renal tubule adenoma or carcinoma (combined) (single and step sections) – 5/50 (10%) (control), 20/50 (40%) ($P < 0.001$, poly-3 test; [$P = 0.0005$, Fisher exact test] – in the treated groups. There was a significant positive trend in the incidence of mammary gland fibroadenoma ($P < 0.001$, poly-3 trend test; [$P = 0.0061$, Cochran–Armitage trend test]), with the incidence – 5/50 (10%), 5/50 (10%), 15/50 (30%), and 13/50 (26%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 ($P = 0.004$, poly-3 test; [$P = 0.0114$, Fisher exact test]) and 150 mg/kg bw ($P = 0.008$, poly-3 test; [$P = 0.0332$, Fisher exact test]). There was a significant positive trend in the incidence of malignant mesothelioma (all organs) ($P < 0.001$, poly-3 trend test;

[$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 1/50 (2%), 3/50 (6%), 5/50 (10%), and 12/50 (24%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 150 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 7/402 (1.7%); range, 0–6%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P = 0.041$, poly-3 test) the incidence of malignant mesothelioma (all organs) in the treated group – 1/50 (2%) and 5/50 (10%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant increase in the incidence – 1/50 (2%), 9/50 (18%), 8/50 (16%), and 5/50 (10%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – of skin (subcutaneous) fibroma at 37 ($P = 0.006$, poly-3 test; [$P = 0.0078$, Fisher exact test]) and 75 mg/kg bw ($P = 0.011$, poly-3 test; [$P = 0.0154$, Fisher exact test]), with the incidence exceeding the upper bound of the range observed in historical controls from this laboratory – 17/402 (4.2%); range, 0–12%. There was a significant positive trend in the incidence of skin (subcutaneous) fibroma or fibrosarcoma (combined) ($P = 0.037$, poly-3 trend test), with the incidence – 1/50 (2%), 12/50 (24%), 8/50 (16%), and 8/50 (16%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased ($P < 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test]) at 37 mg/kg bw; ($P = 0.011$, poly-3 test; [$P = 0.0154$, Fisher exact test]; ($P = 0.005$, poly-3 test; [$P = 0.0154$, Fisher exact test] at 75 and 150 mg/kg bw, respectively), and exceeding the upper bound of the range observed in historical controls (20/402 (5.0%); range, 0–12%) from this laboratory.

In females, there was a significant positive trend in the incidence of hepatocellular adenoma ($P \leq 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 1/50 (2%), 8/50 (16%), 11/49 (22%), and 33/49 (67%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at all doses ($P = 0.017$, poly-3 test; [$P = 0.0154$, Fisher exact test]; $P = 0.002$, poly-3 test; [$P = 0.0017$, Fisher exact test]; $P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test], respectively), and exceeding the upper bound of the range observed in historical controls from this laboratory – 1/401 (0.3%); range, 0–2%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular adenoma in the treated group – 1/50 (2%), 43/50 (86%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of hepatocellular carcinoma ($P < 0.001$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 0/50, 4/49 (8%), and 8/49 (16%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 150 mg/kg bw per day ($P = 0.002$, poly-3 test; [$P = 0.0026$, Fisher exact test]), and exceeding the incidence observed in historical controls from this laboratory (0/401). Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.001$, Fisher exact test]) the incidence of hepatocellular carcinoma in the treated group – 0/50, 22/50 (44%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence (1/50 (2%), 8/50 (16%), 14/49 (29%), 34/49 (69%) for the 0 (control), 37, 75, and 150 mg/kg bw, respectively) being significantly increased at 37 mg/kg bw per day ($P = 0.017$, poly-3 test; [$P = 0.0154$, Fisher exact test]); 75 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test]); and 150 mg/kg bw per ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]); and exceeding the upper bound of the

range observed in historical controls from this laboratory – 1/401 (0.3%); range, 0–2%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular adenoma or carcinoma (combined) in the treated group – 1/50 (2%), 43/50 (86%), at 0 (control) and 300 mg/kg bw per day, respectively. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P = 0.003$, poly-3 test; [$P = 0.0029$, Fisher exact test]) the incidence of hepatocholangioma in the treated group – 0/50, 8/50 (16%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend ([$P = 0.010$, poly-3 trend test; $P = 0.003$, Cochran–Armitage trend test]) in the incidence of hepatocholangiocarcinoma – 0/50, 0/50, 0/49, and 3/49 (6%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P = 0.0013$, Fisher exact test]) the incidence of hepatocholangiocarcinoma in the treated group – 0/50, 9/50 (18%), at 0 (control) and 300 mg/kg bw per day, respectively. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocholangioma or hepatocholangiocarcinoma (combined) in the treated group – 0/50, 17/50 (34%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of benign neuroendocrine tumours of the glandular stomach ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 0/50, 13/50 (26%), and 9/50 (18%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) and 150 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P = 0.0013$, Fisher exact test]). Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P = 0.0029$, poly-3 test; [$P = 0.0287$, Fisher exact test]) the incidence of benign neuroendocrine tumours of the glandular stomach in the treated group – 0/50, 5/50 (10%), at 0 (control) or 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of malignant neuroendocrine tumours of the glandular stomach ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 1/50 (2%), 12/50 (24%), and 26/50 (52%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 and 150 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]). Exposure to methyleugenol at 300 mg/kg bw for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of malignant neuroendocrine tumours of the glandular stomach in the treated group – 0/50, 36/50 (72%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of benign or malignant neuroendocrine tumours of the glandular stomach (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 1/50 (2%), 25/50 (50%), and 34/50 (68%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 and 150 mg/kg bw ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]). Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of benign or malignant neuroendocrine tumours of the glandular stomach (combined) in the treated group – 0/50, 41/50 (82%), for 0 (control) or 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of mononuclear cell leukaemia (all organs) ($P = 0.007$, poly-3 trend test; [$P = 0.012$, Cochran–Armitage trend test]), with the incidence – 17/50 (34%), 20/50 (40%), 19/50 (38%), and 28/50 (56%) in the groups

at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased (P = 0.008, poly-3 test; [P = 0.0219, Fisher exact test]) at 150 mg/kg bw per day. [The Working Group noted that hepatocholangiocarcinoma is a rare neoplasm of the liver, that mesothelioma is a rare neoplasm, and that neuroendocrine tumours are rare neoplasms of the glandular stomach in experimental animals of this strain and age. The Working Group also noted that in the main and stop-exposure studies, there was consistency in the tumour response for cancers of the liver and the glandular stomach in male and female rats, and for renal tubule tumours in male rats.]

Regarding preneoplastic and non-neoplastic lesions, significant increases in incidence were observed in the liver (bile duct hyperplasia, oval cell hyperplasia, hypertrophy, cystic degeneration, eosinophilic foci, basophilic foci, mixed cell foci), glandular stomach (atrophy, neuroendocrine cell hyperplasia) in treated groups for both sexes. In males, an increased incidence of preneoplastic lesions was observed in the kidney (renal tubule hyperplasia). [The Working Group noted that this was a well-conducted GLP study using an adequate number of animals (randomly allocated in groups), both males and females, and multiple doses, and with a duration of most of the lifespan.]

3.2.2 Initiation–promotion

In a study performed by Williams et al. (2013), groups of 12 male F344 rats (age, 5–6 weeks) were treated with methyleugenol (purity, > 98%; in 0.5% methylcellulose) by gavage at a dose of 0 (vehicle control), 62, 125, or 250 mg/kg bw on 3 days per week for 16 weeks, followed by either control diet or diet containing phenobarbital at 500 ppm for 24 weeks. Three deaths occurred in the treated groups immediately after dosing and were attributed to the gavage procedure. The first death occurred on the second day of dosing, and the other two deaths occurred later; the animals were not replaced. The body weights of rats selected for inclusion in the study did not vary by more than 15% from the mean body weight.

Statistically significant increases in the incidence of hepatocellular adenoma were observed only in the groups treated with methyleugenol alone at 250 mg/kg bw [P < 0.001, Fisher exact test] alone or together with phenobarbital at 500 ppm. No hepatocellular carcinoma was observed in treated or control groups. Statistically significant increases in the incidence of hepatocellular altered foci were observed in the groups treated with methyleugenol at 125 mg/kg bw and 250 mg/kg bw [P < 0.001, Fisher exact test] alone or together with phenobarbital at 500 ppm.

[The Working Group noted that this study used multiple doses. However, it also used a small number of animals, only one sex, and a limited number of organs was examined (which was appropriate for the assay being conducted). The short duration may have precluded the formation of carcinomas.]

3.3 Evidence synthesis for cancer in experimental animals

The carcinogenicity of methyleugenol has been assessed in one well-conducted GLP study in male and female B6C3F$_1$ mice by oral administration (gavage) (NTP, 2000; also reported by Johnson et al., 2000), an intraperitoneal injection study in neonate male B6C3F$_1$ mice Miller et al. (1983), in one well-conducted GLP study in male and female F344 rats by oral administration (gavage) (NTP, 2000; also reported by Johnson et al., 2000), and in an initiation–promotion experiment in male F344 rats (Williams et al., 2013).

In male and female B6C3F$_1$ mice treated by gavage, there were increases in the incidence of hepatocellular adenoma, hepatocellular carcinoma, hepatocellular adenoma or carcinoma

(combined), hepatoblastoma, hepatocellular carcinoma or hepatoblastoma (combined), and hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma (combined). In male B6C3F$_1$ mice treated as neonates by intraperitoneal injection, there was an increased incidence of liver hepatoma. In male and female F344 rats treated by gavage, there were increases in the incidence of hepatocellular adenoma, hepatocellular carcinoma, hepatocellular adenoma or carcinoma (combined), hepatocholangioma, hepatocholangiocarcinoma, hepatocholangioma or hepatocholangiocarcinoma (combined), malignant neuroendocrine tumours of the glandular stomach, and benign or malignant neuroendocrine tumours of the glandular stomach. In male F344 rats treated by gavage, there were increases in the incidence of renal tubule adenoma, renal tubule adenoma or carcinoma (combined), malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma or fibrosarcoma (combined). There was a significant increase in the incidence of mononuclear cell leukaemia (all organs) and benign neuroendocrine tumours of the glandular stomach in females. In male F344 rats treated in an initiation–promotion assay, there was a dose-dependent induction of hepatocellular adenoma. In male B6C3F$_1$ mice treated with 1′-hydroxymethyleugenol by intraperitoneal injection, there was a significant increase in the incidence of hepatocellular adenoma.

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Absorption, distribution, and excretion

(a) Humans

Only the absorption of methyleugenol has been investigated in a few studies in exposed humans, after oral or dermal exposure.

(i) Oral exposure

Schecter et al. (2004) examined serum concentrations of methyleugenol in nine fasted (overnight) volunteers (five men; four women; body weight, 63–74 kg) who ingested ginger-snap cookies containing approximately 216 µg of methyleugenol. The mean serum concentration of methyleugenol before exposure was 16.2 ± 4.0 pg/g wet weight (LOD, 3.1 ng/pg; range, < LOD to 37 pg/g). After exposure, the mean peak serum concentration in the nine participants rose to 53.9 ± 7.3 pg/g wet weight (range, 25–100 pg/g) within 15 minutes. [The Working Group noted that the mean serum concentration of methyleugenol measured before exposure probably reflects background exposure via dietary sources.] A half-life of approximately 90 minutes was calculated. The maximum concentration measured by Schecter et al. (2004) was within the range of concentrations of methyleugenol (i.e. < 3.1 to 390 pg/g; mean, 24 pg/g) measured in the serum of 206 non-fasting participants in the United States National Health and Nutrition Examination Survey (NHANES III, 1988–1994) (Barr et al., 2000).

(ii) Dermal exposure

Using a sorptive tape (absorbent polydimethylsiloxane tape) extraction technique, Sgorbini et al. (2010) quantified methyleugenol on the skin surface of two volunteers after the application of a skin cream containing

methyleugenol at 50 ppm. One hour after application, the amount of methyleugenol detected on the stratum corneum decreased by approximately 90.1%. On the basis of its reported boiling-point (255 °C), methyleugenol is a volatile/semi-volatile organic compound and a proportion of loss from the skin surface is probably caused by volatilization. In one volunteer, Sgorbini et al. (2010) estimated that methyleugenol had a permeation rate of 14.5%, 30 minutes after exposure.

(b) Experimental systems

The National Toxicology Program (NTP) investigated the toxicokinetics of methyleugenol using ^{14}C-labelled and unlabelled methyleugenol in F344 rats and B6C3F$_1$ mice (NTP, 2000). The doses administered ranged from 11.8 to 300 mg/kg bw, and exposure methods included a single gavage exposure, a single intravenous exposure, and repeated gavage exposures (e.g. 6, 12 and 18 months; 5 days per week). Overall, methyleugenol is absorbed extensively within minutes. For example, the time taken to reach maximum concentration (T_{max}) values of approximately 5 minutes were observed in male and female rats and mice, and > 70% of the orally administered radioactivity was found in the urine of male rats and female mice 72 hours after a single gavage exposure to [^{14}C]methyleugenol at a dose of between 25 and 150 mg/kg bw. Notably, most of the radioactivity in rats and mice exposed orally to [^{14}C]methyleugenol was detected within the first 24 hours after dosing, with very little excretion between 24 and 72 hours. Of the administered radioactivity, < 13% was recovered in the faeces and < 0.1% in the expired air. The absolute bioavailability of the parent compound after oral exposure was low and increased in a dose-dependent manner, ranging from 6% to 20% in rats and from 4% to 19% in mice. [The Working Group noted that the low percentage values for absolute bioavailability probably reflect substantial first-pass metabolism and that the dose-dependent increase in absolute bioavailability suggests saturation of metabolism at higher doses.]

After a single intravenous exposure (11.8 mg/kg bw in male F344 rats) or oral exposure (118 mg/kg bw in male F344 rats and female B6C3F$_1$ mice) to [^{14}C]methyleugenol, methyleugenol was eliminated in the urine within 12 hours, with most of the radioactivity being excreted within the first 24 hours. At 72 hours after dosing, most residual radioactivity was found in the liver; in fact, the tissue-to-blood ratio of radioactivity was 2:3 in the liver, 0.9:1.4 in the kidney, and < 1 in all other tissues (i.e. brain, fat, heart, large intestine, lungs, muscle, skin, small intestine, spleen, stomach, and testes). [The Working Group noted that the urinary bladder was not included in the analysis.]

After repeated oral exposures (at doses ranging from 37 to 300 mg/kg bw per day in F344 rats and from 37 to 150 mg/kg bw per day in B6C3F$_1$ mice), the terminal half-life of methyleugenol was 1–2 hours, and elimination of methyleugenol appeared to be similar in young adult and aged rodents. However, values for the area under the curve (AUC) appeared to decrease in males after repeated exposures and to increase with age in males and females. [The Working Group noted that these observations suggest metabolic induction, but also the attenuation of methyleugenol metabolism with ageing.]

Hong et al. (2013) explored dose-, sex- and species-dependent effects on various toxicokinetic parameters after exposure to methyleugenol by gavage or intravenous administration in male and female rats and mice. In contrast to the studies described in NTP (2000), Hong et al. (2013) used corn oil, not methylcellulose, as the vehicle. Non-fasted F344 rats (21 males and 21 females, age, ~13 weeks) were exposed by gavage to methyleugenol as a single dose at 37, 75, or 150 mg/kg bw. A separate group of 18 male and 18 female non-fasted rats was exposed intravenously to methyleugenol as a single dose at 37 mg/kg bw. Similarly, B6C3F$_1$ mice (42 males and 42 females, age, ~13 weeks) were exposed by

gavage to methyleugenol as a single dose at 37, 75, or 150 mg/kg bw. A separate group of 36 male and 36 female mice was exposed intravenously to methyleugenol as a single dose at 37 mg/kg bw. After exposure by gavage at 37 mg/kg bw, the absolute bioavailability of methyleugenol was ~4% in rats and 7–9% in mice). [The Working Group noted that the volume of distribution (> 10 L/kg) in rats and mice exceeded the total body water volume (~0.7 L/kg), suggesting extensive distribution, high tissue binding, and/or plasma protein binding.] In addition, it was observed that after exposure by gavage, the plasma concentration-versus-time curves in rats and mice showed secondary peaks. [The Working Group considered that the secondary peaks in the plasma concentration-versus-time curves were caused by the vehicle used (e.g. corn oil acted as a reservoir in the gut that retarded systemic absorption).]

In general, rats showed higher AUC_{∞} values than did mice. After intravenous exposure (37 mg/kg bw) or exposure by gavage (at doses ranging from 37 to 150 mg/kg bw), methyleugenol was rapidly cleared from the systemic circulation in rats and mice. After intravenous exposure (37 mg/kg bw), systemic clearance of methyleugenol from the plasma appeared to be biphasic in rats and mice; a rapid initial phase was followed by a slower terminal phase.

[The Working Group noted that the clearance values (> 59 mL/minute per kg in rats and > 182 mL/minute per kg in mice after intravenous exposure; and > 293 mL/minute per kg in rats and > 717 mL/minute per kg in mice after gavage) were greater than the hepatic blood flow rates (~55 mL/minute per kg in rats and 90 mL/minute per kg in mice), especially after gavage, suggesting significant first-pass metabolism.] No sex-specific differences in toxicokinetic parameters were noted in rats and mice exposed by gavage at doses ranging from 37 to 150 mg/kg bw.

Kreutz et al. (2018) quantified methyleugenol in skin samples after the application of *Aniba canelilla* oil (containing 16.2% methyleugenol), using the porcine ear skin and Franz-type diffusion cell system. The permeation of methyleugenol from the oil increased in a dose-dependent manner, and the order of retention of methyleugenol was receptor fluid > dermis > epidermis > stratum corneum, indicating that methyleugenol is capable of deep tissue penetration. Using the Franz-type diffusion cell system and heat-separated human epidermis, Schmitt et al. (2009) determined that the apparent permeability of methyleugenol ranged from 1.08×10^{-5} to 2.06×10^{-5} cm/second.

The kinetics of methyleugenol administered as part of an herbal extract may differ from those of pure methyleugenol. For example, an estimated T_{max} of approximately 7 hours and an elimination half-life of approximately 4 hours were observed in the plasma of male Sprague-Dawley rats (body weight, 240 ± 10 g; n = 6) exposed orally to an *Asarum* extract containing methyleugenol at approximately 766 mg/kg bw (Fan et al., 2021). Conversely, Meng et al. (2013) reported shorter T_{max} (10 minutes) and half-life (~68 minutes) values in the plasma of male Sprague-Dawley rats after oral exposure to 0.2 g/kg of an extract of *Acorus tatarinowii* Schott containing 0.5% methyleugenol (equivalent to a dose of 1 mg/kg bw).

4.1.2 Metabolism

(a) Exposed humans

Tremmel et al. (2017) investigated the role of the sulfotransferase SULT1A1 enzyme in the formation of methyleugenol–DNA adducts and the influence of genetic variance in the SULT1A1 gene on SULT1A1 phenotypes in the human liver. Liver and blood tissue samples (n = 121) collected from 121 Caucasian [White] patients undergoing liver surgery were analysed for: (i) blood sample genotyping, i.e. SULT1A1 copy number

variation; (ii) SULT1A1 mRNA expression levels; (iii) SULT1A1 protein expression levels; and (iv) concentration of methyleugenol–DNA adducts, i.e. N^2-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyguanosine (N^2-MIE-dG). Methyleugenol-specific DNA adducts were detected in all the liver samples, and the levels measured positively correlated with both mRNA and protein expression levels of SULT1A1. Additionally, a positive association was noted between SULT1A1 copy number variations and levels of methyleugenol–DNA adducts (see also Section 4.2.1(a)).

(b) Metabolism in human tissue-derived microsomes and cell lines

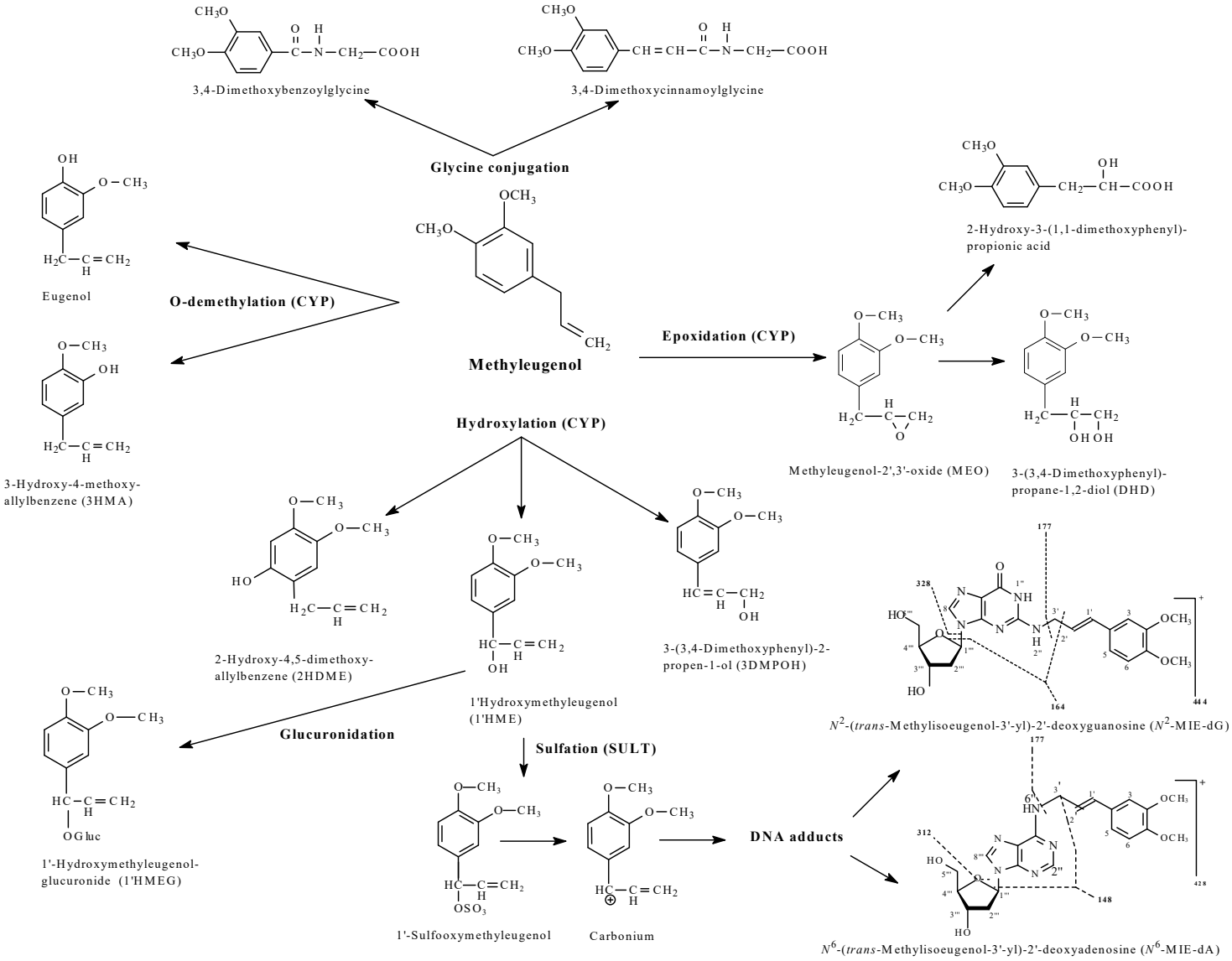
Al-Subeihi et al. (2012) investigated the metabolism of methyleugenol by microsomal preparations from the human liver, lung, kidney, or small intestine. Using pooled microsomal preparations, it was observed that only microsome preparations from the liver were capable of metabolizing methyleugenol and that six metabolites were formed (see Fig. 4.1), of which 1′-hydroxymethyleugenol and its glucuronide conjugate were the most abundant.

When examining human liver microsomes, Jeurissen et al. (2006) reported that the average rate of 1′-hydroxylation of methyleugenol by cytochrome P450 (CYP) in microsomes from 15 individual livers was 2.45 ± 0.34 nmol min^{-1} nmol CYP^{-1} and that activities varied 5-fold (from 0.89 to 4.30 nmol min^{-1} nmol CYP^{-1}). In comparison, Gardner et al. (1997) reported that the mean rate of 1′-hydroxylation of methyleugenol in microsomes from 13 individual human liver samples (~0.50 nmol min^{-1} nmol CYP^{-1}) varied by as much as 27-fold (from 0.05 to 1.33 nmol min^{-1} nmol CYP^{-1}). Al-Subeihi et al. (2015) reported an average rate of oxidation of 1′-hydroxymethyleugenol to 1′-oxomethyleugenol of 5.21 nmol min^{-1} nmol CYP^{-1} in microsomal 9000 × *g* (S9) preparations from 20 individual human liver samples and a difference of approximately 3-fold between the lowest and highest reported activities (from 3 to 8.3 nmol min^{-1} nmol CYP^{-1}). Al-Subeihi et al. (2015) also reported an average rate of sulfonation of 1′-hydroxymethyleugenol of 0.12 nmol min^{-1} nmol CYP^{-1} and an approximate 8-fold difference between the lowest and highest reported activities (0.03–0.25 nmol min^{-1} nmol CYP^{-1}). Gardner et al. (1997) reported that the variation in 1′-hydroxylation activities of the human liver samples was not correlated with CYP content.

[The Working Group noted the substantial variability in the rate of metabolism, i.e. 1′-hydroxylation of methyleugenol, observed in human liver microsomal preparations; however, it was also acknowledged that the results from studies using liver tissue biopsies may have limited relevance to the general population, since the livers of patients at the time of the biopsy probably did not exhibit normal function.]

Al-Subeihi et al. (2013) investigated the effects of nevadensin on the formation of methyleugenol–DNA adducts in human hepatocellular carcinoma cells (HepG2) exposed to 1′-hydroxymethyleugenol. The cells were incubated in the presence of 1′-hydroxymethyleugenol at 100 μM and nevadensin at 0, 0.002, 0.02, 0.2, or 2 μM for approximately 14 hours. Co-incubation with 1′-hydroxymethyleugenol at 100 μM and pentachlorophenol at 12.5 μM was used to model SULT enzyme inhibition. Co-exposure with 1′-hydroxymethyleugenol and nevadensin (from ≥ 0.02 μM nevadensin) resulted in a dose-dependent decrease (up to 88%) in the formation of DNA adducts. Co-exposure with 1′-hydroxymethyleugenol and pentachlorophenol resulted in nearly complete inhibition of the formation of DNA adducts. [The Working Group noted that a previous study identified nevadensin as a potent SULT inhibitor (Alhusainy et al., 2010), which is probably the reason for the observed reduction in methyleugenol–DNA adduct formation with nevadensin at concentrations of ≥ 0.02 μM. Alhusainy et al. (2014) also observed a 24-fold

Fig. 4.1 Proposed metabolic pathways for methyleugenol and its metabolites



CYP, cytochrome P450.
Adapted from Solheim and Scheline (1976); Al-Subeihi et al. (2012); Herrmann et al. (2012).

reduction in DNA adduct formation in male F344 rats after co-administration of nevadensin and methyleugenol (see Section 4.2.1).]

(c) Experimental systems

(i) Non-human mammals in vivo

Solheim and Scheline (1976) exposed male Wistar rats (body weight, 250–300 g) to methyleugenol at a dose of 100, 200, or 400 mg/kg bw by gavage or intraperitoneal injection. Urine and bile were sampled and analysed using GC-MS. No parent molecule was detected in the urine or bile of rats exposed by gavage or by intraperitoneal injection. Twenty-four hours after exposure by gavage, the predominant urinary metabolites were: 2-hydroxy-3-(3,4-dimethoxyphenyl)-propionic acid (20% of the administered dose); 3,4-dimethoxybenzoylglycine (32%); and 3,4-dimethoxycinnamoylglycine (22%). Similar urinary metabolites were observed after intraperitoneal exposure. The major metabolite detected in the bile after oral and intraperitoneal exposure (after treatment of the samples with β-glucuronidase and sulfatase) was 1′-hydroxymethyleugenol. Solheim and Scheline (1976) also noted that 4-dihydroxypropylbenzene is a urinary metabolite of methyleugenol; however, this metabolite was not detected in the urine of rats pre-exposed to neomycin. [The Working Group noted that the absence of 4-dihydroxypropylbenzene in the urine of neomycin-treated rats suggested that rat intestinal microflora might play a role in the metabolism of methyleugenol.]

As previously reported in Section 4.1.1(b), methyleugenol undergoes substantial first-pass metabolism when administered orally. To characterize the urinary metabolites, male F344 rats (n = 3) and female B6C3F$_1$ mice (n = 3) were exposed by gavage to [^{14}C]methyleugenol in corn oil as a single dose at 118 mg/kg bw (NTP, 2000). Three male F344 rats were also exposed by intravenous injection to [^{14}C]methyleugenol in ethanol:Emulphor:saline at a dose of 11.8 mg/kg bw. Urine samples were collected at various intervals up to 72 hours after exposure. Because of the low amounts of radioactivity detected 24 hours after exposure, samples from 24 hours and later were not characterized. Reverse-phase HPLC analysis of pooled urine samples at up to 24 hours revealed that no parent molecule was detected in the urine of male rats exposed intravenously or by gavage or in the urine of female mice after exposure by gavage. The metabolites detected in the urine of both species included hydroxylated, sulfated, and glucuronidated forms.

Feng et al. (2018) developed a method for detecting methyleugenol–DNA adducts (i.e. N^6-(methylisoeugenol-3′-yl)-2′-deoxyadenosine, N^6-MIE-dA) in rat urine. The analytical technique was validated by exposing groups of male Sprague-Dawley rats by gavage to pure methyleugenol (single and repeated doses) and various herbal extracts containing methyleugenol (i.e. *Asari* radix extract, *Acori tatarinowii* rhizoma extract, *Myristicae* semen extract, or Shi San Xiang extract). The doses of methyleugenol administered to each rat ranged from 1 to 25 mg/kg bw for pure methyleugenol and approximately 0.3 to 1.9 mg/kg bw as part of an herbal extract preparation. The amount of N^6-MIE-dA excreted in the urine increased in a dose-responsive manner after the administration of pure methyleugenol. N^6-MIE-dA was also detected in the urine of all rats exposed to the herbal extracts. Another DNA adduct form, N^2-(methylisoeugenol-3′-yl)-2′-deoxyguanosine, N^2-MIE-dG, was also detected in the rat urine; however, only at a dose of 25 mg/kg bw. [The Working Group noted that N^2-(methylisoeugenol-3′-yl)-2′-deoxyguanosine was the type of DNA adduct predominantly detected in the liver tissues of patients studied by Tremmel et al. (2017).] In an earlier study by Yao et al. (2016), several glutathione and related cysteine conjugates were detected in the urine and bile of rats exposed to methyleugenol at 100 mg/kg bw. [The Working Group noted that Yao et al. (2016)

detected three glutathione conjugates in the bile after either gavage or intraperitoneal administration. The conjugates were possibly formed from the α,β-unsaturated aldehyde, carbonium ion, or quinone methide of methyleugenol, e.g. reactive metabolites that may be associated with the putative metabolic pathway for genotoxicity of methyleugenol in rodents.]

(ii) Non-human mammalian cells in vitro

The NTP (2000) investigated the formation of methyleugenol metabolites in rat and mouse hepatocytes and compared it with that in human cells. The cells were incubated with various concentrations of methyleugenol for 18 hours, and the metabolites were characterized by mass spectrometry. Although no definitive structures were assigned, five common metabolites were identified, i.e. a glycine conjugate with a molecular weight of 239 Da; a demethylated and sulfated metabolite (molecular weight, 243 Da); a hydroxylated and sulfated metabolite (molecular weight, 273 Da); a sulfated diol metabolite (molecular weight, 291 Da) in mouse and human cells, but not in rat hepatocytes; and a hydroxylated glucuronide (molecular weight, 369 Da) in rat and mouse but not human hepatocytes.

Al-Subeihi et al. (2011) investigated the metabolism of methyleugenol in microsomal preparations from the liver, lung, and kidney of male Sprague-Dawley rats. The metabolism of methyleugenol in the liver of rats is very similar to that described for humans (Al-Subeihi et al., 2012); 1′-hydroxymethyleugenol is the most abundant metabolite formed. Metabolism in the lung of male rats appeared to be predominantly via O-demethylation; no 1′-hydroxymethyleugenol was formed in incubations with either the lung or the kidney. Only one metabolite was identified after exposure of kidney cells to methyleugenol, i.e. methyleugenol-2′3′-oxide. Male rat liver microsomes were also shown to form glucuronide conjugates, 1′-sulfooxymethyleugenol, and 1′-oxomethyleugenol. Cartus et al. (2012) and Gardner et al. (1997) described similar metabolism in rat liver microsomes.

Gardner et al. (1997) suggested that, at low concentrations, CYP2E1 enzyme but not CYP3A, CYP1A2, CYP2D1, or CYP2C11 catalysed 1′-hydroxylation of methyleugenol in rat hepatocytes in vitro. Additionally, Gardner et al. (1997) showed that pre-treatment of rats with methyleugenol at doses of between 30 and 300 mg/kg bw per day for 5 days increased the 1′-hydroxylation capacity of the harvested hepatocytes in vitro; this suggested that repeated oral exposure to high doses of methyleugenol may induce other enzymes responsible for 1′-hydroxylation (e.g. CYP2B and CYP1A2). Notably, Gardner et al. (1997) did not observe induction of 1′-hydroxylation in rat livers after pre-treatment with methyleugenol at 10 mg/kg bw per day for 5 days. In addition, the increases observed at higher doses (e.g. 30, 100, and 300 mg/kg bw per day) ranged from 38% to 67% higher than control levels. Carlsson et al. (2022) observed significantly higher levels of methyleugenol–DNA adducts (i.e. N^2-MIE-dG and N^6-MIE-dA) in V79 hamster fibroblast cells expressing human SULT1A1 (see also Section 4.2.1).

(iii) Enzymes involved in methyleugenol metabolism in human cells or humanized model systems

Jeurissen et al. (2006) identified human CYP enzymes involved in the metabolism of methyleugenol (i.e. methyleugenol 1′-hydroxylation) using microsomes expressing individual recombinant human CYP genes stably transfected into either insect or human lymphoblastoid cell lines. The activities of CYPs 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, and 3A4 in insect and human lymphoblastoid cells were investigated and compared with the activities of pooled microsomes from 15 samples of human liver. The enzyme activities of the human lymphoblastoid cells were roughly similar to those of human liver microsomes, but the activities of the

enzymes from the insect cells were much higher. After 20 minutes of incubation with 200 µM methyleugenol at 37 °C, insect cells expressing CYPs 1A2, 2A6, 2C9, 2C19, and 2D6 as well as human lymphoblastoid cells expressing CYPs 1A2, 2C9, 2C19, and 2D6 were able to 1′-hydroxylate methyleugenol. Co-incubating the human liver microsomes with various enzyme inhibitors showed that the CYP1A2 inhibitor α-naphthoflavone and the CYP2C9 inhibitor sulfaphenazole were most effective at inhibiting methyleugenol 1′-hydroxylation, with inhibitions of approximately 54% and 70% of control activities, respectively. For all other inhibitors investigated, the inhibition of 1′-hydroxylation was < 12%. This indicates that CYP1A2 and CYP2C9 are important enzymes for the 1′-hydroxylation of methyleugenol. However, at physiologically relevant concentrations of methyleugenol, CYP1A2 showed a significantly higher enzyme efficiency (i.e. $k_{cat}/K_m = 169$) than did CYP2C9 ($k_{cat}/K_m = 5$), CYP2C19 ($k_{cat}/K_m = 3$), and CYP2D6 ($k_{cat}/K_m < 3$). Jeurissen et al. (2007) suggested that related alkylbenzenes, such as estragole, may compete for binding sites on the relevant hydroxylation enzymes (e.g. CYP1A2), affecting methyleugenol metabolism. Additionally, lifestyle factors (e.g. smoking and use of barbiturates) or polymorphisms that may induce or inhibit these enzymes may contribute to interindividual differences in metabolism and, potentially, toxicity (Jeurissen et al., 2006, 2007).

Al-Subeihi et al. (2015) also used insect cells expressing human CYPs (i.e. CYPs 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, or 3A4), and UDP-glucuronosyltransferase (i.e. UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, or 2B17) to evaluate the metabolism of methyleugenol and the bioactivation of its metabolite (i.e. 1′-hydroxymethyleugenol). The results from the insect cell experiments suggested that: (i) CYP1A2 is the main enzyme involved in 1′-hydroxylation of methyleugenol; (ii) CYP2B6 is predominantly responsible for epoxidation of 1′-hydroxymethyleugenol; and (ii) UGT1A9 and UGT2B7 are predominantly responsible for glucuronidation of 1′-hydroxymethyleugenol.

Herrmann et al. (2014) conducted a study using genetically modified strains of FVB/N mice. Four mouse strains were studied, i.e. (i) wildtype (WT) for mouse Sult1a1; (ii) deficient in mouse Sult1a1 (knockout; KO); (iii) expressing human SULT1A1/2 (transgenic; TG); or (iv) hemizygous for human SULT1A1/2 (KO/TG). Mice were exposed to methyleugenol at doses ranging from 0.28 to 280 µmol/kg bw (from 0.05 to 50 mg/kg bw) via gavage or an equimolar high dose of 1′-hydroxymethyleugenol (280 µmol/kg bw; 54.5 mg/kg bw) via intraperitoneal injection. All animals were killed 6 hours after dosing, according to the optimal interval for detecting DNA adduct formation in the liver. Herrmann et al. (2014) found that, after exposure to methyleugenol, the formation of DNA adducts in the livers of KO mice was reduced by ≥ 97% compared with WT mice and that TG mice showed enhanced DNA adduct formation compared with WT mice. Additionally, there was significantly greater DNA adduct formation in WT, TG, and KO/TG mice after equimolar intraperitoneal dosing with 1′-hydroxymethyleugenol than after dosing with methyleugenol exposure by gavage. These results confirmed that SULT1A is a key enzyme responsible for the bioactivation of methyleugenol. Subsequent analysis of extrahepatic tissue from a similar experiment using these transgenic mice showed that an increase in the formation of DNA adducts was also detected in the extrahepatic tissues in the following order: liver > caecum > kidney > colon > stomach > small intestine > lung > spleen (Herrmann et al., 2016). Closer examination of SULT1A activities in the liver, caecum, kidney, and stomach suggested that the formation of DNA adducts in extrahepatic tissue after methyleugenol exposure is dependent on levels of SULT1A enzymes in the tissue. For example, high expression levels of human SULT1A1/2 and mouse Sult1a1 are observed in

the liver and caecum, but extremely low levels of both enzymes are observed in the stomach (Herrmann et al., 2016) (see also Section 4.2.1).

Herrmann et al. (2012) investigated whether recombinant *S. typhimurium* bacteria strain TA100 expressing human sulfotransferases (i.e. SULT1A1, 1C2, 1A2, 1E1, 1A3, 1C1, 1C3, 2A1, and 2B1b) were capable of activating hydroxylated metabolites of methyleugenol to mutagens in the Ames assay. For this experiment, three hydroxylated forms of methyleugenol (i.e. (+)-1′-hydroxymethyleugenol, (–)-1′-hydroxymethyleugenol, and (*trans*)-3′-hydroxymethylisoeugenol) were synthesized and incubated with *S. typhimurium* expressing various individual human sulfotransferases versus those deficient in sulfotransferases. *S. typhimurium* expressing SULT1A1 (expressed in eukaryotes in many tissues) and SULT1C2 (expressed primarily in fetal tissue) showed higher revertant frequencies with very low concentrations of hydroxylate than did the respective deficient TA100 strain. No activation was observed for strains expressing SULT1A3, SULT1C1, SULT1C3, SULT2A1, or SULT2B1b. Some activation was observed for SULT1A2 and SULT1E1 but at a much lower level than observed for SULT1A2 and SULT1E1. [The Working Group noted that since the level of protein expression in bacteria differs significantly from that in eukaryotes, the relevance of these results to the metabolism of methyleugenol by humans should be considered cautiously.]

Herrmann et al. (2012) also investigated the effectiveness of murine sulfotransferases (Sult1a1 or Sult1d1) to activate hydroxylated metabolites of methyleugenol (i.e. (+)-1′-hydroxymethyleugenol, (–)-1′-hydroxymethyleugenol, and (*trans*)-3′-hydroxymethylisoeugenol) using recombinant bacteria (*S. typhimurium* strain TA100) and the Ames assay. Murine Sult1a1 required higher concentrations of all three hydroxylated methyleugenol metabolites to cause a noticeable, but much lower, increase in revertant frequencies compared with its human orthologue (SULT1A1). Murine Sult1d1 did not activate any of the hydroxylated methyleugenol metabolites. In a similar experiment, Honda et al. (2016) used modified TA100 strains of *S. typhimurium* in the Ames assay to investigate the activities of human SULT1C2. Using optimized in vitro conditions (i.e. protein content, 1.2 mg/plate; pre-incubation time, 2 hours), methyleugenol was found to increase the number of revertants significantly and reproducibly in the modified TA100 strains (Honda et al., 2016) (see also Section 4.2.2).

(iv) Acellular systems

Sipe et al. (2014) investigated the metabolism of methyleugenol using horseradish peroxidase and electron paramagnetic resonance spectroscopy. [The Working Group noted that the study provided evidence (through spectral analysis) that methyleugenol underwent peroxidative metabolism in vitro to form free radicals and that these radicals were not good substrates for catalase (see also Section 4.2.5).]

4.1.3 Physiologically based kinetic modelling

The evaluation of the toxicokinetics of methyleugenol has also been approached through physiologically based kinetic (PBK) modelling. For example, the NTP developed PBK models for methyleugenol in rats and mice on the basis of in vivo toxicokinetic parameters determined in F344 rats and B6C3F$_1$ mice and on information available in the literature (NTP, 2000). More recently, Al-Subeihi et al. developed a series of PBK models for methyleugenol. One PBK model in rats was based on in vitro metabolic parameters, in silico-derived partition coefficients, and information on physiological parameters identified in the literature (Al-Subeihi et al., 2011). A second PBK model accounted for SULT inhibition by nevadensin (Al-Subeihi et al., 2013) A third PBK model was developed specifically for humans and was based on in vitro (i.e. phase I

and II metabolism by human cell microsomes) and in silico-derived parameters (Al-Subeihi et al., 2012). The human PBK model was further refined by including: (i) CYP1A2-catalysed bioactivation of methyleugenol; (ii) CYP2B6-catalysed epoxidation of methyleugenol; (iii) the apparent kinetic constants for oxidation of 1′-hydroxymethyleugenol; and (iv) the apparent kinetic constants for sulfation of 1′-hydroxymethyleugenol (Al-Subeihi et al.,2015).

4.2 Evidence relevant to key characteristics of carcinogens

This section reviews the mechanistic data for the key characteristics of carcinogens (Smith et al., 2016) encompassed by methyleugenol. Evidence was available on whether methyleugenol exhibits the key characteristics "is electrophilic or can be metabolically activated to an electrophile", "is genotoxic", "induces oxidative stress", "induces chronic inflammation", "is immunosuppressive", and "modulates receptor-mediated effects", "causes immortalization", and "alters cell proliferation, cell death, or nutrient supply". The evidence for the key characteristics of carcinogens "alters DNA repair or causes genomic instability" and "induces epigenetic alterations" was sparse. The exposure assessments for the mechanistic studies in humans are reported in Section 1.6.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

(a) Humans

(i) Exposed humans

See Table 4.1 and Fig. 4.1.

Evidence of electrophilicity in exposed humans was reported in three studies. Herrmann et al. (2013) investigated the formation of DNA adducts in liver tissue samples collected from surgical liver interventions from a group of 30 individuals (18 males and 12 females) at the Campus Virchow, University Medical Center Charité, Berlin, Germany. Of the 30 surgical non-tumour liver samples, 29 were found to contain the major DNA adduct formed by methyleugenol, namely N^2-MIE-dG, at levels in the range of 1.1–36.2 adducts/10^8 nucleosides, detected by isotope dilution UPLC-MS/MS (Herrmann et al., 2013). The analogous adenosine adduct, N^6-MIE-dA, was also detected in most samples, although at a level that was lower by 60-fold. The maximum and median levels of both adducts combined were 37 and 13 adducts/10^8 nucleosides, respectively. In a further study from the same research group, Tremmel et al. (2017) detected specific methyleugenol–DNA adducts in 121 human liver tissue specimens from subjects undergoing surgery in the department of general, visceral, and transplantation surgery at the Charité centre (Campus Virchow, University Medical Center Charité, Humboldt University Berlin, Germany). The levels of adducts varied by 122-fold (Tremmel et al., 2017). These levels correlated significantly with mRNA and protein levels of human sulfotransferase SULT1A1, indicating the role of this isozyme in the metabolic activation of methyleugenol, as also mentioned in Section 4.1.

In another study, Monien et al. detected the most common DNA adduct formed by methyleugenol, N^2-MIE-dG, in the range of 1.7–23.7/10^8 nucleosides, as measured by UPLC-MS/MS in non-tumour pulmonary tissue samples from 4 men and 6 women with lung cancer, provided by Biopredic International, Rennes, France (Monien et al., 2015). The less frequent adduct, N^6-MIE-dA, was detected in the range of 0.31–1.18 adducts/10^8 nucleosides in 5 out of 10 lung tissue samples. The levels of N^6-MIE-dA were on average 27-fold lower than the levels of N^2-MIE-dG. [The Working Group noted that, although these studies did not investigate specific sources of exogenous exposure of methyleugenol (see Section 1.6), the results might

Table 4.1 End-points relevant to electrophilicity in humans exposed to methyleugenol

End-point (assay)	Biological sample type	Location	No. of exposed and controls	Response[a] (No. of individuals with/without DNA adducts)	Covariates controlled	Comments	Reference
DNA adducts (^{32}P-postlabelling)	Surgical, non-tumourous liver	Germany	18 men, 12 women	+ (29/30)	Patients with hepatitis, cirrhosis, or chronic excessive alcohol use	Source of exposure not documented.	Herrmann et al. (2013)
DNA adducts (^{32}P-postlabelling)	Surgical, non-tumourous liver	Germany	121 participants	+ (121/121)		Source of exposure not documented. Inverse relationship between adducts levels and age.	Tremmel et al. (2017)
DNA adducts (^{32}P-postlabelling)	Surgical, non-tumourous lung	France	4 men, 6 women	+ (10/10)		Source of exposure not documented.	Monien et al. (2015)

[a] +, positive.

demonstrate evidence of universal human exposure to methyleugenol.]

(ii) Human cells in vitro

See Table 4.2.

DNA adducts, analysed by ^{32}P-postlabelling, were formed in a dose-dependent manner in metabolically competent human hepatocellular carcinoma HepG2 cells when exposed to methyleugenol at increasing concentrations (50–450 µM) (Zhou et al., 2007).

N^2-MIE-dG and *N*^{6}-MIE-dA adducts were also formed in HepG2 cells treated with 1′-hydroxymethyleugenol (25–150 µM), as the main phase I metabolite of methyleugenol (Carlsson et al., 2022).

(b) Experimental systems

(i) Humanized animals

The role of sulfotransferases (SULTs) in the metabolism of methyleugenol has been demonstrated in genetically modified mice (see also Section 4.1). The formation of DNA adducts in the liver induced by oral exposure to methyleugenol or by intraperitoneal exposure to 1′-hydroxymethyleugenol and 3′-hydroxymethylisoeugenol was greatly reduced in the liver of *Sult1a1* knockout mice, compared with wildtype mice.

In mice transgenic for human *SULT1A1/2*, adduct levels were higher by about 10-fold than in wildtype mice, and were similar whether or not the mouse gene was also present (i.e. the combination of both human and mouse genes) (Herrmann et al., 2014).

SULT1A forms also dominate metabolic activation in several extrahepatic tissues. Using the same knockout and transgenic mouse strains, Herrmann et al. (2016) found that DNA adduct formation by methyleugenol in mouse caecum and kidney was almost entirely dependent on the presence of mouse *Sult1a1* or human *SULT1A1/2*. In the stomach, however, adduct formation was independent of SULT1A status.

(ii) Non-human mammals in vivo

See Table 4.3.

Intraperitoneal exposure of adult female CD-1 mice to methyleugenol at a dose of 2 or 10 mg/mouse in 0.1 mL of trioctanoin induced the formation of DNA adducts in the liver at levels of 150 ± 15 or 646 ± 88 adducts/10^7 nucleotides, respectively, as assessed by ^{32}P-postlabelling (Randerath et al., 1984).

Methyleugenol was the most potent DNA adduct-forming compound out of the seven compounds tested in a study designed to test the carcinogenicity of alkenylbenzenes. Male C57B1 × C3H/He F_1 (B6C3F_1) mice were injected with methyleugenol at a dose of 0.25, 0.5, 1.0, or 3.0 µmol on days 1, 8, 15, and 22 after birth, respectively. Liver DNA was isolated on days 23, 29, and 43 and analysed by ^{32}P-postlabelling. DNA adducts were observed at levels of 72.7 ± 10.7 pmol/mg DNA on day 23, 37.1 ± 9.7 pmol/mg DNA on day 29, and 25.6 ± 6.2 pmol/mg DNA on day 43 (Phillips et al., 1984).

DNA adducts were also detected, but not quantified, by HPLC-^{32}P-postlabelling analysis of the livers of mice treated intraperitoneally with methyleugenol at a dose of 2000 µmol/kg (Levy and Weber, 1988).

Male Sprague-Dawley rats exposed to methyleugenol at a dose of 1, 5, or 25 mg/kg bw by gavage produced the DNA adduct *N*^{6}-MIE-dA, which was detected in urine samples collected at 12-hour intervals. *N*^{6}-MIE-dA was excreted in a time- and dose-dependent manner (Feng et al., 2018).

The potential of methyleugenol to induce DNA-adduct formation was also proven by the co-exposure of male F344 rats to nevadensin (a SULT inhibitor; 120 mg/kg bw per day for 3 days per week) and methyleugenol (250 mg/kg bw per day, three times per week), resulting in a reduction of 24-fold in adduct formation after 8 weeks compared with rats exposed to methyleugenol only (Alhusainy et al., 2014) (see also

Table 4.2 End-points relevant to genotoxicity and related effects in human cells in vitro exposed to methyleugenol

End-point (assay)	Cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA strand breaks (alkaline comet assay)	Human colon carcinoma H29 cells	+	NT	50 µM	Comets also formed by 1′-hydroxymethyleugenol (at 50 µM), 3-oxomethylisoeugenol, and methyleugenol-2′,3′-oxide (at ≥ 25 µM)	Groh et al. (2016)
Micronucleus formation	Human colon carcinoma H29 cells	–	NT	100 µM	Micronuclei also formed by 1′-hydroxymethyleugenol, 3′-oxomethylisoeugenol, and methyleugenol-2′,3′-oxide (at ≥ 10 µM)	Groh et al. (2016)

HIC, highest ineffective concentration; LEC, lowest effective concentration, NT, not tested.

[a] +, positive; –, negative.

Table 4.3 End-points relevant to electrophilicity and genotoxicity in non-human mammals in vivo exposed to methyleugenol

End-point (assay)	Species, strain (sex)	Tissue or cells	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA adducts	Mouse, CD-1 (F)	Liver	+	2 mg/kg	i.p., for 24 h		Randerath et al. (1984)
DNA adducts	Mouse, B6C3F$_1$ (M)	Liver	+	0.25, 0.5, 1.0, and 3.0 µmol on days 1, 8, 15, and 22 after birth	Injected; DNA isolated on days 23, 29, and 43		Phillips et al. (1984)
DNA adducts	Mouse, C57BL/6J	Liver	+	2000 µmol/kg	i.p, for 3 h		Levy and Weber (1988)
DNA adducts	Mouse, wildtype (FVB/N), *Sult1a1*-knockout, transgenic human *SULT1A1/2*	Liver	+	50 mg/kg	By gavage, for 6 h	Adduct levels greatly reduced in *Sult1a1*-knockout mice; 10-fold higher in human *SULT1A1/2*-transgenic mice than in wildtype mice.	Herrmann et al. (2014)
DNA adducts	Mouse, wildtype (FVB/N), *Sult1a1* knockout, transgenic human *SULT1A1/2*	Caecum, kidney	+	50 mg/kg	By gavage, 1.5–24 h	Adduct formation highly dependent on the presence of mouse *Sult1a1* or human *SULT1A*.	Herrmann et al. (2016)
DNA adducts	Rat, F344 (M)		+	250 mg/kg per day	By gavage, 3×/wk for 8 wk	Adduct levels reduced 24-fold by co-administration of nevadensin, a SULT inhibitor.	Alhusainy et al. (2014)
DNA adducts	Rat, Sprague-Dawley (M)	Urine	+	1, 5, or 25 mg/kg	By gavage; urine collected at 12 h intervals	dA adduct of methyleugenol excreted in the urine.	Feng et al. (2017)
DNA strand breaks (alkaline comet assay)	Rat, F344 (M)	Liver, bladder, kidney, lung	–	2000 mg/kg, at 1–8 h	By gavage, 1, 3, 6 and 8 h	Comet formation observed at 6 h and 8 h. when endonuclease III included in the protocol.	Ding et al. (2011)
		Bone marrow	+/–	2000 mg/kg		Bone marrow gave positive results at 8 h only.	
Gene mutation	Rat, *Gpt* delta (M, F)		+	100 mg/kg per day	By gavage, for 13 wk		Jin et al. (2013)

Table 4.3 (continued)

End-point (assay)	Species, strain (sex)	Tissue or cells	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Gene mutation	Mouse, B6C3F$_1$/N (M, F)	Liver tumour	+	37, 75, or 150 mg/kg bw	By gavage, for 2 years	Analysis of samples from a 2-year cancer study. Exome sequencing revealed a higher mutation burden than found in spontaneous tumours.	Auerbach et al. (2018)
Micronucleus formation	Mouse, B6C3F$_1$/N (M, F)	Peripheral blood erythrocytes	–	1000 mg/kg	By gavage for 14 wk	No increase in percentage of polychromatic erythrocytes.	NTP (2000)

dA, deoxyadenosine; dG, deoxyguanosine; F, female; h, hour(s); HID, highest ineffective dose; i.p., intraperitoneal; LED, lowest effective dose; M, male; NT, not tested; ppm, parts per million; SULT, sulfotransferase; wk, week(s).

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study).

Section 4.1.2). Pentachlorophenol (11 mg/kg per day), included in the study as a positive control, caused a reduction in DNA-adduct formation that was similar to that caused by methyleugenol.

Methyleugenol has also been shown to covalently modify RNA. Yang et al. (2020) detected three adenosine adducts (formed at the 1-, 3-, and 6-positions of adenine, characterized by LC-MS/MS) plus two guanosine adducts and one cytidine adduct in the hepatic tissues of male Kunming mice exposed intraperitoneally to a mixture of chemically synthesized methyleugenol d_0-Me/d_3-Me (1:1) at a dose of 10, 30, or 50 mg/kg.

Intraperitoneal exposure of male Fisher rats to methyleugenol, either as a single dose at 10 or 100 mg/kg or at doses of 10, 30, 100, or 300 mg/kg per day for five consecutive days, induced the formation of covalent protein adducts in the liver, as assessed by enzyme-linked immunosorbent assay (ELISA) using specific antisera raised in rabbits treated with serum albumin conjugated with 1′-acetoxymethyleugenol (Gardner et al., 1996). Adducts were formed in a dose-dependent manner, with the highest levels found in microsomal fractions and lesser amounts in the nuclear, mitochondrial, and cytosolic fractions. Methyleugenol was also shown to interact with glutathione and proteins (see Section 4.1.2(b)). In fact, biliary glutathione and urinary cysteine conjugates were observed in male Sprague-Dawley rats exposed to methyleugenol at 100 mg/kg (Yao et al., 2016).

(ii) Non-human mammalian cells in vitro

See Table 4.4.

Incubation of primary hepatocytes isolated from male Wistar rats with methyleugenol and some of its metabolites formed by rat, bovine and human liver microsomes, such as 1′-hydroxymethyleugenol, 3′-hydroxymethylisoeugenol, and 3′-oxomethylisoeugenol, induced the formation of the deoxyguanosine adduct, N^2-MIE-dG. The deoxyadenosine adduct N^6-MIE-dA was also detectable in some incubations at levels 50 times as low as the deoxyguanosine adduct. The levels of adducts formed by 1′-hydroxymethyleugenol were higher than those formed by methyleugenol itself, suggesting the importance of this metabolite in the pathway of metabolic activation of the parent compound (Cartus et al., 2012).

Carlsson et al. observed a significant increase in both N^2-MIE-dG and N^6-MIE-dA DNA adducts in V79 hamster fibroblasts expressing the human SULT1A1 enzyme and exposed to methyleugenol, thus proving the role of SULT enzyme in the metabolic activation of 1′-hydroxymethyleugenol (Carlsson et al., 2022).

As reported in studies in vivo, reactive metabolites of methyleugenol have also been shown to modify RNA in vitro. Yang et al. (2020) used LC-MS/MS to detect and characterize three adenosine adducts, formed at the 1-, 3-, and 6-positions of adenine, in RNA from primary mouse hepatocytes incubated with methyleugenol at 100 μM.

(iii) Non-mammalian experimental systems

See Table 4.5.

In studies that investigated the potential effects of methyleugenol in a turkey egg genotoxicity assay, it was observed that the injection of methyleugenol (2–4 mg/egg) into turkey eggs containing 22–24-day-old fetuses induced DNA adducts in the fetal liver, as assessed by ^{32}P-postlabelling analysis (Kobets et al., 2016, 2018). In a follow-up study, Kobets et al. (2019) confirmed the formation of N^2-guanine and N^6-deoxyadenine derivatives by mass spectrometry and also observed DNA adduct formation in fetuses contained in chicken eggs treated with methyleugenol (Kobets et al., 2019).

As mentioned in Section 4.1.2(b), hydroxylated metabolites of methyleugenol induced the formation of DNA adducts, determined by UPLC-MS/MS, in *S. typhimurium* strain TA100 expressing either human or murine sulfotransferases (Herrmann et al., 2012). Specifically, (+)-1′-hydroxymethyleugenol, (–)-1′-

Table 4.4 End-points relevant to electrophilicity and genotoxicity in non-human mammalian cells in vitro exposed to methyleugenol

End-point (assay)	Species, strain, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA adducts (mass spectrometry)	Rat, Wistar (M), primary hepatocytes	+	NT	250 µM	dG adduct detected, plus dA adduct at 50-times lower level. Adducts also formed by 1′-hydroxymethyleugenol, 3′-hydroxymethylisoeugenol and 3′-oxomethylisoeugenol	Cartus et al. (2012)
DNA strand breaks (comet assay)	Hamster, Chinese, lung fibroblasts, V79	+	NT	25 µM		Groh et al. (2012)
Unscheduled DNA synthesis	Rat, F344 (M), primary hepatocytes	+	NT	1000 µM		Howes et al. (1990); Chan and Caldwell (1992)
Unscheduled DNA synthesis	Rat, F344 (M), primary hepatocytes	+	NT	10 µM	One concentration tested	Burkey et al. (2000)
	Mouse, $B6C3F_1$ (F), primary hepatocytes	+	NT	10 µM		
Micronucleus formation	Hamster, Chinese, lung fibroblasts, V79	–	NT	100 µM	Although methyleugenol gave negative results, several metabolites gave positive results	Groh et al. (2012)
Sister-chromatid exchange	Hamster, Chinese, ovary, CHO cells	–	+	17 µg/mL	Toxic at 233 µg/mL –S9	NTP (2000)
Chromosomal aberrations	Hamster, Chinese, ovary, CHO cells	–	–	233 µg/mL	Toxic at 500 µg/mL	NTP (2000)

CHO, Chinese hamster ovary; dA, deoxyadenosine; dG, deoxyguanosine; HIC, highest ineffective concentration; LEC, lowest effective concentration, NT, not tested; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative.

Table 4.5 End-points relevant to genotoxicity and related effects of methyleugenol and metabolites in non-mammalian experimental systems

Test system (species, strain)	End-point (assay)	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Turkey embryos, age 22–24 days	DNA adducts (^{32}P-postlabelling)	+	NA	3 doses totalling 4 mg/egg	Adducts confirmed by mass spectrometry	Kobets et al. (2016, 2018, 2019)
Chicken embryos, age 9–11 days	DNA adducts (^{32}P-postlabelling)	+	NA	3 doses totalling 2 mg/egg	Adducts confirmed by mass spectrometry	Kobets et al. (2019)
Turkey embryos, age 22–24 days	DNA strand breaks (comet assay)	–	NA	3 doses totalling 4 mg/egg		Kobets et al. (2016, 2018)
Saccharomyces cerevisiae, RS9	Recombination	+	+	0.68–2.04 mM [121.3–363.75 µg/mL]	Intrachromosomal (*HIS*$^+$) and interchromosomal (*ADE*$^+$) recombination in a dose-related manner	Schiestl et al. (1989)
Saccharomyces cerevisiae, RS112	Recombination	+	NA	0.6 mg/mL	DEL recombination; nonlinear dose–response relation	Brennan et al. (1996)
Salmonella typhimurium TA97, TA100, TA1535, TA1537	Mutation	–	–	6 µM/plate	Highest dose is lower than OECD 471 recommendations because of toxicity	Schiestl et al. (1989)
Salmonella typhimurium TA98, TA98, TA100, TA102	Mutation	–	–	333 µg/plate	Highest dose is lower than OECD 471 recommendations due to toxicity	NTP (2000)
Salmonella typhimurium TA100-hSULT1A1, -hSULT1A1, -hSULT1A2, -hSULT1E1	Mutation	+	NA	~3 nmol (+)- and (–)-1′-hydroxymethyleugenol, 3′-hydroxymethylisoeugenol	Metabolites tested	Herrmann et al. (2012)
Salmonella typhimurium TA100-hSULT1A3, -hSULT1C1, -hSULT1C3, -hSULT2A1, -hSULT2B1b	Mutation	–	NA	100 nmol (+)- and (–)-1′-hydroxymethyleugenol, 3′-hydroxymethylisoeugenol	Metabolites tested	Herrmann et al. (2012)
Salmonella typhimurium TA100-hSULT1A1	Mutation	+	NA	31 µM 1′-hydroxymethyleugenol	Metabolite tested	Berg et al. (2016)
Escherichia coli WP2 *uvrA*	Mutation	–	–	300 µg/plate	Highest dose is lower than OECD 471 recommendations due to toxicity	Sekizawa and Shibamoto (1982)

Table 4.5 (continued)

Test system (species, strain)	End-point (assay)	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium strain with hSULT1C2	Mutation	–	+	0.391–62.5 µg/plate	Dose–response relation up to 62.5 µg/plate +S9	Honda et al. (2016)
Bacillus subtilis rec H17 Rec$^+$) and M45 (Rec$^-$) strains	DNA damage (rec assay)	+	NA	1.0 mg/disk		Sekizawa and Shibamoto (1982)

HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not applicable; NR, not reported; OECD, Organisation for Economic Co-operation and Development; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative.

hydroxymethyleugenol, and (*trans*)-3′-hydroxymethylisoeugenol induced the formation of DNA adducts in bacteria expressing human SULT1A1; low levels of DNA adducts were also detected in bacteria expressing human SULT1A2 and SULT1E1 and murine Sult1a1. No DNA adduct formation was observed in bacteria expressing other human SULT forms (1A3, 1C1, 1C3, 2A1, and 2B1b) or murine Sult1d1, or in the unmodified parent TA100 strain. The main adduct formed was N^2-MIE-dG, with much lower levels of the minor adduct N^6-MIE-dA detectable in some cases.

(iv) Acellular systems

Methyleugenol labelled with ^{14}C was reported to bind to calf thymus DNA both in the presence of Araclor 1254-induced rat and mouse liver S9 and in the presence of uninduced rat, mouse, and human liver S9 (NTP, 2000). Methyleugenol (100 μM) was also reported to modify protein cysteine residues in mouse liver microsomes, forming three types of modification derived from the elctrophilic intermediates, α,-β-unsaturated aldehyde and the 1′-carbonium ion of methyleugenol, as characterized by LC-MS/MS (Feng et al., 2017).

4.2.2 Is genotoxic

(a) Humans

(i) Exposed humans

No data on genotoxicity-associated endpoints in exposed humans were available to the Working Group.

(ii) Human cells in vitro

See Table 4.2.

Methyleugenol and three of its metabolites (1′-hydroxymethyleugenol, methyleugenol-2′,3′-epoxide, and 3′-oxomethylisoeugenol) induced alkali-labile lesions in the DNA of human colon carcinoma H29 cells, as measured by the alkaline comet assay (Groh et al., 2016). The parent compound and 1′-hydroxymethyleugenol gave positive results at 50 μM, the other two metabolites at 25 μM and above. Methyleugenol did not induce micronucleus formation at up to 100 μM, although methyleugenol-2′,3′-epoxide at 100 μM and 3′-oxomethylisoeugenol at ≥ 10 μM gave positive results. Micronuclei were CREST-positive (kinetochore-containing), indicating an aneugenic mode of action (Groh et al., 2016).

A DNA damage response, including induction of γH2AX, was observed in human liver HepG2 cells treated with the methyleugenol metabolite 1′-hydroxymethyleugenol at 25–150 mM, but strand breaks, measured by the comet assay, were not detected (Carlsson et al., 2022).

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.3.

DNA damage was assessed by the comet assay conducted on male F344 rats exposed to methyleugenol as a single oral dose at 400 or 1000 mg/kg bw (Ding et al., 2011). There was no evidence of increased DNA damage in the liver, bone marrow, bladder, kidney, and lung at 3 and 24 hours after treatment. After a higher dose of 2000 mg/kg bw, the liver, bone marrow, and bladder did not exhibit DNA damage at 1, 3, 6, and 8 hours after treatment, with the exception of the bone marrow at 8 hours. However, when a modified comet assay (including endonuclease III in the protocol for liver samples) was used (Table 4.6), comet formation was detected in methyleugenol-exposed rats at 6 and 8 hours, suggesting that methyleugenol induced oxidative damage in DNA.

When methyleugenol was investigated in the F344 *gpt* delta transgenic rat model, in male and female rats treated orally at 0, 10, 30, or 100 mg/kg for 13 weeks, a significant increase in *gpt* and Spi^- mutation frequencies was observed in the liver at the highest dose (a carcinogenic dose) (Jin et al., 2013). [The Working Group noted that

Table 4.6 End-points relevant to oxidative stress in experimental systems exposed to methyleugenol

End-point	Assay	Species, strain (sex), cell line, system	Results[a]	Dose or concentration (LEC/LED or HIC/HID)	Comments	Reference
Non-human mammals in vivo						
Oxidative DNA damage	Endo III-comet assay	Rats, F344 (M), liver	+	2000 mg/kg, gavage, 1, 3, 6 and 8 h	Comet formation was observed at 6 h and 8 h when endonuclease III was included in the protocol.	Ding et al. (2011)
ROS	Hydroethidine	Rats, SD (M)	↓	100 mg/kg, i.p.	ROS ↑ after experimentally induced ischaemia (visualized microscopically). The increase was partially reversed by methyleugenol. No control using methyleugenol with non-ischaemic tissue.	Choi et al. (2010)
GSH/GST GSH content GST content SOD CAT NO Lipid peroxidation	GSH content GST content Enzyme activity Enzyme activity Griess reagent TBARS	Intestinal mucosal tissue, homogenized from adult Wistar rats, treated with methyleugenol, hypoxia (in parentheses), or both, respectively	NC, (↓), ↑ NC, (↓), ↑ NC, (↓), ↑ NC, (↓), ↑ NC, (↑), ↓ NC, (↑), ↓	100 mg/kg per day, gavage, 30 days	Methyleugenol treatment of rats with and without ischaemia induced by surgical ligation of intestinal tissue for 30 min followed by perfusion. In most cases, methyleugenol treatment of ischaemic tissue changed the measured parameters to control values.	Saleh and El-Shorbagy (2017)
DSBs Oxidative DNA damage	Fpg-comet assay	V79 Chinese hamster lung fibroblasts	+	25 µM	Enhanced formation of DSBs	Groh et al. (2012)
Non-human mammalian cells in vitro						
ROS	DCFDA	Mixed glial cells cultured from prefrontal cortex of SD rats (M), age 1 day	↓	10 µM	ROS ↑ after hypoxia (damage visualized microscopically and by LDH release) or stimulation with IFNγ/LPS. Partially reversed by methyleugenol exposure. No methyleugenol control experiments without hypoxia or IFNγ/LPS, respectively.	Choi et al. (2010)

Table 4.6 End-points (continued)

End-point	Assay	Species, strain (sex), cell line, system	Results[a]	Dose or concentration (LEC/LED or HIC/HID)	Comments	Reference
MnSOD, CAT	Enzyme activity	Primary cortical neuronal cultures from SD (M) rats, day 17–18 of gestation	↑	10 μM	Increase demonstrated after hypoxia treatment (but methyleugenol concentration unclear). methyleugenol (10 μM) partially reversed increases in NO and iNOS gene expression induced by IFNγ/LPS. No control experiments without hypoxia or IFNγ/LPS treatments, respectively.	Zhou et al. (2017)
ROS	DCF fluorescence	Mouse macrophage cell line RAW 264.7	NC, (↑), ↓	140 μM [25 μg/mL] methyleugenol, tBHP (alone), or both, respectively	Cells were exposed to methyleugenol alone, tBHP, or tBHP with increasing concentrations of methyleugenol. tBHP increased ROS and decreased GSH and SOD activity. Methyleugenol was not cytotoxic (MTT assay) but 2 mM tBPH was. Co-incubation of 2 mM tBHP and increasing methyleugenol concentrations partially reversed tBPH cytotoxicity and impact on ROS generation and SOD activity.	
GSH	Protein content, tBHP	Mouse macrophage cell line RAW 264.7	↑, (↓), ↑			
SOD	Protein content, tBHP	Mouse macrophage cell line RAW 264.7	↑, (↓), ↑			
HO-1 GCLC GCLM NQO1	Protein expression – western blot analysis	Mouse macrophage cell line RAW 264.7	↑ ↑ ↑ ↑	LEC, 70 μM LEC, 35 μM LEC, 35 μM LEC, 70 μM		
Nrf2 Keap1/β-actin	Protein expression – western blot analysis	Mouse macrophage cell line RAW 264.7	↑ ↓	140 μM 140 μM		
ARE response	Luciferase activity	Mouse macrophage cell line RAW 264.7	↑	140 μM		
ROS	DCF fluorescence	Mouse macrophage cell line J774A.1	NC, (↑), ↓	140 μM methyl-eugenol, tBHP (alone), or both, respectively		

Table 4.6 End-points (continued)

End-point	Assay	Species, strain (sex), cell line, system	Results[a]	Dose or concentration (LEC/LED or HIC/HID)	Comments	Reference
Nrf2	Transcription activity (ARE luciferase) and qRT-PCR	NIH 3T3 cell line HEK293 cell line	No transcription ↑ Transcription	25 µg/mL		Ma et al. (2021)
Non-mammalian species						
Lipid peroxidation	TBARS	*Candida albicans*	↑	LEC, 56 µM		Khan et al. (2011)
SOD, CAT, GPX			↑	LEC, 56 µM		
GSH			↓	LEC, 56 µM		
Acellular systems						
GSH activity	In vitro reaction of GSH with 1-chloro-2,4-dinitrobenzene	GST isozymes isolated from male Wistar rat liver			Concentration that inhibited 25% of the reaction for various isozymes: GST 1-1, 0.6 mM GST 1-2, > 2.5 mM GST 3-3, 0.5 mM GST 4-4, 1.3 mM GST 7-7, 1.2 mM	Rompelberg et al. (1996)

ARE, antioxidant response element; CAT, catalase; DCF, dichlorofluorescein; DCFDA, 2′,7′-dichlorofluorescein diacetate; DSB, double-strand break; Endo III, endonuclease type III; ESR, electron spin resonance; Fpg, formamidopyrimidine DNA glycosylase (also known as 8-oxoguanine DNA glycosylase); GCLC, glutamate cysteine ligase catalytic/modifier subunit; GSH, glutathione; GST, glutathione *S*-transferase; h, hour(s); HEK293 human embryonic kidney cell line; HIC/HID, highest ineffective concentration/dose; HO-1, haem oxygenase 1; IFNγ, interferon gamma; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; LDH, lactate dehydrogenase; LEC/LED, lowest effective concentration/dose; LPS, lipopolysaccharide; M, male; MnSOD; manganese-dependant (mitochondrial) superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIH 3T3, transformed mouse fibroblast cell line; NO, nitric oxide; NQO1, quinone oxidoreductase; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species (cytoplasmic unless otherwise specified); SD, Sprague-Dawley; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; tBHP, *tert*-butylhydroperoxide.

[a]↓, decrease; ↑, increase; NC, no change; +, positive.

these results suggested that the hepato-carcinogenic potential of methyleugenol might involve a genotoxic mechanism.]

Exome sequence analysis of DNA extracted from samples of hepatocellular carcinoma induced in B6C3F$_1$/N mice (n = 3) exposed daily by gavage to methyleugenol at 0 (vehicle control), 37, 75, or 150 mg/kg for 2 years revealed a higher mutation burden than was found in spontaneous tumours from untreated controls, and the mutation frequency increased linearly with dose (Auerbach et al., 2018). When compared with the Catalogue of Somatic Mutations in Cancer (COSMIC) 30-signature data set, the trinucleotide motif signature most closely resembled signature 4 (attributed to benzo[*a*]pyrene) and signature 24 (attributed to aflatoxin B$_1$).

However, in male and female B6C3F$_1$ mice exposed to methyleugenol at doses of up to 1000 mg/kg for 14 weeks, no increase in the percentage of micronucleated normachromatic erythrocytes was observed, nor was there an increase in the percentage of polychromatic erythrocytes (NTP, 2000).

(ii) Non-human mammalian cells in vitro

See Table 4.4.

Methyleugenol induced unscheduled DNA synthesis (UDS) in primary cultures of freshly isolated hepatocytes from male F344 rats (Howes et al., 1990). In a subsequent study (Chan and Caldwell, 1992), the inclusion of 1′-hydroxymethyleugenol indicated that this metabolite was more potent in inducing UDS than was its parent compound. In a subsequent study, methyleugenol induced UDS in cultured primary hepatocytes from male F334 rats and female B6C3F$_1$ mice (Burkey et al., 2000).

Groh et al. (2012) investigated methyleugenol-treated V79 Chinese hamster lung fibroblasts for DNA damage using the comet assay, for micronucleus formation, and for mutation at the *hprt* locus. The metabolites 1′-hydroxymethyleugenol, methyleugenol-2′,3′-epoxide, and 3′-oxomethylisoeugenol, were also tested. All four compounds induced comet formation, with 1′-hydroxymethyleugenol and methyleugenol-2′,3′-epoxide causing greater DNA damage than did methyleugenol. However, only methyleugenol-2′,3′-epoxide and 3′-oxomethylisoeugenol increased the frequencies of micronuclei. Only methyleugenol-2′,3′-epoxide showed marginal mutagenicity at the *hprt* locus.

In Chinese hamster ovary (CHO) cells, methyleugenol at a concentration of 17 µg/mL induced sister-chromatid exchange in the presence of S9 (NTP 2000). In the absence of S9, methyleugenol gave negative results at 50 µg/mL and was toxic at 167 µg/mL. Methyleugenol did not induce chromosomal aberrations in CHO cells when tested at up to 233 µg/mL in either the presence or absence of S9 and was toxic at 500 µg/mL (NTP, 2000).

(iii) Non-mammalian experimental systems

See Table 4.5.

In studies that investigated methyleugenol by injecting it into turkey eggs containing fetuses aged 22–24 days, the compound did not induce DNA damage, as detected by the comet assay. These studies are also described in Section 4.2.1 (Kobets et al., 2016, 2018).

In diploid *Saccharomyces cerevisiae* strain RS9, methyleugenol induced intrachromosomal (*HIS*$^+$) and interchromosomal (*ADE*$^+$) recombination in a dose-related manner (Schiestl et al., 1989). In another diploid strain, RS112, methyleugenol induced DEL recombination (Brennan et al., 1996).

Methyleugenol was inactive in inducing mutations in *S. typhimurium* strains TA100, TA1535, TA98, TA1537, and TA1538, and in *Escherichia coli* WP2 *uvrA* (Sekizawa and Shibamoto, 1982), at up to 300 µg/plate. [The Working Group noted that toxicity precluded testing at higher doses.]

Similarly, Schiestl et al. (1989) found methyleugenol to be inactive in *S. typhimurium* strains TA97, TA98, TA100, and TA102 when tested at

up to 6 µM/plate. It was inactive when tested with either hamster or rat S9 in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 at up to 333 µg/mL and was toxic at the next concentration tested, 666 µg/mL (NTP, 2000).

In a modified strain of *S. typhimurium* TA100 expressing human sulfotransferase SULT1C2, methyleugenol induced mutations in the presence of rat liver S9 (Honda et al., 2016). The same investigators had previously demonstrated that 1′-hydroxymethyleugenol was mutagenic in this strain and with human SULT1A1 (Herrmann et al., 2012) (see also Section 4.1.2). The latter was also reported in a study in which 1′-hydroxymethyleugenol in TA100-hSULT1A1 and TA100-hSULT1C2 without rat liver S9 served as a positive control (Berg et al., 2016).

Methyleugenol gave positive results in the absence of S9 in the DNA repair test in *Bacillus subtilis* (Sekizawa and Shibamoto, 1982).

4.2.3 Alters DNA repair or causes genomic instability

(a) Humans

No studies relative to DNA repair or genomic instability in humans or in human cells exposed to methyleugenol were available to the Working Group.

(b) Experimental systems

Yafune et al. (2013) investigated cell cycle effects in the liver of rats treated with methyleugenol for 28 days (1000 mg/kg bw per day, by gavage). Methyleugenol was reported to increase the number of liver cells overexpressing $p21^{Cip1}$, a cyclin-dependent kinase inhibitor playing a key role in the cell cycle checkpoint in G_1 phase. Methyleugenol also increased the number of cells overexpressing Aurora B or Incenp proteins. The authors considered these findings to be an indication of cell population growth, in line also with an increase in the percentage of cells expressing nuclear antigen Ki-67 cell proliferation marker (see also Section 4.2.4(b)).

Methyleugenol reduced the expression of several genes involved in the DNA repair and damage response in the livers of male F344 rats 3 hours after an oral dose of 2000 mg/kg bw (Ding et al., 2011). Among the affected genes were DNA damage-binding genes *Brca1* and *Rad1*; base excision repair genes *Mpg* and *Ogg1* (an oxidative DNA damage repair gene that encodes 8-oxoguanine DNA glycosylase); the double-strand break repair gene *Rad52*; the mismatch repair genes *Pms1*, *Pms2*, and *Pold3*; and the cell cycle check point gene *Rad9*. Expression levels recovered at 6 hours and 8 hours after treatment, except for *Brca1*, *Ogg1*, and *Pold3*.

4.2.4 Induces epigenetic alterations

(a) Humans

(i) Exposed humans

No studies relative to the induction of epigenetic alterations on exposed humans were available to the Working Group.

(ii) Human cells in vitro

Groh et al. (2013) treated human colon carcinoma HT29 cells for 24 hours with methyleugenol and three of its metabolites, 1′-hydroxymethyleugenol, methyleugenol-2′,3′-epoxide, and 3′-oxomethylisoeugenol at 10–100 µM. Methyleugenol and 1′-hydroxymethyleugenol did not inhibit histone deacetylase (HDAC) activity, but the other two metabolites (methyleugenol-2′,3′-epoxide and 3′-oxomethylisoeugenol) did. HDAC inhibition was not accompanied by changes in the levels of HDAC1 protein. [The Working Group noted that growth inhibition was observed after treatment. The growth inhibitory potency of the investigated alkylbenzenes in HT29 cells was ranked as follows: 3′-oxomethylisoeugenol > methyleugenol-2′,3′-epoxide > 1′-hydroxymethyleugenol ≈ methyleugenol.]

(b) Experimental systems

Yafune et al. (2013) observed an increase in the number of cells positive for phosphorylated histone H3 (p-H3) and for heterochromatin protein 1 α (HP1α⁺) in the livers of rats treated with methyleugenol compared with rats in untreated control groups and in groups treated with non-carcinogens. The data suggested a role for histone modification in cell transition through different cell cycle phases, such as M-phase arrest through G_1/S. (see also Sections 4.2.3 and 4.2.8(b)).

4.2.5 Induces oxidative stress

(a) Humans

No studies relative to the induction of oxidative stress in humans or in human cells exposed to methyleugenol were available to the Working Group.

(b) Experimental systems

See Table 4.6.

Methyleugenol alone was tested in few studies. Oxidative stress was not observed in homogenized small intestinal tissue of male Wister rats treated with methyleugenol at 100 mg/kg bw per day by gavage (Saleh and El-Shorbagy, 2017). In adult male Wistar rats treated with methyleugenol at 100 mg/kg per day for 30 days, there was no impact on levels of nitric oxide (NO) or oxidized lipids (thiobarbituric acid reactive substances, TBARS), cellular glutathione (GSH), or the activity of glutathione-*S*-transferase (GST), superoxide dismutase (SOD), or catalase (CAT) in homogenized intestinal tissue. Surgical ligation of the mesenteric arterial blood supply was used to induce ischaemia. The resulting ischaemia increased NO and TBARS and reduced GSH levels and GST, SOD, and CAT activities. Treating the rats with methyleugenol before surgical ligation prevented these changes in the markers of the oxidative stress response, and levels remained similar to those in sham-operated animals.

In rats, prior administration of methyleugenol at 100 mg/kg was partially protective against the induction of reactive oxygen species (ROS) and ischaemic damage in cerebral tissue caused by blocking the cerebral blood supply in adult male Sprague-Dawley rats. It was also protective against the induction of intracellular ROS and cytotoxicity caused by hypoxia in mixed glial cells cultured from the prefrontal cortex of male Sprague-Dawley rats (age, 1 day) (Choi et al., 2010). No experimental animals or cells were exposed to methyleugenol in the absence of hypoxia in this study.

Methyleugenol (50 or 100 µg/mL) increased transcription of nuclear factor erythroid 2-related factor 2 (NRF2) in a human embryonic kidney cell line (HK293) and a mouse embryonic fibroblast cell line (NIH 3T3), but no increase in NRF2 protein was evident by western blot in the human embryonic kidney cell line (Ma et al., 2021). Methyleugenol (25 µg/mL) did not increase intracellular ROS detected by 2′,7′-dichlorodihydrofluorescein diacetate (DCDFA) fluorescence in a murine macrophage cell line but did increase intracellular GSH and SOD activity. Treating the cells with *t*-butyl hydroperoxide depressed intracellular GSH and SOD activity; these changes were partially reversed in a concentration-dependent manner by co-treatment with methyleugenol (Zhou et al., 2017). [The Working Group noted that, in these studies, methyleugenol altered biomarkers of oxidative stress only after the induction of oxidative stress by another treatment, i.e. hypoxia or *t*-butylhydroperoxide.]

Khan et al. (2011) found that growing *Candida albicans* to mid-exponential phase with as little as 10 µg/mL (56 µM) methyleugenol in the growth medium produced evidence of oxidative stress. This was demonstrated by an increase in lipid peroxidation (TBARS) and antioxidant activity (SOD and CAT activity), as well as a decrease in cytosolic GSH. [The Working Group noted that

these results may be of limited direct relevance to humans, because the studies were conducted in non- mammalian species versus mammalian cells.]

(c) Acellular systems

Rompelberg et al. (1996) reported reversible inhibition of each of the several GST isozymes isolated from rat and human liver cytosol. Methyleugenol has been reported to have weak free radical scavenging activity in aqueous solution, on the basis of monitoring its reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH). The concentration of methyleugenol that caused 50% radical scavenging (IC_{50}) was 80 mol/mol DPPH at 4–400 mM (Nenadis et al., 2021) and 13.7 µM (about 25% as active as ascorbic acid), when tested at concentrations of 0.1–100 µM (Choi et al., 2010).

The electron paramagnetic resonance spectrum of the product of oxidation of methyleugenol by H_2O_2 and horseradish peroxidase is consistent with conversion of the 1-methoxy group to an oxy radical (Sipe et al., 2014). Perhaps more significantly, these authors noted an unexpected electron paramagnetic resonance signal in the methyleugenol source material. The results of additional experiments suggested that the source material lots were contaminated with 10–30 ppm of an unidentified hydroperoxide formed by methyleugenol auto-oxidation. This hydroperoxide was found to be a substrate for horseradish peroxidase (in the absence of H_2O_2) but not for catalase. The authors suggested that this auto-oxidation may have implications for understanding the metabolism of methyleugenol. [The Working Group noted that the presence of this hydroperoxide, particularly if formed in larger amounts, may also have an impact on toxicological test results for synthetic methyleugenol.]

4.2.6 Induces chronic inflammation

(a) Humans

(i) Exposed humans

No studies relative to chronic inflammation in humans exposed to methyleugenol were available to the Working Group.

(ii) Human cells in vitro

Methyleugenol, administered at 50, 100, and 200 µM, caused a non-significant reduction in matrix metallopeptidase 9 (MMP-9) activity in BEAS-2A cells induced with tumour necrosis factor alpha (TNFα) and interleukin 4 (IL-4) (Kim et al., 2014). [The Working Group noted that MMP-9 is a known mediator of inflammation through its involvement in the processing of various chemokines or cytokines.]

(b) Experimental systems

(i) Non-human mammals in vivo

As described in Section 3, evidence of inflammation was reported in the 2-year studies in rodents (NTP, 2000). Inflammation was also reported in subchronic studies in male and female $B6C3F_1$ mice and F344/N rats treated with methyleugenol at doses of 0, 10, 30, 100, 300, or 1000 mg/kg bw in 0.5% aqueous methylcellulose by gavage, 5 days per week, for 14 weeks (NTP, 2000; Abdo et al., 2001).

In the 14-week subchronic study in rats, the incidence of atrophy and chronic inflammation of the mucosa of the glandular stomach was significantly increased (NTP, 2000). Sustained inflammation and atrophy were observed at 300 mg/kg bw (minimal to mild lesions) and 1000 mg/kg bw (mild to moderate lesions) in both male and female rats. Inflammation, although not atrophy, was reported in the glandular stomach at a lower dose of 100 mg/kg bw in female rats only. The inflammation was of mild severity, consisting of fibrosis and diffuse infiltration of the lamina propria by lymphocytes, neutrophils, and macrophages. However, in the 2-year gavage

study, chronic inflammation was not reported in the glandular stomach or the liver of male or female rats. [The Working Group noted that consideration of non-neoplastic inflammatory lesions at the 2-year time point may have limited value in terms of carcinogenesis.]

Atrophy of the glandular stomach (100%, 5/5 exposed versus 0/5 controls), but not residual inflammatory changes, was reported at the 6- or 12-month interim kill of male and female rats at the highest dose (300 mg/kg, stop-exposure study). Of note, the incidence of splenic fibrosis in female rats at 150 and 300 mg/kg was also significantly increased (NTP, 2000). [The Working Group noted that chronic tissue inflammation often leads to tissue fibrosis (Koyama and Brenner, 2017; Tanwar et al., 2020), suggesting that the splenic fibrosis observed after 2 years resulted from chronic inflammation, even though there was no evidence of inflammation at the end of the 2-year study in exposed rats. The diagnosis of inflammation, especially chronic inflammation in the spleen, is notably challenging because of the constituent cell types in splenic parenchyma.]

In mice, chronic inflammation in the liver was not reported in the 14-week study in mice, although subacute inflammation in the liver was observed in male (1000 mg/kg) and female (300 mg/kg) mice (NTP, 2000), and significant increases in the incidence of atrophy, degeneration, and mitotic alterations of the glandular stomach were seen in female mice at 300 mg/kg. In the 2-year gavage study, a dose-related increase in chronic active inflammation was observed in the glandular stomach of 51% and 66% of male mice at 75 and 150 mg/kg, respectively. Similarly, at these two concentrations, non-proliferative chronic active inflammation was observed in the liver of 56% of treated male mice (NTP, 2000).

Of note, oral administration of methyleugenol at doses of 3 and 10 mg/kg bw in male ddY strain mice did not affect the activity of cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2), which are important mediators of inflammation (Yano et al., 2006).

(ii) Non-human mammalian cells in vitro

One in vitro study was identified in which methyleugenol at non-cytotoxic doses (ranging from 5 to 100 μM) attenuated the immune response elicited by exposure of a rat basophilic leukaemia mast cell line (RBL-2H3) to immunoglobulin E (IgE) and 2,4-dinitrophenol–bovine serum albumin. Methyleugenol markedly suppressed the release of TNFα and IL4 in a dose-dependent manner and inhibited the formation of prostaglandin E_2 (PGE2), prostaglandin D_2 (PGD2), and leukotriene C_4 (LTC4). Similarly, methyleugenol suppressed the formation of leukotriene B_4 (LTB4) (Tang et al., 2015). [The Working Group noted that these results collectively suggest that methyleugenol may induce immunosuppression by inhibiting the release of pro-inflammatory cytokines TNFα and IL4 and the allergic inflammatory response mediated by PGE2, PGD2, LTC4, and LTB4.]

4.2.7 Modulates receptor-mediated effects

(a) Humans

(i) Exposed humans

No studies relative to the modulation of receptor-mediated effects by methyleugenol in exposed humans were available to the Working Group.

(ii) Human cells in vitro

One study reported that methyleugenol selectively activated human transient receptor potential ankyrin 1 (hTRPA1), with a half-maximal effective concentration (EC_{50}) value of 160.2 ± 21.9 in exposed hTRPA1-Flp-In 293 stably transfected cells or in HEK293T cells transiently expressing hTRPA1. These results suggest agonism on TRPA1, which is a mediator of several downstream signalling pathways involved in sensory neural processes (Moon et al., 2015).

(b) Experimental systems

(i) Non-human mammals in vivo

Methyleugenol at dose of 3 or 10 mg/kg significantly inhibited the duration of pain-related behaviours (biting or licking the tail and hind paws) induced by 0.25 nM intrathecal injection of *N*-methyl-D-aspartic acid (NMDA) in mice. Bicuculline (3 mg/kg, via subcutaneous injection), a known antagonist of the gamma-aminobutyric acid type A ($GABA_A$) receptor, was shown to suppress the behaviours reported after oral administration of methyleugenol at 10 mg/kg, suggesting $GABA_A$ agonism (Yano et al., 2006).

(ii) Non-human mammalian systems ex vivo

Three studies conducted in mammalian models ex vivo provided evidence that methyleugenol interacts with GABA receptors. Ding et al. (2014) studied $GABA_A$ receptor agonism and reported that methyleugenol enhanced GABA-induced ionic currents in primary cultures of mouse hippocampal neurons harvested from pregnant C57BL/6J mice. In another study, methyleugenol demonstrated agonist activity in ionotropic $GABA_A$ receptors when infused bilaterally into transverse central lateral brain slices from C57BL/6J mice (Zhu et al., 2018). Similarly, GABAergic inhibitory activity in the central amygdala was significantly increased by methyleugenol infused bilaterally into the central amygdala brain slices, acting on the $GABA_A$ receptor and reducing anxiety in mice (Liu et al., 2019a).

4.2.8 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

(i) Exposed humans

No studies relative to alterations in cell proliferation, cell death, or nutrient supply with methyleugenol in exposed humans were available to the Working Group.

(ii) Human cells in vitro

Few studies in human cells in vitro were available to the Working Group. Of these, most investigated the potential beneficial effects of methyleugenol as a cytostatic, antiproliferative, and antioxidant agent, limiting their relevance to the key characteristic under evaluation.

Kuang et al. (2021) showed that although methyleugenol alone (0–40 µmol/L) did not induce any changes in the viability of human immortalized kidney cells (HK-2), pre-treatment with methyleugenol at 0–40 µmol/L in an in vitro model of hypoxia/reoxygenation-exposed HK-2 cells promoted the expression of NRF2 and haem oxygenase-1 (HO-1) and translocation of NRF2 to the nucleus, and downregulated the expression of NADPH oxidase 4 (NOX4), reducing apoptosis. The effect of methyleugenol was reversed by treatment with the NRF2 inhibitor ML385. [The Working Group considered that the study was of limited informativeness, since no results on NRF2, HO-1, or NOX4 expression levels after treatment with methyleugenol alone were reported.]

Yi et al. (2015) evaluated the potential effects on cell growth and apoptosis of methyleugenol treatment alone or in combination with cisplatin (a known antiproliferation anticancer drug) in human cervical cancer cells (HeLa). Methyleugenol treatment at 10, 20, 40, 60, 80, and 100 µM induced a concentration-dependent increase in the growth inhibition rate, as measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A synergistic effect on growth inhibition was observed when HeLa cells were treated simultaneously with methyleugenol (60 µM) and cisplatin (1 µM) for 48 hours. However, methyleugenol treatment at 60 µM for 48 hours was reported to shift 15% of the cell population to G_0/G_1 phase, compared with 9% observed in untreated cells and to induce apoptosis, as shown by a significant increase in the activity of

apoptotic mediator factor caspase 3 and reduction in mitochondrial membrane potential. [The Working Group considered that the study had some limitations since the duration of methyleugenol treatment was not reported clearly across all the tests performed.]

Yin et al. (2018) investigated the potential antiproliferative effects of methyleugenol in a human retinoblastoma cell line, RB355. Methyleugenol treatment (0–200 μM) for 48 hours induced a significant concentration-dependent decrease in cell viability (IC_{50} = 50 μM) as measured by the MTT assay. The vital staining of cells treated with methyleugenol (50 μM) with the autophagolysosome marker monodansylcadaverine (MDC) showed induction of autophagy, which was confirmed by a concentration-dependent (25, 50, 100 μM) increase in the expression of autophagy protein LC3-II and a decrease in LC3-I and p62, but no change in BECLIN-1 or VSP4. In addition, a concentration-dependent (25, 50, 100 μM) increase in the proportion of cells arrested in G_2/M cell cycle phase was observed; this was accompanied by downregulation of PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), m-TOR (mechanistic target of rapamycin kinase), AKT (AKT serine/threonine kinase 1) and respective phosphorylated forms, as measured by western blot analysis of levels of the three proteins. [The Working Group noted that the relevance of this study was limited by the lack of detailed statistical analysis of the data.]

Groh and Esselen (2017), while investigating the potential of methyleugenol and its metabolites (1′-hydroxymethyleugenol, 3′-oxomethylisoeugenol, methyleugenol-2′,3′-epoxide) to trigger the DNA damage response in human colon adenocarcinoma HT29 cells, observed that it was mostly the metabolites that induced the accumulation of HT29 cells in G_2 phase. The DNA damage response was characterized by a time-delayed phosphorylation of ATM (ataxia-telangiectasia, mutated)/ATR (ATM- and Rad3-related) kinases and checkpoint kinase 1 after 2 hours of incubation, and the tumour suppressor protein P53 after 24 hours of incubation. The test compounds induced apoptotic cell death, as indicated by cleavage of caspase 3 and poly-(ADP-ribose)-polymerase after 72 hours.

Deng et al. (2021) studied the effects of methyleugenol and one of its metabolites, 2-allyl-4,5-dimethoxyphenol (DMP), on cell proliferation and growth in two human cell lines (breast cancer MDA-MB-231 and fetal lung fibroblast HFL1 cells). Cells were incubated with methyleugenol or DMP at concentrations of 15.625, 31.25, 62.5, 125, 250, 500, or 1000 μg/mL in the culture medium for 24 hours. Methyleugenol and DMP significantly inhibited the cell proliferation rate, as measured by the MTT assay. The IC_{50}s for methyleugenol and DMP were 454.0647 and 62.7670 μg/mL, respectively, in MDA-MB-231 cells and 687.9166 μg/mL and 539.6506 μg/mL, respectively, in fetal lung fibroblast HFL1 cells. [The Working Group considered this study to be of limited relevance because the metabolite DMP investigated is normally formed in non-mammalian species (i.e. Insecta). In addition, methyleugenol was used as the reference compound.]

(b) Experimental systems

(i) Non-human mammals in vivo

In a repeated-dose toxicity study, B6C3F_1 female mice were exposed to methyleugenol in the diet at a dose of 75 mg/kg per day, 5 days per week, for 2 weeks. After treatment, no evidence of liver tissue alterations was observed by histopathology; however, in liver tissue homogenates methyleugenol was shown to induce alterations in the expression of several genes and expressed sequence tags previously identified by the authors as potential early biomarkers of mouse liver carcinogenesis. Specifically, methyleugenol induced upregulation of cell cycle genes encoding cyclin G1 (Ccng1) and p21 (Cdkn1a) proteins, as well as strong upregulation of growth arrest and

DNA-damage-inducible, beta (Gadd45b), upregulation of the transcription factor early growth response 1 (Egr1), the jun B proto-oncogene (Junb), deoxyribonuclease IIa (Dnase2a), and transforming growth factor beta stimulated clone 22 (Tsc22), a transcriptional repressor and putative tumour suppressor gene. As assessed by oligonucleotide array and quantitative reverse transcription-polymerase chain reaction (qRT-PCR), methyleugenol also induced the downregulation of two tumour suppressor genes encoding fragile histidine triad diadenosine triphosphatase (Fhit) and WW domain-containing oxidoreductase (Wwox), which are involved in apoptotic evasion, as well as the gene encoding cytokine inducible SH2-containing protein (Cish), a member of a family of intracellular proteins that regulates the response of immune cells to cytokines (Iida et al., 2005).

Characterization of the events leading to hepatocarcinogenesis in male F344/NSlc rats (age, 5 weeks) after exposure to methyleugenol at 1000 mg/kg per day by gavage for 28 days was also performed in a series of studies carried out by Taniai et al. (2012), Yafune et al. (2013), and Kimura et al. (2016). Taniai et al. (2012) observed that methyleugenol caused diffuse distribution of cytomegalic liver cells with anisokaryosis and scattered centrilobular necrosis, as assessed by histopathology. Immunohistochemical analysis of liver tissue samples showed that methyleugenol, at the dose tested, significantly increased the number [percentage] of cells that stained positive for the cell proliferation marker Ki-67 compared with untreated or non-carcinogen-treated controls [α-naphthyl isothyocianate or acetaminophen] and the number of cells that were positive for minichromosome maintenance complex component 3 (Mcm3) compared with α-naphthyl isothiocyanate but not untreated controls. Methyleugenol also caused a significant increase in the number of cells that stained positive for DNA topoisomerase II alpha (TopoIIα); as well as an increase in the number of cells staining positive for ubiquitin D (Ubd) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) compared with untreated or non-carcinogen-treated controls.

In the study by Yafune et al. (2013), it was reported that exposure to methyleugenol at 1000 mg/kg bw per day for 2 days significantly decreased body weight but increased liver weight, compared with untreated animals. The authors also investigated several proteins involved in cell proliferation and apoptosis in liver tissues; the corresponding genes had been previously selected by a global gene screen using microarrays as those most upregulated in the liver of rats receiving thioacetamide (a representative cytomegaly-inducing hepatocarcinogen) as repeated oral doses (400 ppm in the diet). Liver tissue samples from methyleugenol-treated animals showed a significant increase in the percentage of immunoreactive cells expressing $p21^{Cip1}$[Cdkn1a], a cycle-dependent kinase CDK inhibitor; nuclear Cdc2 (cell division cycle 2), which drives the G_2/M transition; Aurkb (aurora kinase B); Incenp (inner centromere protein); p-H3; HP1α (heterochromatin protein 1 alpha); Klf6 (Kruppel like transcription factor 6); and p53, compared with the untreated and non-carcinogen controls, thus sustaining cell proliferation and apoptosis in response to G_2/M cycle arrest (see also Section 4.2.4).

In a follow-up study, Kimura et al. (2016) further investigated the onset of carcinogen-specific cell cycle-related alterations during the early time course of repeated carcinogen administration, studying the effects of methyleugenol after 3 and 7 days of treatment compared with 28 days. Time-dependent significant bodyweight decreases and liver-weight increases were observed in treated animals compared with untreated controls. No morphological alterations in the liver were observed after 3 days or 7 days of treatment, although centrilobular liver cell hypertrophy associated with a cytoplasmic ground glass appearance was reported; after

28 days of treatment, the presence of liver cells exhibiting cytomegaly and anisokaryosis was confirmed, as assessed by histopathology. At day 3, the number of cells that were positive for TopoIIα, p-H3, and Mad2 (a spindle checkpoint protein) significantly decreased compared with untreated controls; lower levels were observed for Ubd+ cells, and the numbers of cells expressing $p21^{Cip1}$ and p-Mdm2 (phosphorylated Mdm2) were significantly increased. The number of cells expressing TopoIIα, p-H3, Mad2, Ubd, γH2AX, and Ubd decreased on day 7 compared with the increased levels in untreated controls. Instead, the number of Ki-67-positive cells remained unchanged until day 28 of treatment, and cleaved caspase 3-positive cells increased starting from day 7. The number of TopoIIα+, p-H3+, Mad2+, Ubd+, γH2AX+, $p21^{Cip1}$+ and p-Mdm2+ cells was significantly increased after 28 days of treatment. In addition, modulation of the transcripts of cyclins (Cdkn1a and Cdkn2a), retinoblastoma 1 and 2 (Rb1 and Rb2), and p53 were observed (by RT-PCR) from day 3 to day 28, confirming the involvement of G_1/S checkpoint-related genes starting from earlier time points, and of genes related to spindle checkpoints, M phase, and DNA damage at later time points.

Abdo et al. (2001) reviewed the NTP studies (NTP, 2000) investigating the effects of methyleugenol in groups of 10 female rats at a dose of 0, 37, 75, or 150 mg/kg per day, 5 days per week, for 30 or 90 days, or 300 or 1000 mg/kg per day for 30 days, and in groups of male mice exposed to a dose of 0, 9, 18.5, 37, 75, 150, or 300 mg/kg per day for 30 or 90 days. They observed a significant increase in cell proliferation, indicated by an increase in the percentage of cells labelled with bromodeoxyuridine (BrdU), mainly in the fundic glands of the glandular stomach in male mice at 150 or 300 mg/kg for 30 days or 18.5 37, or 75 mg/kg for 90 days. The treatment induced non-neoplastic lesions in the liver, glandular stomach, and nose of male and female mice. Cytological alterations, necrosis, subacute inflammation (see also Section 4.2.6), and bile duct hyperplasia were observed in male mice at 1000 mg/kg and in female mice at 300 and 1000 mg/kg. Significant increases in the incidence of atrophy, degeneration, and mitotic alterations of the glandular stomach were seen in female mice at 300 mg/kg. Serum gastrin levels were significantly increased in male mice at 150 and 300 mg/kg after 30 days but not after 90 days of exposure. In male and female rats, there were increases in the incidence of cytological alterations, cytomegaly, Kupffer cell pigmentation, and mixed foci of cellular alteration in the liver at 1000 mg/kg. In addition, increases in the incidence of atrophy and chronic inflammation in the glandular stomach were observed in male and female rats at 300 and 1000 mg/kg. There were significant increases in serum gastrin levels in female rats at all doses after 30 days and after higher (150, 33, 1000 mg/kg) exposures at 90 days. The BrdU labelling index showed significant increases in cell proliferation in the fundic glands of the glandular stomach in female rats in almost all dosed groups at both 30 days (37, 150, 300 and 1000 mg/kg) and 90 days (37, 75 and 150 mg/kg). In addition, increased BrdU labelling was also observed in the liver in rats at 150, 300, and 1000 mg/kg at 30 days and 150 mg/kg at 90 days.

Williams et al. (2013) studied the effects of methyleugenol administered at a dose of 62–250 mg/kg bw for 24 weeks in male F344 rats. Methyleugenol produced diffuse hepatocellular hypertrophy and enlargement of the cholangiole plexus in the periportal regions, midzonal macrovesicular phanerosis, periportal infiltrate of mononuclear cells, and multiple foci of hepatocellular necrosis. The hepatocellular replicating fraction values (cells positive for proliferating cell nuclear antigen, PCNA) increased more than twofold.

While investigating genotoxicity in F344 *gpt* delta transgenic rats exposed to methyleugenol at the dose of 10, 30 or 100 mg/kg for 13 weeks, Jin et al. (2013) observed increases in the number and

area of foci positive for the placental form of GST in the liver and increases in cell proliferation, (as measured by the ratio of PCNA-positive hepatocytes) in male and female rats at 100 mg/kg.

In the 2-year cancer study conducted by the NTP (Johnson et al., 2000; NTP, 2000; see also Section 3 of the present monograph), male and female F344/N rats and B6C3F$_1$ mice were treated with methyleugenol at doses of 37, 75, or 150 mg/kg per day by gavage, 5 days per week, for 105 weeks (rats) or 104 weeks (mice). A stop-exposure group of rats received methyleugenol at 300 mg/kg per day by gavage for 53 weeks and then vehicle only for the remaining 52 weeks. Tissues were examined microscopically. In rats, methyleugenol increased the incidence of hepatocyte hypertrophy and oval cell hyperplasia (except for males at 37 mg/kg per day) and also increased the incidence of bile duct hyperplasia in groups of males at 37, 75, and 150 mg/kg per day and females at 150 and 300 mg/kg per day. Increases in the incidence of oval cell hyperplasia, hypertrophy, and mixed cell foci were also noted at the 6- and 12-month interim evaluations in male and female rats at 300 mg/kg per day. In addition, atrophy of the glandular stomach was observed at the 6- and 12-month interim evaluations in male and female rats at 300 mg/kg per day. Atrophy of the glandular stomach was observed in male and female rats in all dose groups. The incidence of neuroendocrine cell hyperplasia in the fundic region of the glandular stomach was increased in groups at 150 and 300 mg/kg per day and was more prevalent and severe in females than in males. In mice, the incidence of bile duct and oval cell hyperplasia increased, starting at 37 mg/kg per day for males and 75 mg/kg per day for females. The incidence of haematopoietic cell proliferation increased, starting at 37 mg/kg per day. Atrophy of the glandular stomach was observed in male and female mice at 75 and 150 mg/kg per day. Hyperplasia of the glandular stomach increased at 75 mg/kg per day in male and female mice.

In a further analysis of the lesions observed in rats from the above study (NTP, 2000; see also Section 3.2), Janardhan et al. (2015) reviewed by microscopy the original haematoxylin and eosin-stained tissue sections of the stomach with a diagnosis of neuroendocrine cell hyperplasia and benign and malignant neuroendocrine tumour. In addition, haematoxylin and eosin-stained slides of metastatic neuroendocrine tumours in other tissues were reviewed. Hyperplasia and non-neoplastic lesions in the neuroendocrine cells were observed mainly in the fundus region of the stomach and showed positive immunoreactivity for chromogranin A and synaptophysin.

(ii) Non-human mammalian cells in vitro

Wang et al. (2021b) investigated the potential protective effects of methyleugenol on liver ischaemia reperfusion injury (LIRI) and whether this effect was regulated by the PI3K/Akt signalling pathway. They used a partially warm LIRI model in which C57BL/6J mice underwent 60 minutes of ischaemia, followed by reperfusion for 6 hours. They also used also used a hypoxia–reoxygenation injury cell model, mouse liver cell line (AML12), which underwent 24 hours of hypoxia, followed by 18 hours of normoxia. The extent of liver injury was assessed by serum transaminase concentrations, haematoxylin and eosin staining, quantitative real-time PCR, myeloperoxidase activity, and TUNEL analysis. Apoptosis was detected using flow cytometry. Protein levels of p-PI3K, PI3K, p-Akt, Akt, p-Bad, Bad, Bcl-2, Bax, and cleaved caspase 3 were detected by western blotting. LY294002 (an inhibitor of PI3K/Akt signalling) was used to elucidate the relationship between methyleugenol and PI3K/Akt signalling. The results showed that methyleugenol alleviated the LIRI-induced liver injury, the inflammatory response, and the apoptosis induced by hypoxia–reoxygenation.

Deng et al. (2021) studied the cytotoxicity of methyleugenol and its metabolite, DMP, in mouse embryonic hepatocytes (BNL-CL.2). The

concentrations studied were 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL, and cytotoxicity was measured by the MTT assay after 24 hours of exposure. The authors reported IC_{50}s of 114.5169 and 1171.5902 µg/mL, respectively. [The Working Group considered the study to be of limited relevance because the metabolite investigated is mainly formed in non-mammalian species (i.e. Insecta). In addition, methyleugenol was used as the reference compound.]

4.2.9 Data relevant to multiple key characteristics

(a) Results from microarrays and omics

(i) Non-human mammals in vivo

See Table 4.7.

Yafune et al. (2013) (see also Section 4.2.8(b)) aimed to clarify the regulatory molecular mechanisms behind cell cycle aberrations related to the early stages of hepatocarcinogenesis. Gene expression analysis using microarrays and qRT-PCR was performed on RNA samples from the liver of rats exposed to several hepatocarcinogens, including methyleugenol, for 28 days. There were increases in cell proliferation (Ki-67-positive cells) and apoptosis (TUNEL-positive cells) after exposure to methyleugenol and consistent increases in several marker genes and proteins involved in cell proliferation. Increased staining for p53, $p21^{Cip1}$, Cdc2, Aurkb, Incenp, p-H3, and HP1α proteins was observed in the livers of mice exposed to methyleugenol. qRT-PCR showed increased expression of Klf6 but not Ndrg1. [The Working Group noted that the study had some limitations because no description of pathway analyses was provided, or comparisons of gene profiles between the different chemicals examined or between results of microarray and qRT-PCR.]

To understand gene expression patterns produced after exposure to hepatocarcinogens with different mechanisms of action, Iida et al. (2005) used qRT-PCR and oligonucleotide microarray analysis to identify genes that were altered in $B6C3F_1$ mouse liver after treatment with different known carcinogens, including methyleugenol (75 mg/kg per day, in the diet, for 2 weeks). Liver gene expression of 20 842 genes was assessed by oligonucleotide microarray. Several cancer-related genes, including those involved in apoptosis (*Fhit*, *Wwox*) and those involved in the cell cycle (*Tsc22*, *Gadd45b*) were induced or repressed in unique patterns for specific carcinogens but were not altered by the non-carcinogens. The authors noted that even if molecular alterations in the tumours were similar, such as in the case of oxazepam and methyleugenol, early gene expression changes appeared to be carcinogen-specific and involved apoptosis and cell cycle-related genes. [The Working Group noted that pathway analysis was not performed.]

To identify hepatocarcinogens using computational approaches, Auerbach et al. (2010) developed a series of classification prediction models based on gene expression in the male F344 rat liver and generated using full genome microarrays after 2, 14, or 90 days of exposure to a collection of hepatocarcinogens, including methyleugenol. Methyleugenol increased the expression of *Mybl2* and *Adam8* and downregulated *Wwox* and *Fhit* after 90 days of treatment. All four of these genes play mechanistic roles in cell proliferation or apoptosis. [The Working Group noted that pathway analysis was not performed.]

(ii) Human mammalian cells in vitro

Kreuzer et al. (2020) performed experiments to identify genes that would be predictive of DNA damage in vitro. Metabolically active human HepaRG hepatocarcinoma cells were exposed to five food-relevant genotoxic carcinogens, including methyleugenol. Transcriptomic responses were analysed using RNA sequencing technology and validated by real-time RT-PCR. Various biostatistical approaches revealed a characteristic transcript signature of 37 genes

Table 4.7 Microarray and omics results for multiple key characteristics in non-human mammalian systems in vivo and in vitro after exposure to methyleugenol

End-point	Normalization and statistics	Curation	Results	Tissue, cell type, or cell line	Relevant KCs[a]	Exposure concentration or range and duration	Comments	Reference
Transcriptomics Agilent Mouse two-colour oligonucleotide array of ≈ 20 000 genes	Agilent Feature Extraction software: Rosetta Resolver (version 3.2, build 3.2.2.0.33) Expression analysis performed with GeneSpring 6.2	None provided	Microarray: 47 genes altered; cell cycle-related genes Microarray and qRT-PCR: upregulated genes: *Cdkn1a*, *Ccng1*, *Gadd45b*, *Junb*, *Dnase2*; reduced or absent *Fhit* and *Wwox* expression	Liver	KC10 (cell proliferation and apoptosis)	Female $B6C3F_1$ mice (age 6 weeks) dosed with methyleugenol at 75 mg/kg per day	No pathway analysis	Iida et al. (2005)
Transcriptomics Agilent Rat Whole Genome oligonucleotide microarrays in 4 × 44K format	Agilent Feature Extraction software (v9.5); normalized using quantile normalization followed by per chip median centring; significant changes determined using a *t*-test (Benjamini and Hochberg multiple testing correction); GSEA performed using GeneSpring GX 10	NIEHS CEBS [not confirmed]	Increased mitosis at 2 and 14 days; reduced or absent *Fhit* and *Wwox* expression	Liver	KC10 (cell proliferation and apoptosis)	Male F344/N rats dosed with methyleugenol at 150 mg/kg per day by gavage in methylcellulose (training set); 35.6 or 356 mg/kg per day in corn oil (test set); killed on days 3, 15, 91	No pathway analysis on methyleugenol, only on hepatocarcinogens versus non-hepatocarcinogens	Auerbach et al. (2010)

Table 4.7 (continued)

End-point	Normalization and statistics	Curation	Results	Tissue, cell type, or cell line	Relevant KCs[a]	Exposure concentration or range and duration	Comments	Reference
Transcriptomics RNA-Seq	Alignment to the human genome (hg19) performed using STAR (version 2.5.2b); differentially expressed genes (DEGs) identified using DESeq2 A false discovery rate-adjusted *P*-value (*Q* value) < 0.05 (Benjamini-Hochberg correction) was defined as threshold for statistical significance Ingenuity pathway analysis used	GSE14654	Data set overlap using Venn diagrams showed that 37 genes were commonly regulated by all five test chemicals including methyleugenol; several of the 37 genes were functionally linked to cell cycle progression, DNA damage response, and/or cell death, e.g. *FHIT*, *FOSL1*, *GDF15*, *MACROD2*, *NINL*, *PAK7*, *SRC*, and *WWOX*; upstream regulators downregulated	Human hepatoma, HepaRG cells	KC2; KC10 (apoptosis; nutrient supply)	250 µM; 24-hour treatment	Methyleugenol was used as a reference genotoxic agent	Kreuzer et al. (2020)

Table 4.7 (continued)

End-point	Normalization and statistics	Curation	Results	Tissue, cell type, or cell line	Relevant KCs[a]	Exposure concentration or range and duration	Comments	Reference
Transcriptomics RNA-Seq (cont.)			(*HNF4A*, *SCAP*, *SREBF2*, *SREBF1*, *INSR*). Several toxicity functions/ pathways were perturbed (necrosis of liver, apoptosis of hepatocytes, apoptosis of liver cells, focal necrosis of liver, cell death of liver cells); diseases and biofunctions (fatty acid metabolism, transport of molecule, export of molecule, synthesis of lipid, transport of lipid)					

CEBS, Chemical Effects in Biological Systems database; GSEA, gene set enrichment analysis; KC, key characteristic of carcinogens; NIEHS, National Institute of Environmental Health Sciences; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

[a] KC2, "is genotoxic"; KC10, "alters cell proliferation, cell death, or nutrient supply".

that exhibited a similar expression pattern after exposure to the test chemicals. The 37-gene signature could differentiate between genotoxic and nongenotoxic carcinogens. The genes in the signature were shown by pathway analyses to be involved in the DNA damage response and p53 activation. In addition, methyleugenol treatment was predicted by IPA ingenuity pathway analysis to lead to the suppression of several metabolic transcription factors, metabolic diseases, and metabolic biofunctions (Kreuzer et al., 2020). [The Working Group noted that this study was not targeted towards understanding the mechanisms of methyleugenol action but used methyleugenol as a reference agent for DNA damage.]

(iii) Summary

In summary, the four studies demonstrated effects on one or more of the key characteristics. The studies in mice and rats (Iida et al., 2005; Auerbach et al., 2010; Yafune et al., 2013) provided evidence that in the livers of exposed rodents methyleugenol causes increases in hepatocyte proliferation and suppression of hepatocyte apoptosis. In human HepaRG cells, there was evidence for increases in DNA damage and weak evidence for alteration of nutrient supply.

(b) Evaluation of high-throughput in vitro toxicity screening data

The analysis of the in vitro bioactivity of the agents reviewed in *IARC Monographs* Volume 134 was informed by data from high-throughput screening assays generated by the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes of the government of the USA (Thomas et al., 2018). Methyleugenol was one of thousands of chemicals tested across the large assay battery of the Tox21 and ToxCast research programmes. Detailed information about the chemicals tested, assays used, and associated procedures for data analysis is publicly available (US EPA, 2023).

The ToxCast/Tox21 high-throughput screening results are presented according to the assays that have been mapped to the key characteristics of carcinogens (Reisfeld et al., 2022). The detailed results are available in supplementary information for this volume (Annex 4, Supplementary material for Section 4, Mechanistic Evidence, online only, available from: https://publications.iarc.who.int/627). Here, for brevity, assays for which there is a positive "hit call" are referred to as "active" assays. A summary of these results is given below as the number of active assays (without any caution flags) out of the total number of key characteristic-related assays for the chemical.

Among the 288 assays in which methyleugenol was tested, it was found to be active and without caution flags in four assays relevant to the key characteristics of carcinogens. Methyleugenol was active in four assays mapped for key characteristic 8, "modulates receptor-mediated effects" (Reisfeld et al., 2022). Three of the assays were cell-based, multiplexed-readout assays in HepG2 (human hepatocellular carcinoma cell line), and measurements were taken 24 hours after chemical dosing in a 24-well plate. The assays that were positive included ATG_RXRb_TRANS_up, ATG_PPARg_TRANS_up, and ATG_PXR_TRANS_up, which measure the ability of the compound to activate a reporter gene through the ligand-binding domain of the nuclear receptors retinoid X receptor beta (RXRβ), peroxisome proliferator-activated receptor gamma (PPARγ), and the pregnane X receptor (PXR), respectively. The AC_{50} (50% of maximal activity) values were 68.11, 10.77, and 35.11 μM, respectively.

The fourth assay, LTEA_HepaRG_CYP2B6_up, is described as a cell-based, multiplexed-readout assay that uses HepaRG (a human liver cell line), and measurements were taken at 48 hours after chemical dosing on a 96-well plate. The assay measures the expression of the *CYP2B6* gene, which is known to be regulated by the constitutive androstane receptor (CAR) and

PXR. In a screen of 1060 compounds, including methyleugenol, *CYP2B6* was found to be the second most frequently altered gene (Franzosa et al., 2021).

The activity of methyleugenol was tested in two (TOX21_RXR_BLA_Agonist_ratio, ATG_RXRb_TRANS_up), six (ATG_PPARg_TRANS_up, ATG_PPRE_CIS_up, OT_PPARg_PPARgSRC1_0480, OT_PPARg_PPARgSRC1_1440, TOX21_PPARg_BLA_Agonist_ratio, TOX21_PPARg_BLA_antagonist_ratio), and three (ATG_PXR_TRANS_up, ATG_PXRE_CIS_up, LTEA_HepaRG_CYP2B6_up) assays for modulation of RXRβ, PPARγ, and PXR, respectively. [The Working Group considered that because methyleugenol was active in only one or two of the total number of assays for each nuclear receptor, the link between methyleugenol and activation of these receptors might be tenuous and should be confirmed with additional appropriate studies in vitro and in vivo.]

5. Summary of Data Reported

5.1 Exposure characterization

Methyleugenol is a liquid with a clove-like odour that can be produced by methylation of eugenol. It occurs naturally in essential oils of various herbs and spices, e.g. basil, lemongrass, and fennel. The compound as such was used as a flavouring agent in various food products and in consumer products such as cosmetics and personal care products, insect attractants, and as an anaesthetic agent in the veterinary context. However, its use for flavouring purposes was prohibited in the European Union (EU) in 2008 and in the USA in 2018. In the EU, methyleugenol must not be added to cosmetics; however, it is still present in various foods and consumer products that contain herbs, spices, or their essential oils. In this case, maximum concentrations of the substance are established in specific legislations.

In occupational settings, workers handling products containing methyleugenol (e.g. aromatherapists and those using essential oils for body massages, workers from food processing industries) are potentially exposed to methyleugenol by dermal and/or inhalation routes. The general population is ubiquitously exposed, mainly through the ingestion of food, or dermally using personal care products containing methyleugenol. The exposure levels of the general population are expected to be less than 1 μg/kg bw per day for each source of exposure.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

Treatment with methyleugenol caused an increase in the incidence of malignant neoplasms or an appropriate combination of benign and malignant neoplasms in two species (mouse and rat).

Methyleugenol was administered by oral administration (gavage) in one study in male and female $B6C3F_1$ mice. In both sexes, methyleugenol caused an increase in the incidence of liver tumours (hepatocellular adenoma; hepatocellular carcinoma; hepatocellular adenoma, carcinoma, or hepatoblastoma (combined); combined hepatocellular carcinoma or hepatoblastoma). In females methyleugenol caused an increase in incidence of hepatoblastoma.

Methyleugenol was administered by oral administration (gavage) in one study in male and female F344 rats. In both sexes, methyleugenol caused an increase in the incidence of liver tumours (hepatocellular adenoma; hepatocellular carcinoma; hepatocellular adenoma or carcinoma (combined); hepatocholangioma, and hepatocholangiocarcinoma) and benign and malignant neuroendocrine tumours of the

glandular stomach. In male rats, methyleugenol caused an increase in the incidence of renal tubule adenoma of the kidney, fibroadenoma of the mammary gland, skin fibroma, skin fibroma or fibrosarcoma (combined), and malignant mesothelioma (all organs).

5.4 Mechanistic evidence

There is sparse evidence on the absorption, distribution, metabolism, and excretion of methyleugenol in exposed humans. Methyleugenol is rapidly absorbed after oral exposure and is capable of dermal permeation. There are no data on inhalation exposure. In rodents, methyleugenol is rapidly absorbed, extensively metabolized in the liver, and predominantly excreted in the urine as sulfate or glucuronide conjugates after oral exposure. Methyleugenol undergoes phase 1 oxidation followed quickly by phase 2 conjugation in experimental animals. Human microsomes produce similar metabolites to those found in rodents. Metabolic activation of methyleugenol in rodents involves 1′-hydroxylation followed by sulfonation to form 1′-sulfooxymethyleugenol, which subsequently undergoes sulfate elimination to form a reactive carbonium ion that can react with DNA, RNA, and proteins. Cytochrome P450 (CYP) 1A2, 2E1, and 2C9 are the predominant enzymes for 1′-hydroxylation of methyleugenol in experimental systems. SULT1A isoforms (i.e. SULT1A1 and SULT1A2) are key enzymes involved in the bioactivation of 1′-hydroxymetheugenol to form the reactive carbonium ion.

Data were available for methyleugenol for the following key characteristics of carcinogens: "is electrophilic or can be metabolically activated to an electrophile", "is genotoxic", "alters DNA repair or causes genomic instability", "induces oxidative stress", "induces chronic inflammation", "modulates receptor-mediated effects" and "alters cell proliferation, cell death, or nutrient supply".

There is consistent and coherent evidence that methyleugenol exhibits key characteristics of carcinogens.

Methyleugenol can be metabolically activated to an electrophile. Three studies in humans detected methyleugenol–DNA adducts in the liver and lung. The major adduct is formed at the N^2 position of guanine, with a minor adduct formed at the N^6 position of adenine. These adducts have also been detected in the livers of mice treated with methyleugenol, and the N^6 adenine adduct has been detected in the urine of rats. In genetically modified mice, DNA adduct formation was dependent on the presence of mouse Sult1a1 or human SULT1A1/2 in the liver, caecum, and kidney, but not the stomach. DNA adduct formation in rat liver was also shown to be SULT-dependent. Methyleugenol formed DNA adducts in turkey and chicken embryos. It has also been shown to bind covalently to RNA in the liver of mice and to protein in the liver of rats.

Methyleugenol is genotoxic. No data were available in humans or human primary cells exposed to methyleugenol. There is consistent and coherent evidence for the genotoxicity of methyleugenol in experimental systems. In human cell lines in vitro, methyleugenol and its metabolite 1′-hydroxymethyleugenol caused DNA strand breaks. Methyleugenol induced mutations in rat and mouse liver in vivo. Methyleugenol did not induce micronucleus formation in a human cell line in vitro. Methyleugenol did not increase micronucleated normachromatic erythrocytes in mice. It induced unscheduled DNA synthesis in hepatocytes of male rats and female mice. Methyleugenol and its metabolite 1′-hydroxymethyleugenol caused DNA damage in Chinese hamster lung fibroblasts but did not increase the frequency of micronuclei. In Chinese hamster ovary cells, methyleugenol induced sister-chromatid exchanges in the presence of rat liver S9 (9000 × *g* supernatant) but did not

induce chromosome aberrations. Methyleugenol induced recombination in yeast.

Methyleugenol and 1′-hydroxymethyleugenol were mutagenic in the presence of rat liver S9 in a strain of *Salmonella typhimurium* genetically modified to express human SULT isoforms. Methyleugenol gave positive results in the absence of S9 in a DNA repair test in *Bacillus subtilis*.

Methyleugenol induces cell proliferation, cell death, or alters nutrient supply. No data were available in humans or primary human cells exposed to methyleugenol. There is consistent and coherent evidence for cell proliferation in experimental systems. A series of repeated-dose toxicity studies (3-day, 7-day, 28-day, 90-day, up to 2-year) in mice and rats showed that methyleugenol was able to cause cell proliferation and alter related biomarkers in the liver. In addition, methyleugenol induced hyperplasia in the glandular stomach, nose, and bile duct in mice, and stomach in rats. Gene expression changes in the livers of treated rodents demonstrated modulation of genes involved in cell cycle and cell proliferation.

There is suggestive evidence that methyleugenol induces chronic inflammation. No data were available in humans or human primary cells exposed to methyleugenol. One study showed that methyleugenol reduces matrix metalloproteinase 9 (MMP-9) activity in a human cell line. In 14 weeks of exposure, methyleugenol increased chronic inflammation of the mucosa of the glandular stomach and atrophy in male and female rats. Increased atrophy in rats was also observed at the 6- or 12-month interim kill after a single dose. No evidence of inflammation was observed at the 2-year treatment with methyleugenol in rats; however, a dose-related increase in chronic active inflammation in the glandular stomach was observed in male but not female mice exposed for 2 years.

For the other key characteristics, "alters DNA repair or causes genomic instability", "induces epigenetics alterations", "induces oxidative stress", and "modulates receptor-mediated effects", there is a paucity of data.

Methyleugenol was found to be mostly without effects relevant to the key characteristics of carcinogens in the assay battery of the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes. However, the cells used in the assay battery are not metabolically competent.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of methyleugenol.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of methyleugenol.

6.3 Mechanistic evidence

There is *strong evidence* that methyleugenol exhibits key characteristics of carcinogens in experimental systems, including humanized mice, and supported by studies in humans.

6.4 Overall evaluation

Methyleugenol is *probably carcinogenic to humans* (Group 2A).

6.5 Rationale

The Group 2A evaluation for methyleugenol is based on *sufficient evidence* for cancer in experimental animals and *strong* mechanistic evidence in experimental systems, including humanized mice and supported by studies in humans. The

sufficient evidence for cancer in experimental animals is based on an increase in the incidence of malignant neoplasms and a combination of benign and malignant neoplasms in two species (mouse and rat) in two studies that complied with GLP. There is *strong evidence* that methyleugenol exhibits multiple key characteristics of carcinogens; methyleugenol is electrophilic; it is genotoxic; and it alters cell proliferation, cell death, or nutrient supply. The supporting data that methyleugenol exhibits these key characteristics come primarily from experimental systems and is supported by evidence of pro-mutagenic methyleugenol-specific DNA adducts in liver and lung of exposed humans and in livers of humanized mice. The evidence regarding cancer in humans was *inadequate* because no studies were available.

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ISOEUGENOL

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 97-54-1 (*E/Z*); 5912-86-7 (*Z*); 5932-68-3 (*E*) (Chemical Abstracts Service, 2022a)

EC/List No.: 202-590-7 (*E/Z*); 227-633-7 (*Z*); 227-678-2 (*E*) (ECHA, 2023a, b, c)

Chem. Abstr. Serv. name: 2-methoxy-4-(1-propenyl)phenol; *cis*-isoeugenol (*Z*); *trans*-isoeugenol (*E*) (O'Neil, 2006; Chemical Abstracts Service, 2022a)

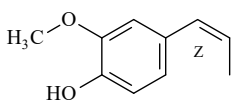
IUPAC systematic name: 2-methoxy-(4-prop-1-enyl)phenol (*E/Z*); 2-methoxy-4-[(*Z*)-prop-1-enyl]phenol (*Z*); 2-methoxy-4-[(*E*)-prop-1-enyl]phenol (*E*) (NCBI, 2022a, b)

Synonyms: 2-methoxy-4-(1-propen-1-yl)phenol (ACI); phenol, 2-methoxy-4-(1-propenyl)-(9CI); phenol, 2-methoxy-4-propenyl- (8CI); 1-(3-methoxy-4-hydroxyphenyl)-1-propene; 2-methoxy-4-(1-propenyl)phenol; 2-methoxy-4-propenylphenol; 3-methoxy-4-hydroxy-1-propenylbenzene; 4-(1-propenyl) guaiacol; 4-hydroxy-3-methoxy-1-propenylbenzene; 4-hydroxy-3-methoxy-β-methylstyrene; 4-propenyl-2-methoxyphenol; 4-propenyl-guaiacol; *iso*-eugenol; isoeugenol; NSC 6769 (Chemical Abstracts Service, 2022c)

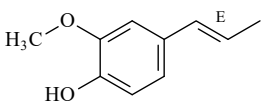
1.1.2 Structural and molecular information

Relative molecular mass: 164.20 (Chemical Abstracts Service, 2022a)

Chemical structure: (Chemical Abstracts Service, 2022a)



Z-(*cis*-) isomer



E-(*trans*-) isomer

Molecular formula: $C_{10}H_{12}O_2$ (Chemical Abstracts Service, 2022a).

1.1.3 Chemical and physical properties

In both nature and commerce, isoeugenol is usually a mixture of *cis*- and *trans*-isomers in the approximate ratio of 1:7 (NTP, 2010). The information in the present monograph pertains to the mixture, unless stated otherwise. [The Working Group noted that there is no evidence to suggest that the *cis*- and *trans*-isomers of isoeugenol have significantly different environmental occurrences.]

Description: The mixture of the isomers is a pale yellow, viscous liquid with a floral odour reminiscent of carnation (Burdock, 2010; NTP, 2010). The *cis*-isomer is a liquid (O'Neil, 2006), but the *trans*-isomer is crystalline, and its odour is more delicate (Fahlbusch et al., 2003).

Odour threshold: 22.54 µg/L in ethanol (46% volume) (Fan and Xu, 2011)

Boiling-point: 266 °C (Chemical Abstracts Service, 2022a)

Melting-point: –10 °C (mixture); 33 °C (*trans*-isomer) (O'Neil, 2006; Chemical Abstracts Service, 2022a)

Density: 1.0869 g/cm^3 at 20 °C (Chemical Abstracts Service, 2022a)

Solubility: slightly soluble in water; soluble in most fixed oils and ether [diethyl ether]; 1:5 in 50% alcohol; insoluble in glycerine (O'Neil, 2006; Burdock, 2010)

Flash-point: > 100 °C (NCBI, 2022a)

Vapour pressure: 0.01–0.02 mm Hg (NCBI, 2022a)

Viscosity: 7.476 cP at 20 °C (NCBI, 2022a)

Octanol/water partition coefficient (P): log K_{ow} = 3.04 (Griffin et al., 1999)

Dissociation constant: pK_a = 9.88 at 25 °C (NCBI, 2022a).

1.1.4 Commercial products and impurities

The commercial product is a mixture of *cis*- and *trans*-isomers (Burdock, 2010), but the pure *trans*-isomer is also available commercially (NTP, 2010). The *trans*-isomer dominates because it is thermodynamically more stable (Panten and Surburg, 2016). Commercial qualities with purities in the range of 90–94%, 95–98%, and ≥ 99% are available (Chemical Abstracts Service, 2022b). [The Working Group noted that there is no publicly available information on impurities. Depending on the specific manufacturing process (see Section 1.2.1), it can be deduced that eugenol or other phenolic compounds such as guaiacol may be present as impurities, especially in lower-purity grades. Possible inorganic impurities include metals used as catalysts during the manufacturing process.]

1.2 Production and use

1.2.1 Production process

Isoeugenol is produced by the alkaline isomerization of eugenol obtained from essential oils rich in eugenol (Burdock, 2010). The conversion involves heating eugenol with potassium hydroxide (Larrañaga et al., 2016) with catalysts such as various metals (Červený et al., 1987; Fahlbusch et al., 2003) or more environmentally friendly hydrotalcite (Kishore and Kannan, 2002). In particular, eugenol – the starting material for the production of isoeugenol – is often obtained from the leaf oil of clove (*Syzygium aromaticum*) (Panten and Surburg, 2015). Another synthetic route starts with the esterification of guaiacol and propionic acid, followed by a Fries rearrangement of the resulting guaiacyl propionate in the presence of aluminium chloride into 4-hydroxy-3-methoxypropiophenone, which is reduced to the corresponding secondary alcohol, and removal of water finally yields isoeugenol (Fahlbusch et al., 2003). [The Working Group was unable to find information about which process is currently preferred to produce isoeugenol. The Working Group noted that, in addition to chemical synthesis, isoeugenol may also be directly extracted from a variety of plant materials by steam distillation or with organic solvents.]

1.2.2 Production volume

In 1983, information from five isoeugenol producers indicated that approximately 21 000 pounds [9 tonnes] were produced (NTP, 2010). [The Working Group noted that NTP (2010)

did not state where the production occurred, presumably in the USA.] In recent years, USA national aggregate production volumes were below 1 000 000 pounds [454 tonnes] for 2016, 2017, 2018, and 2019. One company in the USA reported annual production volumes of 8818 pounds [4.0 tonnes] in 2016, 8818 pounds [4.0 tonnes] in 2017, 6613 pounds [3.0 tonnes] in 2018, and 4409 pounds [2.0 tonnes] in 2019 (US EPA, 2023). In 1990, four isoeugenol importers imported between 12 000 pounds [5.4 tonnes] and 122 000 pounds [55.3 tonnes] (data from US EPA; NTP, 2010). [The Working Group noted that NTP (2010) did not state where the importation occurred, presumably only into the USA.] In 1992, the USA imported approximately 730 000 pounds [330 tonnes] of eugenol or isoeugenol (NTP, 2010).

The total use of isoeugenol in Europe was estimated to be 26 000 kg/year (HERA, 2005).

In 2004, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) assumed an annual production volume of 817 kg for the USA and 327 kg for Europe (data extracted from references for 1970–1999), whereas the annual volume in naturally occurring foods was assumed to be 2162 kg for the USA (no data for Europe were available). The ratio between consumption via natural occurrence in foods and use as a flavouring substance was 7 (WHO, 2004a). [Considering this ratio, the Working Group noted that relying solely on production data may lead to an underestimation of the total amount of isoeugenol use. Therefore, it is important to consider both the production and importation data and the amount of isoeugenol naturally contained in foods to obtain a more accurate estimate of overall exposure.]

1.2.3 Uses

The presence of isoeugenol in products can derive from the addition of the isolated or synthesized agent, but also from its natural occurrence in a wide variety of plants (see Section 1.4). Therefore, in a substantial proportion of the products in which isoeugenol has been detected, the presence of isoeugenol may be unintentional.

Additional fragrance materials can be produced by esterification or etherification of the hydroxy group of isoeugenol (Panten and Surburg, 2016).

Isoeugenol is used as a reagent in the synthesis of compounds such as isoindolo quinolines (Merchán-Arenas et al., 2020). Isoeugenol can be biotechnologically converted to vanillin using several different types of microorganism (Priefert et al., 2001; Ma et al., 2022). For example, a method has been described that gave an 81% yield of vanillin, without overoxidation to vanillic acid or accumulation of undesirable by-products such as acetaldehyde (Yamada et al., 2008). However, although the process of making vanillin by oxidizing isoeugenol was historically significant, this method is no longer commonly used today and has been replaced by more efficient and cost-effective methods (Panten and Surburg, 2016).

The sweet, spicy, floral fragrance of isoeugenol leads to its use as a fragrance or flavouring agent in perfumes, cosmetics, personal hygiene products, household cleaning agents, and foods. Tasting like anise or liquorice, isoeugenol is added to non-alcoholic drinks, baked foods, candies, and chewing gums (NTP, 2010).

Isoeugenol exhibits antibacterial and antifungal properties, inhibiting the growth of a broad range of bacteria and moulds, including *Listeria monocytogenes, Escherichia coli*, *Bacillus licheniformis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Salmonella* type B, *Staphylococcus aureus, Aspergillus oryzae, Penicillium camemberti*, and *Penicillium roqueforti* (Faith et al., 1992; Wendorff et al., 1993; Mansour et al., 1996; Hyldgaard et al., 2015). [The Working Group noted that it was not clear from the information provided whether the doses of isoeugenol

commonly used in foods were sufficient to exhibit bacteriostatic and fungistatic effects.]

The Consumer Product Information Database (CPID) lists the use of isoeugenol in 86 products, including disinfectant cleaners, laundry detergents, toilet cleaners, air and fabric fresheners, fragrance diffusers, candles, leather lotions, shoe-cleaning creams, moisturizing oils, eau-de-cologne sprays, sunscreen lotions, roll-on deodorants, and various automobile cleaners and fresheners (DeLima Associates, 2022). In Europe, about 60% of the total use of isoeugenol was for household laundry and cleaning products, including laundry detergents, laundry pre-treatment products, fabric softeners, hard-surface cleaners, hand dishwashing products, and toilet cleaners (HERA, 2005).

In medicine, isoeugenol is used as a test reference allergen in epicutaneous patch tests, which are indicated for use in the diagnosis of allergic contact dermatitis (NCBI, 2022a).

Isoeugenol is used as the active ingredient in so-called zero-withdrawal anaesthetics used in the culture and management of finfish and shellfish (NTP, 2010). Isoeugenol-containing anaesthetics prevent struggling and thus maintain muscle quality during the "rested harvesting" of king salmon (NTP, 2010). Isoeugenol can be used as a feed additive for fattening cattle, pigs, or chickens (EFSA, 2012).

[The Working Group noted that the literature is often unclear as to whether isoeugenol itself is used, or whether it has been added indirectly via the use of various plant materials and essential oils.]

1.3 Detection and analysis

Methods for the sampling, identification, and quantification of isoeugenol in air, water, herbs, cosmetics, and food have been developed and used in research and practice. No methods were found for the measurement of isoeugenol in the soil. Some methods are described in a recent review (Dang and Quirino, 2021), and selected publications containing information on sample preparation and method performance data, including the limit of detection (LOD), when reported, are presented in Table 1.1. A European Standard (EN 16274) method for the analysis of isoeugenol in consumer products was approved in 2012 (CEN, 2012). Most studies report the sum of *cis*- and *trans*-isoeugenol, or only the *trans*-isomer. However, there are methods capable of distinguishing between the two isomers (Wisneski et al., 1988; Rodríguez-Bencomo et al., 2008; Martínez-Gil et al., 2018).

1.3.1 Air

Stanfill and Ashley (2000) developed a method combining solid-phase extraction with analysis by selected ion monitoring-gas chromatography-mass spectrometry (SIM-GC-MS) to quantify isoeugenol in mainstream cigarette smoke particulate. Mainstream smoke from a smoking machine was captured into a glass fibre filter and extracted with hexane and 3′,4′-methylenedioxyacetophenone. The LOD was 20.1 ng/cigarette.

Kuo et al. (2009) developed a diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) method to measure isoeugenol in aerosol particles of essential oils in indoor air. The aerosol was collected on aluminium foil for 30 minutes. The DRIFTS results were similar to those of gas chromatography (GC) analysis.

1.3.2 Water

Martínez et al. (2013) developed an analytical method based on headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) for the simultaneous determination of 76 micropollutants in water samples. The LOD for isoeugenol was 0.100 ng/mL. An immunoassay for the determination of isoeugenol was developed

Table 1.1 Analytical methods for the measurement of isoeugenol in various matrices

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Air				
Indoor air	No extraction; collection of aerosol on aluminium foil	DRIFTS (42 ng/mL)	Aerosols of ylang essential oils	Kuo et al. (2009)
Tobacco smoke particulate	Smoking machine; glass fibre (Cambridge) filter; hexane/MDAP extraction, SPE	SIM-GC-MS (20.1 ng/cigarette)		Stanfill and Ashley (2000)
Water				
Surface water, sea water, waste water	Headspace SPME	GC-MS (0.100 ng/mL)		Martínez et al. (2013)
Water	No pre-treatment	EFM-based LFIA (6.02 µg/kg)		Lei et al. (2023)
Food				
Herbs/spices, sauces	Ultrasound extraction of finely chopped samples in methanol, maceration at 50°C for 12 hours	Capillary LC (0.148 mg/L)		Avila et al. (2009)
Peppers	Ultrasound extraction with ethyl acetate	GC-HRMS (10 µg/kg)	*trans*-Isoeugenol validated for black pepper according to SANTE/11813/2017 guidelines	Rivera-Pérez et al. (2020); European Commission (2017b)
Fish fillet	Homogenization, ultrasound extraction in hexane, SPE with ethyl acetate	GC-MS/MS (1.2 µg/kg)		Ke et al. (2016)
Fish fillet	Homogenization in dry ice, four sequential extractions with acetonitrile, SPE	LC (4–14 µg/kg)	LOD range is for 9 freshwater fish species	Meinertz et al. (2008)
Fish and shrimp	Ultrasound extraction in acetonitrile; dispersive SPE with polystyrene-glycidylmethacrylate microspheres, primary secondary amines, and C18; DMSO-assisted concentration	HPLC-UV (13 µg/kg)		Shi et al. (2022)
Smoked sausage, smoked fish	Homogenization in phenol solution, membrane-based microextraction using PVDF-*co*-PTFE membrane impregnated with choline chloride	HPLC-FLD (0.6 µg/kg)		Shishov et al. (2020)
Fish	Homogenization, ultrasound extraction with acetonitrile, air drying in room temperature, dissolved in methanol solution	LFIA (5.9 µg/kg)		Lei et al. (2023)
Medicinal herbs or plants				
Anemopsis californica root	Supercritical fluid extraction (methanol)	GC-MS (NR)		Medina-Holguín et al. (2008)
Myrtus communis L.	Headspace SPME, headspace single-drop microextraction	GC-MS (NR)	*cis*-Isoeugenol	Moradi et al. (2012)
Consumer products				
Creams	Direct contact sorptive tape extraction	GC-MS (190 µg/kg)		Sgorbini et al. (2010)

Table 1.1 (continued)

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Creams, perfumes, anti-hair loss products, etc.	Headspace-programmable temperature vapourizer	Fast GC-MS (0.014 µg/mL)		del Nogal Sánchez et al. (2010)
Perfumes	Acetonitrile dilution	HPLC (0.13 µg/mL)		Soo Lim et al. (2018)
Insect repellent, massage oil, cream, hair conditioner	Acetonitrile dilution	HPLC (0.10 µg/mL)		Villa et al. (2007)
Perfumes, colognes, toilet waters	NaOH/isooctane extraction	LC-FLD (16 µg/mL for *cis*-isoeugenol; 38 µg/mL for *trans*-isoeugenol)		Wisneski et al. (1988)
Creams and lotions	Dispersive SPE-PLE	GC-MS [0.075 µg/g]		Lamas et al. (2010)

DMSO, dimethyl sulfoxide; DRIFTS, diffuse reflectance infrared Fourier transform spectroscopy; EFM, europium-fluorescent microspheres; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; FLD, fluorescence detection; GC-HRMS, gas chromatography-high resolution mass spectrometry; LC, liquid chromatography; LFIA, lateral-flow immunoassay; LOD, limit of detection; MDAP, 3′,4′-methylenedioxyacetophenone; MeOH, methanol; MS/MS, tandem mass spectrometry; NaOH, sodium hydroxide; NR, not reported; PLE, pressurized liquid extraction; PVDF-co-PTFE, poly(vinylidene fluoride-*co*-tetrafluoroethylene; SIM, selected ion monitoring; SPE, solid-phase extraction; SPME, solid-phase microextraction; UV, ultraviolet.

recently. The LOD was 6.02 µg/kg in water (Lei et al., 2023).

1.3.3 Medicinal herbs or plants

Medina-Holguín et al. (2008) used supercritical fluid extraction and GC-MS to quantify isoeugenol in the roots of the medicinal plant *Anemopsis californica* (yerba mansa or lizard tail). Moradi et al. (2012) measured *cis*-isoeugenol in *Myrtus communis* L. (common myrtle) with GC-MS. In sample preparation, HS-SPME and headspace single-drop microextraction (HS-SDME) were not superior to hydrodistillation.

1.3.4 Consumer products

Sgorbini et al. (2010) determined isoeugenol content in cosmetic creams spread on skin by direct contact sorptive tape extraction and GC-MS. del Nogal Sánchez et al. (2010) determined isoeugenol content in cosmetic products by headspace-programmed temperature vapourization-fast gas chromatography-quadrupole mass spectrometry (HS-PTV-fast GC-MS). The LOD was 0.014 µg/mL.

Villa et al. (2007) and Soo Lim et al. (2018) analysed perfumes and other cosmetics by acetonitrile dilution high-performance liquid chromatography (HPLC) and reported LODs of 0.10 and 0.13 µg/mL, respectively. Lamas et al. (2010) developed a solid-phase dispersion pressurized liquid extraction method followed by GC-MS for the analysis of isoeugenol and other compounds in creams and lotions. For isoeugenol, the LOD was [0.075 µg/g].

1.3.5 Food

Several methods for the quantification of isoeugenol in food have been described. Before extraction, Avila et al. (2009) homogenized herbal samples and macerated them for 12 hours at 50 °C. On-column preconcentration-capillary LC was used to determine isoeugenol in herbs, spices, and sauces. The LOD was 13 ng/mL. With the direct determination method (without preconcentration), the LOD was 0.148 mg/L.

Rivera-Pérez et al. (2020) used gas chromatography coupled to high-resolution mass spectrometry (GC-HRMS-Q-Orbitrap) in combination with a simple ultrasound-assisted extraction method with ethyl acetate to determine simultaneously eight alkenylbenzenes – including *trans*-isoeugenol – in peppers. For *trans*-isoeugenol, the LOD was 10 µg/kg and the limit of quantitation (LOQ) was 200 µg/kg.

For the analysis of isoeugenol in fish, samples were homogenized at room temperature or in dry ice. Hexane (Ke et al., 2016; Shishov et al., 2020) or acetonitrile (Meinertz et al., 2008; Shi et al., 2022) were used as extraction solvents. The use of ultrasound improved extraction yield.

Meinertz et al. (2008) developed an SPE-LC-based method to quantify isoeugenol residue in fillet tissue from 10 species of freshwater fish. The LODs for isoeugenol were between 4 and 14 µg/kg.

Ke et al. (2016) determined isoeugenol content in fish fillets using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS). The LOD was 1.2 µg/kg and the LOQ was 4 µg/kg.

Shishov et al. (2020) used membrane-based microextraction and HPLC-FLD to quantify isoeugenol in smoked sausage and fish. The LOD was 0.6 µg/kg.

Shi et al. (2022) established a dispersive solid-phase extraction method, combined with HPLC-UV, for the simultaneous determination of seven anaesthetics, including isoeugenol, in fish and shrimp. For isoeugenol, the LOD was 13 µg/kg.

An HPLC method with fluorescence detection for the determination of residues of isoeugenol in muscle or skin of Atlantic salmon was developed and validated in a laboratory that was approved for Good Laboratory Practice (GLP). The LOQ for the method was 0.25 mg/kg. The method was sufficiently validated (according to the

requirements of Volume 8 of the Rules governing veterinary medicinal products in the European Union, EU) and verified by the relevant European reference laboratory, which confirmed the suitability of the method (EMA, 2020). Isoeugenol remained quantifiable in salmon plasma (LOD, 25 ng/L) for up to 12 hours after the end of exposure but was no longer detectable in plasma at 24 hours (EMA, 2020).

An immunoassay for the determination of isoeugenol has been developed. The LOD was 5.9 µg/kg in fish (Lei et al., 2023).

1.3.6 Biological specimens

cis-Isoeugenol was measured in human serum by GC-MS (Wang et al., 2010). [The Working Group noted that this study lacked a detailed description of sampling, sample preparation, and detection methods.]

A method based on solid-phase extraction followed by analysis using GC-MS has been reported for measuring isoeugenol in urine (Dills et al., 2001, 2006). The LODs were approximately 0.004 µg/mL.

1.4 Occurrence and exposure

1.4.1 Environmental occurrence

Isoeugenol is a compound that occurs naturally in the essential oils of more than 500 plant species, including cloves (*Syzygium aromaticum*), sweet flag (*Acorus calamus)*, sweet wormwood (*Artemisia annua)*, Ceylon cinnamon (*Cinnamomum verum)*, coffee (*Coffea arabica)*, nutmeg (*Myristica fragrans)*, basil (*Ocimum basilicum)*, perilla (*Perilla frutescens)*, and ylang-ylang (*Cananga odorata*) (USDA, 2021; HMDB, 2022; Wishart et al., 2022). An overview of the occurrence of isoeugenol in plants is given in Table 1.2.

Isoeugenol occurs naturally in foods and beverages and can be added to foods as a flavouring agent. It is present in smoked food products since it is a component of wood smoke. Isoeugenol also occurs in cosmetics and personal care products, and some tobacco and cannabis products. The production and use of isoeugenol can result in its release to the environment through various waste streams. When wood is burned, isoeugenol is released into the air. The direct release of isoeugenol to the environment is anticipated because of its use as a sedative or anaesthetic for fish (NCBI, 2022a).

(a) Air

Isoeugenol is expected to exist almost exclusively as a vapour in the ambient atmosphere (NCBI, 2022a). In the vapour phase, isoeugenol is degraded in the atmosphere by reaction with photochemically generated hydroxyl radicals and with ozone. The atmospheric half-life is estimated to be between 3 and 4 hours (NCBI, 2022a). Occurrence in air is expected in smoke from wood combustion (NCBI, 2022a).

Isoeugenol is primarily produced during high-temperature torrefaction of woody biomass (González Martínez et al., 2018). The emission rate of isoeugenol in wood smoke from the combustion of oak, eucalyptus, and pine wood was reported to be 1.0, 0.5, and 17 mg/kg, respectively (Nolte et al., 2001). The emission rate of isoeugenol in wood smoke from the combustion of pine wood logs, oak wood logs, and synthetic logs was reported to be 8.04 mg/kg, 0.16 mg/kg, and not detected, respectively (Rogge et al., 1998). Isoeugenol, acetoguaiacone, and 4-vinylguaiacol were the dominant phenolic compounds identified in the pyrolysis of *Tectona grandis* (teak) biomass (Balogun et al., 2014). According to a technical specification of the European Committee for Standardization, isoeugenol is one of the most abundant single organic compounds in the product gases of biomass gasification (CEN, 2006).

Table 1.2 Concentrations of isoeugenol in various plant parts

Plant	Part	Concentration (mg/kg)
Acorus calamus	Rhizome	228–12 510
Anethum graveolens	Plant	[a]
Artemisia dracunculus	Plant	[a]
Artemisia annua	Leaf	1–45
Cananga odorata	Flower	[a]
Cinnamomum verum	Stem bark	[a]
Cinnamomum verum	Leaf essential oil	[a]
Cinnamomum verum	Bark	2–8
Cinnamomum aromaticum	Plant	[a]
Coffea arabica	Seed	[a]
Myristica fragrans	Seed essential oil	1000–3800
Myristica fragrans	Seed	40–320
Psiadia argute	Essential oil	565 000
Nicotiana tabacum	Leaf	[a]
Ocimum basilicum	Plant	8–95
Origanum sipyleum	Shoot	3–5
Oryza sativa	Plant	[a]
Perilla frutescens	Leaf essential oil	2500
Pimenta racemosa	Leaf	[a]
Pimenta dioica	Plant	[a]
Pimenta dioica	Leaf essential oil	[a]
Santalum album	Wood	[a]
Satureja parvifolia	Shoot	[a]
Satureja odora	Shoot	525
Scutellaria baicalensis	Root essential oil	[a]
Syzygium aromaticum	Plant	[a]
Thymus vulgaris	Plant	[a]
Vaccinium corymbosum	Fruit	[a]
Vaccinium myrtillus	Fruit juice	[a]
Zingiber officinale	Rhizome	1.48–1.68
Laurus nobilis	Leaves	1000–6000
Strychnos spinosa	Peel of the fruits	4762
Salacca zalacca	Fruit	[a]
Myroxylon pereirae	Resin fraction	8500

[a] No quantitative data reported.
Compiled from Janssens et al. (1990), Duke (2001), Sitrit et al. (2003), Kilic et al. (2004), Wijaya et al. (2005), Schaller and Schieberle (2020), USDA (2021).

(b) Water

Two condensate effluents from a bleached softwood pulp mill in Canada contained isoeugenol at concentrations of 10 µg/L and 121 µg/L (Belknap et al., 2006). Isoeugenol was detected at concentrations ranging from 0 (LOD, not reported) to 28 643 µg/L in six effluents collected from 13 pulp and paper mills in Quebec, Canada (Lavallee et al., 1992).

When released to water, isoeugenol is expected to be adsorbed to suspended solids and sediments. Volatilization from water is expected, with half-lives for a model river and model lake of 13 and 100 days, respectively. Isoeugenol can be degraded in natural waters by reaction with photochemically generated hydroxyl radicals, with an estimated half-life of 21 days. The potential for bioconcentration of isoeugenol in aquatic organisms is moderate (NCBI, 2022a).

(c) Soil

On the basis of its physical properties (see Section 1.1), isoeugenol is expected to have low mobility in soil. Volatilization of isoeugenol from moist soil surfaces is expected to be an important process (NCBI, 2022a). Biodegradation may be an important environmental process in soil, with 79% biodegradation achieved after 28 days (NCBI, 2022a). Isoeugenol is not expected to volatilize from dry soil surfaces because of its vapour pressure (NCBI, 2022a). [The Working Group noted that there are limited data available to support or refute theoretical assumptions about the fate of the compound in soil. Some of the information available appears contradictory (e.g. that isoeugenol volatilizes from moist but not from dry soil) and in need of experimental validation.]

(d) Consumer products

The usual and maximum concentrations of isoeugenol in some cosmetic products were 0.03% and 0.3% in soap, 0.003% and 0.03% in detergents, 0.015% and 0.1% in creams and lotions, and 0.4% and 0.8% in perfumes, respectively (Opdyke, 1975). According to labelling, 27 out of 300 evaluated cosmetics (9%) on the market in the United Kingdom (UK) in 2006 contained isoeugenol (Buckley, 2007). However, a study from Sweden noted during a survey of 45 products that isoeugenol was not detected in 67% of fragrances in which it had been declared by the supplier (Bárány and Lodén, 2000).

Rastogi and colleagues measured the isoeugenol content of different consumer products available on the Danish or European markets (Rastogi et al., 1996, 1998, 1999). Out of 42 cosmetic products based on natural ingredients, isoeugenol was found in 3 products (7%) at 0.0127%, 0.027%, and 0.139% (Rastogi et al., 1996). Out of 22 vapour-spray deodorants with fragrance, 9 (41%) contained isoeugenol (mean, 0.0129 g/100 mL; median, 0.0098 g/100 mL; range, 0.0001–0.0458 g/100 mL); 9 out of 22 aerosol deodorant sprays (41%) contained isoeugenol (mean, 0.0031 g/100 mL; median, 0.0024 g/100 mL; range, 0.0001–0.0104 g/100 mL), and 2 out of 28 roll-on deodorants (8%) contained isoeugenol at 0.0241 and 0.0268 g/100 mL (Rastogi et al., 1998). In an investigation of 25 children's cosmetics, isoeugenol was not detected in shampoos and lotions, but was detected at 0.019% and 0.074% in two out of seven (29%) hydroalcoholic products (Rastogi et al., 1999). Among 25 popular perfume brands, isoeugenol was found in 14 products (56%) (mean, 71 mg/L; median, 80 mg/L; range, 48–193 mg/L) (Rastogi et al., 2007). Among 29 international brands of hydroalcoholic perfumes and aftershaves, 16 products (55%) contained isoeugenol (mean, 71 mg/kg; median, 45 mg/kg; range, 27–203 mg/kg) (Rastogi and Johansen, 2008).

In 59 domestic and professional products for which hand exposure would occur (such as soap, cleaners, laundry agents, hand and dish wash, furniture polish, stain remover, and car shampoo), isoeugenol was detected in three products (5%)

[no quantitative data were provided], and it was concluded that isoeugenol occurred much more frequently in cosmetic products than in household products (Rastogi et al., 2001).

In Taiwan, China, four popular types of traditional worship incense based on agarwood (*Aquilaria agallocha*) or sandalwood (*Santalum album*) contained isoeugenol (mainly in the *trans*-form) at a concentration of between 18 and 29 mg/kg of powder. The emissions also contained the *cis*-isomer produced during the burning process, with a total isoeugenol content between 56 and 80 mg/kg of incense (Kuo et al., 2015).

Isoeugenol has been detected in the essential oil of cannabis (*Cannabis sativa*) Turner et al., 1980).

Isoeugenol is a constituent of smoke condensate from Turkish tobacco (approximately 14.7 mg per 1000 cigarettes) (Rodgman and Cook, 1964). Both *cis*- and *trans*-isomers have been detected in cigarette smoke condensates (Arnarp et al., 1989). In a study of eight commercial cigarette brands in the USA, seven brands were found to contain isoeugenol (mean values, 265–4050 ng/cigarette) in smoke particles from unblocked cigarettes (Stanfill and Ashley, 2000). The effect of blocking the ventilation holes in the cigarette filter was investigated in another brand (containing isoeugenol at 188 ng/cigarette). Isoeugenol was detected in the smoke of the unblocked cigarette and in the smoke when the holes were partially or completely blocked (226, 525, and 1030 ng in the smoke particulate of one cigarette, respectively) (Stanfill and Ashley, 2000).

Some tobacco flavourings contain alkenylbenzenes, including isoeugenol (Stanfill et al., 2003). Isoeugenol was found in only 1 out of 20 brands of bidi cigarettes (small hand-rolled cigarettes produced mainly in India) purchased in the USA; levels ranged from 71 mg/kg to 93 mg/kg. Lower levels of isoeugenol were found in cigarettes produced in the USA, ranging from 0.055 to 0.44 mg/kg (Stanfill et al., 2003). In a study of 68 cigarette brands on the USA market, isoeugenol was found in 4 brands (5.9%) at levels of 0.068–0.38 mg/kg (Stanfill and Ashley, 1999).

(e) Food

Some of the plant species containing isoeugenol are culinary herbs, spices, and edible fruits, such as blueberry, guava, blackberry, tomato, cinnamon, cloves, nutmeg, mace, thyme, tea, coffee, plum, dill, Chinese quince, pimento, bay leaves, ginger, pepper, fennel, and mate (Kilic et al., 2004; Burdock, 2010; Rivera-Pérez et al., 2020; Schaller and Schieberle, 2020; Suleiman, 2020). Processed foods can be flavoured with plant parts, essential oils, or extracts from specific plants that contain isoeugenol (Burdock, 2010). The concentration of isoeugenol in some edible products has been reported to be about 4 mg/kg in beverages, 4–10 mg/kg in foods, and 0.3–1000 mg/kg in gums (Opdyke, 1975). An overview of concentrations of isoeugenol in foods is given in Table 1.3.

Isoeugenol has been reported to be added as a flavouring agent to alcoholic beverages (0.21–0.35 mg/kg), bakery products (10.88–14.38 mg/kg), chewing gum (0.14–0.17 mg/kg), condiments and relishes (up to 0.04 mg/kg), frozen dairy products (4.25–6.63 mg/kg), gelatins and puddings (4.56–6.42 mg/kg), hard candies (3.60 mg/kg), meat products (4.34 mg/kg), non-alcoholic beverages (3.27–5.61 mg/kg), and soft candies (6.22–9.62 mg/kg) (Burdock, 2010).

Isoeugenol was detected in two samples of barley tea at 1 and 22 µg/kg (Tatsu et al., 2020). It was also found in infusions of green, black, and oolong teas (n = 19) at concentrations ranging between 0.5 and 30 µg/kg (semiquantitative data) (Baba and Kumazawa, 2014).

In wines, isoeugenol may occur because of extraction out of oak wood because of either traditional barrel ageing or treatment with oak chips. The final levels of extracted isoeugenol depend on the toast degree of the wood chips and

Table 1.3 Concentrations of isoeugenol in selected food products

Food, drink, or preparation	Isoeugenol concentration
Smoked sausages	6–76 mg/kg
Fillet tissue freshwater fish species exposed to isoeugenol	19–62 mg/kg
Bakery products[a]	11–14 mg/kg
Soft candies[a]	6.2–9.6 mg/kg
Frozen dairy products[a]	4–7 mg/kg
Gelatins and puddings[a]	4.6–6.4 mg/kg
Non-alcoholic beverages[a]	3.3–5.6 mg/kg
Meat products[a]	~4.3 mg/kg
Hard candies[a]	~3.6 mg/kg
Alcoholic beverages[a]	0.2–0.4 mg/kg
Soluble coffee	0.06–0.3 mg/kg
Chewing gum[a]	0.14–0.17 mg/kg
Roasted coffee beans	0.006–0.1 mg/kg
Condiments and relishes[a]	≤ 0.04 mg/kg
Roasted chicory coffee brews	15–45 µg/L
Wine (wood-aged)	4–34 µg/L
Green, black, and oolong teas	0.5–30 µg/kg
Barley tea	1–22 µg/kg
Brewed coffee	~2 µg/L

[a] Flavoured products.
Compiled from Tressl et al. (1978), Meinertz et al. (2008), Viegas et al. (2008), Burdock (2010), Hernández-Orte et al. (2012), Pöhlmann et al. (2012), Hitzel et al. (2013), Baba and Kumazawa (2014), Herrero et al. (2016), Kalschne et al. (2018), Martínez-Gil et al. (2018), Wu and Cadwallader (2019), Pua et al. (2020), Tatsu et al. (2020).

the time of contact with the wine, and levels may be reached of about 12 µg/L for *trans*-isoeugenol and about 1 µg/L for *cis*-isoeugenol (Rodríguez-Bencomo et al., 2008). During the ageing of Chardonnay and Sauvignon Blanc wines in French oak wood barrels, isoeugenol increased from not detectable levels to 4–8 µg/L (Herrero et al., 2016). Wines aged in Colombian oak wood (*Quercus humboldtii*) were found to have higher concentrations of isoeugenol (32–34 µg/L) than did wines aged in European or American oak wood (*Q. alba* and *Q. petrea*) (6–20 µg/L) (Martínez-Gil et al., 2018). In Tempranillo wine, isoeugenol concentrations increased from 4 µg/L to 15 µg/L during malolactic fermentation, but no significant increase was found during malolactic fermentation of Cabernet Sauvignon wines (isoeugenol, 6–8 µg/L) (Hernández-Orte et al., 2012).

In juice, wine, and vinegar produced from hawthorn fruit (*Crataegus tanacetifolia*), isoeugenol was detected at concentrations of 7.23, 166.3, and 199.6 µg/100 mL (Özdemir et al., 2022).

Isoeugenol occurs in smoke flavour preparations as a result of the pyrolysis of lignin. In 15 samples of liquid and solid smoke preparations, isoeugenol was found to be between 0.1% and 1.2% of the total phenolic fraction in 7 samples, between 2.0% and 2.2% of the total phenolic content in smoked ham, and between 2.1% and 2.3% of the phenolic extract in bacon treated with liquid smoke (Tóth and Potthast, 1984). In six commercial liquid smoke preparations, isoeugenol was detected at concentrations of between 1.4 and 15 mg/L (Giri et al., 2017). The isoeugenol content in 19 frankfurter-type sausages and mini salamis experimentally

smoked with different types of wood ranged from 6 to 76 mg/kg [mean, 24 mg/kg; median, 11 mg/kg], which was significantly higher than the content found in 11 non-smoked mini salamis (4 mg/kg) (Hitzel et al., 2013). In another investigation of 24 frankfurter-type sausages experimentally hot-smoked, isoeugenol content ranged from 9 to 29 mg/kg [mean, 19 mg/kg; median, 19 mg/kg] (Pöhlmann et al., 2012).

One sample of roasted *Coffea arabica* coffee contained isoeugenol at 0.1 mg/kg (Tressl et al., 1978). In roasted coffee beans [species not reported] from Columbia, isoeugenol was detected at 0.12 mg/kg (Ho et al., 1993). A roasted *C. canephora* sample contained isoeugenol at 5.7 µg/kg, increasing to up to 7.7 µg/kg after steam treatment (Kalschne et al., 2018). Roasted *C. arabica* beans from Yunnan province, China, contained 0.15% isoeugenol (Zhou et al., 2013a, b) [the percentage probably refers to the percentage in the aroma extract and is not an absolute percentage in the coffee bean]. *C. arabica* coffee beans processed by monsooning (storage in humid air) contained isoeugenol at about 9 µg/kg, irrespective of subsequent irradiation treatment (Variyar et al., 2003). Isoeugenol was also detected in two out of four samples of brewed *C. arabica* coffee from Brazil and Colombia (2.1 µg/L and 1.7 µg/L, respectively) (Pua et al., 2020). It was also detected in a sample of Brazilian soluble coffee at levels between 0.06 and 0.3 mg/kg, depending on the analytical methodology used (Viegas et al., 2008), and in a sample of Brazilian roasted *C. arabica* coffee oil at 0.81 µg/kg (Böger et al., 2021). Isoeugenol was also found in roasted chicory (*Cichorium intybus*) coffee brews (n = 3) at an average concentration of 45 µg/L (*trans*-isoeugenol) and 15 µg/L (*cis*-isoeugenol) (Wu and Cadwallader, 2019).

In fresh king salmon (*Oncorhynchus tshawytscha*), isoeugenol was detected in fish harvested with isoeugenol-containing anaesthetic [no quantitative data provided] (Wierda et al., 2006). In nine freshwater fish species exposed to isoeugenol at 8.5 mg/L for 60 minutes, the fillet tissue contained isoeugenol at between 19 and 62 mg/kg [mean, 42 mg/kg; median, 39 mg/kg] (Meinertz et al., 2008). In a market survey of fish fillets in China, isoeugenol was detected in two samples of grouper fish fillets (86 and 1032 µg/kg) [the total sample number was not reported; the occurrence was probably caused by the use of isoeugenol or clove oil as a veterinary anaesthetic] (Ke et al., 2016).

1.4.2 Occupational exposure

In the USA, the National Institute for Occupational Safety and Health (NIOSH) conducted the National Occupational Exposure Survey (NOES) in 1981–1983. NOES revealed that 35 171 (95% confidence interval, CI, 28 489–41 853) employees (71% females) were potentially exposed to isoeugenol (CDC, 1990). Of the potentially exposed, 29 918 employees worked as hairdressers or cosmetologists (85%), and the other commonly exposed employees included janitors and cleaners, machine operators (1282), and packaging and filling machine operators (875). Firefighters may be exposed to isoeugenol contained in smoke after using extinguishing agents (Dills et al., 2008). Urinary concentrations of isoeugenol were significantly increased after smoke exposure among wildland firefighters (Neitzel et al., 2009).

[The Working Group noted the lack of comprehensive exposure data in an occupational context. The Working Group also noted that, despite lacking exposure data, occupational exposure to isoeugenol by dermal or inhalation routes may additionally occur through the production and use of products containing isoeugenol as a fragrance (e.g. in professional cleaning settings), the production and use of isoeugenol as a flavouring agent in the food industry, and the production and use of isoeugenol-containing veterinary anaesthetics.]

1.4.3 Exposure of the general population

Exposure to isoeugenol is mainly associated with its presence as a fragrance in household products and cosmetics (Buckley et al., 2000; Temesvári et al., 2002; Tanaka et al., 2004; Bruze et al., 2005; White et al., 2007; Ezendam et al., 2011). Avoiding this exposure could be difficult, considering that fragrances are ubiquitous in consumer products (Sánchez-Pujol et al., 2021). Additionally, exposure to isoeugenol is also possible through the consumption of foods that contain plants and plant extracts, such as savoury basil, clove, mace, dill seed, and nutmeg (Smith et al., 2009; Scheman et al., 2014), or foods that contain isoeugenol as a flavouring agent, such as non-alcoholic beverages, candies, and chewing gums (Smith et al., 2009). Isoeugenol has also been demonstrated to be a pyrolysis product of lignin (a structural component of plants) and to be present in the smoke particulate fraction of seven of the eight US commercial cigarette brands analysed (Stanfill and Ashley, 2000).

Smith et al. (2009) estimated the daily per capita intake of isoeugenol as a flavouring agent to be 0.02 µg/kg bw per day in the USA. Burdock (2010) also reported an estimated individual exposure to isoeugenol in flavourings of 0.78 µg/kg bw per day. Exposure of the human population was also estimated as part of a fragrance ingredient safety assessment by the Research Institute for Fragrance Materials. Exposure by inhalation was estimated to account for 0.1 µg/kg bw per day and total systemic exposure (dermal, oral, and inhalation) was 0.4 µg/kg bw per day (Api et al., 2016). JECFA has estimated exposure of the general population; the estimated daily per capita intake was 120 µg in Europe and 40 µg in the USA (WHO, 2004b). The European Food Safety Authority (EFSA) has estimated a daily per capita intake of 0.012 µg/day, based on the maximized survey-derived daily intake (MSDI) approach, not considering possible natural occurrences in food (EFSA, 2011).

No data on biomonitoring levels of isoeugenol in the general population were available to the Working Group. Two biomonitoring studies measured increased levels of isoeugenol in the urine of volunteers after exposure to wood smoke or smoke-flavoured food ingestion in an experimental setting in Washington state, USA (Dills et al., 2001, 2006). Pre-exposure *trans*-isoeugenol urine levels of the participants with no known smoke exposure in the 48 hours before sampling were 0.14 ± 0.20 µg/mL (mean ± standard deviation) in 21 participants (Dills et al., 2001) and 0.014 ± 0.010 µg/mL in 9 participants (Dills et al., 2006). Urinary *cis*-isoeugenol levels were 0.002 ± 0.003 µg/mL in 9 participants (Dills et al., 2006) with no prior smoke exposure. [The Working Group noted that these small studies with volunteers are probably not representative of exposure levels of other population groups. They however give some indication that commonly consumed smoke-flavoured foods and exposure to wood smoke or barbecues contribute to the general population exposure.]

1.5 Regulations and guidelines

In the European Union, isoeugenol is authorized to be used as a flavouring substance in food. This is in accordance with Commission Implementing Regulation (EU) No. 872/2012 of 1 October 2012, adopting the list of flavouring substances provided for by Regulation (EC) No. 2232/96 and introducing isoeugenol in Annex I to Regulation (EC) No. 1334/2008 (European Commission, 2012).

In the USA, isoeugenol was classified as "generally recognized as safe" (GRAS) as a food ingredient by the US Food and Drug Administration (US FDA) under 21 CFR §172.515 (US FDA, 2004) and was also permitted for direct addition in food for human consumption as a synthetic flavouring substance (US FDA, 2010).

Regarding the use of isoeugenol in cosmetics, Annex III of Regulation 1223/2009/EC on Cosmetic Products as amended by Regulation (EU) 2022/1531, established the conditions for the use of isoeugenol (European Commission, 2009, 2022). For use in oral products and cosmetic products other than oral products, the presence of this substance must be indicated in the list of ingredients when its concentration exceeds 0.001% in leave-on products and 0.01% in rinse-off products (European Commission, 2009, 2022). For cosmetic products other than oral products, a maximum threshold of 0.02% was established (European Commission, 2009, 2022). Isoeugenol use was banned from any toys except olfactory board games, cosmetic kits, and gustative games (European Parliament and Council, 2009).

Commission Implementing Regulation (EU) No. 2017/60 authorizes the use of isoeugenol as a feed additive in animal nutrition for pigs, ruminants, and horses, except those producing milk for human consumption, and pets. The substance was specified in the Annex, belonging to the additive category "sensory additives" and to the functional group "flavouring compounds" (European Commission, 2017a).

According to the Annex of Regulation (EU) No. 363/2011, the maximum residue limit of isoeugenol in fin fish species via its use in veterinary medicine is 6000 µg/kg (European Commission, 2011). Similarly, the Republic of Korea has also implemented a revised maximum residue level (MRL) for isoeugenol for fish (including eels) of 0.01 mg/kg, a limit that was effective from 1 July 2018. In Australia and Japan, the MRL for isoeugenol in fish is 100 mg/kg (Australian Government, 2018; Japan Chemical Research Foundation, 2023). In the USA, isoeugenol has not been approved for use in veterinary medicine; consequently, seafood products containing isoeugenol may not be imported into the USA (US FDA, 2023).

Under the CLP regulation (Classification, labelling and packaging of substances and mixtures, Regulation (EC) No. 1272/2008), isoeugenol is classified as a skin sensitizer 1A (ECHA, 2015)

[The Working Group noted that no threshold has been established for occupational exposure to isoeugenol.]

1.6 Quality of exposure assessment in key mechanistic studies in humans

See Supplementary Table S1.4 (Annex 1, Supplementary material for Section 1, Exposure Characterization, online only, available from: https://publications.iarc.who.int/627).

One study (Sieben et al., 2001) examined T-cell responses using peripheral blood mononuclear cells and T cells from skin lesions of fragrance-allergic patients after exposure to isoeugenol. The study employed patch testing to confirm fragrance allergy and a second patch test to establish a positive reaction to isoeugenol. The second patch test was performed 3–4 weeks after the first positive skin test. Biopsies were taken from skin lesions 48 hours after topical application of a single fragrance from patients with a second positive patch test reaction. Blood for in vitro investigations was drawn 1–2 weeks after the positive patch test, which is sufficient time to detect immune responses to the exposure. [The certainty of exposure in this study is probably high on the basis of the information provided, since exposure to isoeugenol appears to be controlled with standardized exposure levels.] Final concentrations of isoeugenol ranged from 0.1 to 100 µg/mL, allowing for a range of exposures to be tested, covering both low and high concentrations.

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

See Table 3.1.

3.1 Mouse

In a study of chronic toxicity and carcinogenicity that complied with GLP, groups of 50 male and 50 female B6C3F$_1$ mice (age, 5–7 weeks) were exposed by gavage to isoeugenol (purity, ≥ 99%) in corn oil at doses of 0 (control), 75, 150, or 300 mg/kg body weight (bw) per day, 5 days per week, for 104 (females) or 105 (males) weeks (NTP, 2010). The survival rate of males in the group at the highest dose was significantly decreased (P = 0.019, life-table trend test) compared with the control group. The survival rates of all other exposed groups were similar to those of the vehicle controls. The mean body weights of males and females at the highest dose were less (by 10% and 14%, respectively) than those of the control groups at the end of the study.

In male mice, exposure to isoeugenol significantly increased the incidence of hepatocellular adenoma (multiple) in all treated groups – 10/50 (20%), 26/50 (52%), 28/50 (56%), 20/50 (40%) for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively ($P \leq 0.01$, poly-3 test, [P = 0.0008, Fisher exact test]; $P \leq 0.01$, poly-3 test, [P = 0.0002, Fisher exact test]; and $P \leq 0.05$, poly-3 test, [P = 0.0243, Fisher exact test], respectively). There was a significant positive trend (P = 0.012, poly-3 trend test) in the incidence of hepatocellular adenoma (includes multiple) – 24/50 (48%), 35/50 (70%), 37/50 (74%), 33/50 (66%) in the groups at 0 (control), 75, 150 and 300 mg/kg bw per day treated groups, respectively – and the incidence was significantly increased in all treated groups (P = 0.015, poly-3 test, [P = 0.0207, Fisher exact test]; P = 0.010, poly-3 test, [P = 0.0067, Fisher exact test]; and P = 0.009, poly-3 test, respectively), exceeding the upper bound of the range observed in historical controls from this laboratory: gavage – 50/100 (50% ± 2.8%); range, 48–52%; all routes: 544/1146 (47.5% ± 14.9%); range, 14–72%.

There was a significant positive trend (P = 0.027, poly-3 trend test; [P = 0.03, Cochran–Armitage trend test]) in the incidence of hepatocellular carcinoma (includes multiple) – 8/50 (16%), 18/50 (36%), 19/50 (38%), 18/50 (36%) for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively – and the incidence was significantly increased in all treated groups (P = 0.022, poly-3 test, [P = 0.0195, Fisher exact test]; P = 0.017, poly-3 test, [P = 0.0116, Fisher exact test]; and P = 0.012, poly-3 test, [P = 0.0195, Fisher exact test], respectively), exceeding the upper bound of the range observed in historical controls from this laboratory: gavage – 22/100 (22.0% ± 8.5%); range, 16–28%; all routes – 317/1146 (27.7% ± 9.2%); range, 8–48%. There was a significant positive trend ($P < 0.001$, poly-3 trend test, [$P < 0.001$, Cochran–Armitage trend test]) in the incidence of hepatocellular adenoma or carcinoma (combined) – 28/50 (56%), 43/50 (86%), 43/50 (86%), 43/50 (86%) – for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively – and the incidence was significantly increased in all treated groups ($P \leq 0.003$, poly-3 test, [P = 0.0009, Fisher exact test]), exceeding the upper bound of the range observed in historical controls from this laboratory: gavage – 61/100 (61.0% ± 7.1%); range, 56–66%; all routes – 729/1146 (63.6% ± 15.6%); range, 20–84%.

In female mice, there was a significant positive trend (P = 0.015, poly-3 trend test; [P = 0.008, Cochran–Armitage trend test]) in the incidence of histiocytic sarcoma in multiple tissues, and the incidence – 0/49, 1/50 (2%), 1/50 (2%), 4/50 (8%) – was within the range observed in historical controls from this laboratory: gavage – 0/99; all routes – 31/1249 (2.5% ± 2.5%); range, 0–8%. [The Working Group noted that histiocytic sarcoma is a rare non-Langerhans histiocytic neoplastic disorder with unifocal or multifocal extranodal tumours. It shows highly pleomorphic cells or

Table 3.1 Studies of carcinogenicity in experimental animals exposed to isoeugenol

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 5–7 wk 105 wk NTP (2010)	Gavage Purity, ≥ 99% Corn oil 0, 75, 150, 300 mg/kg bw 5 days/wk for 105 wk 50, 50, 50, 50 39, 38, 36, 27	*Liver* Hepatocellular adenoma (multiple) 10/50 (20%), 26/50** (52%), 28/50** (56%), 20/50* (40%)	**$P \leq 0.01$, poly-3 test; [$P = 0.0008$, Fisher exact test] **$P \leq 0.01$, poly-3 test; [$P = 0.0002$, Fisher exact test] *$P \leq 0.05$, poly-3 test; [$P = 0.0243$, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals; adequate duration; males and females used; multiple doses used. Historical controls: hepatocellular adenoma (includes multiple): gavage – 50/100 (50.0% ± 2.8%), range, 48–52%; all routes – 544/1146 (47.5% ± 14.9%), range, 14–72%; hepatocellular carcinoma (includes multiple): gavage – 22/100 (22.0% ± 8.5%), range, 16–28%; all routes – 317/1146 (27.7% ± 9.2%), range, 8–48%; hepatocellular adenoma or carcinoma (combined): gavage – 61/100 (61.0% ± 7.1%), range, 56–66%; all routes – 729/1146 (63.6% ± 15.6%), range, 20–84%.
		Hepatocellular adenoma (includes multiple) 24/50 (48%), 35/50* (70%), 37/50** (74%), 33/50*** (66%)	$P = 0.012$, poly-3 trend test; *$P = 0.015$, poly-3 test; [$P = 0.0207$, Fisher exact test] **$P = 0.010$, poly-3 test; [$P = 0.0067$, Fisher exact test] ***$P = 0.009$, poly-3 test; [$P = 0.0528$, Fisher exact test]	
		Hepatocellular carcinoma (includes multiple) 8/50 (16%), 18/50* (36%), 19/50** (38%), 18/50*** (36%)	$P = 0.027$, poly-3 trend test; [$P = 0.03$, Cochran–Armitage trend test] *$P = 0.022$, poly-3 test; [$P = 0.0195$, Fisher exact test] **$P = 0.017$, poly-3 test; [$P = 0.0116$, Fisher exact test] ***$P = 0.012$, poly-3 test; [$P = 0.0195$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 28/50 (56%), 43/50* (86%), 43/50* (86%), 43/50* (86%)	$P < 0.001$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test] *$P \leq 0.003$, poly-3 test; [$P = 0.0009$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (F) 5–7 wk 104 wk NTP (2010)	Gavage Purity, ≥ 99% Corn oil 0, 75, 150, 300 mg/kg bw 5 days/wk for 103 wk 49, 50, 50, 50 34, 39, 38, 33	*Liver* Hepatocellular adenoma (includes multiple) 11/49 (22%), 10/50 (20%), 9/49 (18%), 4/50 (8%) Hepatocellular carcinoma (includes multiple) 3/49 (6%), 8/50 (16%), 9/49 (18%), 6/50 (12%) Hepatocellular adenoma or carcinoma (combined) 13/49 (26%), 16/50 (32%), 15/49 (30%), 9/50 (18%) *Multiple sites* (including liver, ovary, uterus, spleen, lung, lymph nodes, kidney, thymus, and bone marrow) Histiocytic sarcoma 0/49, 1/50 (2%), 1/50 (2%), 4/50 (8%)	NS NS NS P = 0.015, poly-3 trend test; [P = 0.0088, Cochran–Armitage trend test]	*Principal strengths*: well-conducted GLP study; adequate number of animals; adequate duration; males and females used; multiple doses used. Historical controls: hepatocellular adenoma (includes multiple): gavage – 17/99 (17.2% ± 7.4%), range, 12–22%; all routes – 345/1245 (27.8% ± 17.0%), range, 2–62%; hepatocellular carcinoma (includes multiple): gavage – 4/99 (4.1% ± 2.9%), range, 2–6%; all routes – 131/1245 (10.5% ± 7.7%), range, 0–28%; hepatocellular adenoma or carcinoma (combined): gavage – 20/99 (20.3% ± 8.9%), range, 14–27%; all routes – 419/1245 (33.7% ± 19.1%), range, 8–64%; histiocytic sarcoma: gavage – 0/99; all routes – 31/1249 (2.5% ± 2.5%), range, 0–8%.
Full carcinogenicity Rat, F344/N (M) 5–7 wk 105 wk NTP (2010)	Gavage Purity, ≥ 99% Corn oil 0, 75, 150, 300 mg/kg bw 5 days/wk for 105 wk 50, 50, 50, 50 35, 34, 33, 30	*Mammary gland* Carcinoma 0/50, 0/50, 0/50, 2/50 (4%) *Thymus* Benign or malignant thymoma (combined) 0/50, 0/50, 0/50, 2/50 (4%)	P = 0.042, poly-3 trend test; [P = 0.015, Cochran–Armitage trend test] P = 0.047, poly-3 trend test; [P = 0.015, Cochran–Armitage trend test]	*Principal strengths*: well-conducted GLP study; adequate number of animals; adequate duration; males and females used; multiple doses used. Historical controls: mammary gland carcinoma: gavage – 0/100; all routes – 8/1199 (0.7% ± 1.3%), range, 0–4%; thymomas: gavage – 0/94; all routes – 3/1146 (0.3% ± 0.7%), range, 0–2%.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344/N (F) 5–7 wk 105 wk NTP (2010)	Gavage Purity, ≥ 99% Corn oil 0, 75, 150, 300 mg/kg bw 5 days/wk for 105 wk 50, 50, 50, 50 33, 35, 34, 31	No significant increase in tumour incidence in treated animals		*Principal strengths*: well-conducted GLP study; adequate number of animals; adequate duration; males and females used; multiple doses used. *Other comments*: exposed groups similar to vehicle control groups.

bw, body weight; F, female; GLP, Good Laboratory Practice; M, male; NS, not significant; wk, week(s).

spindle cell cytology, mimicking pleomorphic or spindle cell sarcoma.]

Regarding non-neoplastic lesions, in males, the incidence of clear cell foci of the liver was significantly increased in the groups at the lowest and intermediate doses. The incidence of respiratory metaplasia in olfactory epithelium in all exposed groups in males and females and of atrophy and hyaline droplet accumulation in all exposed groups, except in females at the lowest dose, was significantly greater than that observed in controls. The incidence of hyperplasia of the Bowman glands (olfactory glands) was also increased significantly in all exposed groups. The incidence of minimal to mild necrosis of renal papilla and of mild to moderate necrosis of renal tubules was increased significantly in females at the highest dose, but no renal tumours were observed. In male mice, forestomach tumours were not observed, but the incidence of forestomach squamous hyperplasia, inflammation, and ulceration increased with exposure and was significant in the groups at the highest dose. The incidence of glandular stomach ulcers was low but significantly increased in the groups of males and females at the highest dose. [The Working Group noted that this was a well-described study that complied with GLP, used multiple doses, both sexes (with respective control groups), an adequate duration of exposure and observation, and an adequate number of animals per group.]

3.2 Rat

In a study of chronic toxicity and carcinogenicity that complied with GLP, groups of 50 male and 50 female F344/N rats (age, 5–7 weeks) were exposed by gavage to isoeugenol (purity, ≥ 99%) in corn oil at doses of 0, 75, 150, or 300 mg/kg bw per day, 5 days per week, for 105 weeks. There was no difference in survival rates between exposed male and female rats and unexposed control rats. Mean body weights of male rats at the highest dose were 9% greater than those of the controls at the end of the study (NTP, 2010).

In male rats, there was a significant positive trend in the incidence of mammary gland carcinoma – 0/50, 0/50, 0/50, 2/50 (4%) for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively) (P = 0.042, poly-3 trend test; [P = 0.015; Cochran–Armitage trend test]) – and benign or malignant thymoma (combined) – 0/47, 0/43, 0/49, 2/48 (4%) for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively (P = 0.047, poly-3 trend test; [P = 0.015; Cochran–Armitage trend test]) – and the incidence of benign and malignant thymoma (combined) exceeded the upper bound of the range observed in historical controls from this laboratory: gavage – 0/94; all routes – 3/1146 (0.3% ± 0.7%), range 0–2%. [Thymomas are mediastinal tumours with a lobulated architecture comprised of cellular lobules intersected by fibrous bands, in which the neoplastic cells are the epithelial cells, and the thymocytes are reactive.]

Regarding non-neoplastic lesions, the rates of minimal atrophy and minimal-to-mild respiratory metaplasia of the olfactory epithelium were increased in males at the intermediate dose and in males and females at the highest dose. The incidence of minimal-to-mild olfactory epithelial degeneration in males at the highest dose was similarly increased. [The Working Group noted that lesions in the olfactory epithelium of the nose are unusual, considering that this was not an inhalation study. The Working Group also noted that this was a well-described and well-conducted study that complied with GLP, used multiple doses, both sexes (with respective control groups), an adequate duration of exposure and observation, and an adequate number of animals per group.]

3.3 Evidence synthesis

The carcinogenicity of isoeugenol has been assessed in one well-conducted GLP study in male and female B6C3F$_1$ mice treated by oral administration (gavage) (NTP, 2010) and in one well-conducted GLP study in male and female F344 rats treated by oral administration (gavage) (NTP, 2010).

In the study that complied with GLP in male and female B6C3F$_1$ mice treated by oral administration (gavage), there was a significant increase in the incidence of hepatocellular adenoma (multiple) in males in all treated groups. Also in males, there was a significant positive trend in the incidence of hepatocellular adenoma (includes multiple) with the incidence being significantly increased in all treated groups. There was a significant positive trend in the incidence of hepatocellular carcinoma (includes multiple), and the incidence was significantly increased in all treated groups. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined), and the incidence was significantly increased in all treated groups. In female mice, there was a significant positive trend in the incidences of histiocytic sarcoma in multiple tissues.

In the study that complied with GLP in male and female F344 rats treated by oral administration (gavage), there was a significant positive trend in the incidence of mammary gland carcinoma and benign or malignant thymoma (combined) in males.

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

This section describes the available evidence on the absorption, distribution, metabolism, and excretion of isoeugenol in humans and experimental animals.

4.1.1 Absorption, distribution, and excretion

(a) Exposed humans

The absorption and distribution of isoeugenol in exposed humans were investigated in a few studies of dermal administration.

As they did for methyleugenol (see the monograph on methyleugenol in the present volume), Sgorbini et al. (2010) quantified the amount of isoeugenol (mixture of *cis*- and *trans*-isomers) penetrating the skin after the application of a skin cream containing isoeugenol at 50 ppm, using a sorptive tape (absorbent polydimethylsiloxane tape) extraction technique in two volunteers. The amount of isoeugenol detected on the stratum corneum decreased by approximately 68.6% 1 hour after the exposure. The estimate was based on the boiling-point (266 °C) and the semi-volatile nature of isoeugenol; thus, a proportion of the loss from the skin surface was probably due to volatilization.

Isoeugenol was also tested for absorption and penetration in excised human epidermis in the presence of several cosmetic and ointment vehicles (Jimbo et al., 1983). Radiolabelled isoeugenol (10 mM) was dissolved in liquid vehicles (i.e. ethanol, ethanol/water, propylene glycol, and liquid paraffin), cosmetic vehicles (lotion, milky lotion, oil/water-type cream, water/oil-type cream, oil/water-type foundation, and oil-type foundation) or ointment vehicles (petrolatum and macrogol). Mixtures of ^{14}C-labelled isoeugenol and various vehicles were applied to portions

of human skin (postmortem excised epidermis from the lower abdominal area) under occlusive conditions for 24 hours. Absorption through the epidermis was measured using a liquid scintillation counter. The highest absorption (penetration) of 10.38 ± 0.63% was observed when isoeugenol was dissolved in a cosmetic vehicle (i.e. milky lotion). Notably, penetration was an order of magnitude lower for ethanol, ethanol/water, or propylene glycol vehicles, which showed penetration percentages of < 1%. [The Working Group noted that the identity of the test material was not provided.]

Liu and Hotchkiss (1997) investigated the percutaneous absorption of ^{14}C-labelled isoeugenol in human and rat (see Section 4.1(b)) skin, using a flow-through diffusion cell in vitro model. Approximately 8.4% and 7.5% of the radioactivity was detected in the human skin and receptor fluid, respectively, 72 hours after application of [^{14}C]isoeugenol at 15.5 mg/cm^2. [The Working Group noted that the study was of low informativeness since it did not report sufficient details, including the identity of the test material.]

Skin sensitization in exposed humans

Madsen et al. (2010) assessed the potency of ethosome formulations of isoeugenol (as a mixture of *cis*- and *trans*-isomers) to enhance skin sensitization in human volunteers. Forty-eight patients with a previous positive patch-test reaction to isoeugenol were selected to be tested for an enhanced reaction when isoeugenol was delivered in an ethosome formulation (a formulation containing phospholipid-based elastic nanovesicles with a high ethanol content) or in an ethanol/water vehicle. The study participants underwent first a patch test (n = 8) with isoeugenol at concentrations of 0, 2.80, or 6.54 mg/mL, followed by a repeated open application test (ROAT) (n = 6) with isoeugenol at 5.66 mg/mL. Both tests were conducted using methyldibromo glutaronitrile as the positive control. Delivery of isoeugenol in an ethosome formulation elicited an enhanced reaction compared with delivery in an ethanol/water vehicle. In contrast, in a follow-up in vitro study, the same group reported that the percutaneous absorption and deposition of isoeugenol in a human skin Franz cell model was decreased when isoeugenol was delivered in the ethosome formulation (Madsen et al.,2011). [The Working Group noted that the number of study participants and groupings were not clearly reported. Furthermore, on the basis of the in vitro results, the increased sensitization potency observed for the ethosome formulation of isoeugenol may not be due to penetration or absorption characteristics.]

(b) Experimental systems

(i) Oral and intravenous routes

Badger et al. (2002) studied the absorption, distribution, metabolism (see also Section 4.1.2(a)(ii)), and excretion of ^{14}C-labelled isoeugenol (as a mixture of *cis*- and *trans*-isomers) in male F344 rats weighing 175–250 g, after exposure by gavage (156 mg/kg bw in corn oil) or intravenous administration (15.6 mg/kg bw in emulphor/ethanol/saline). [The Working Group noted that the number of animals used in this study was not reported.] The disposition of radioactivity in the expired air, blood, urine, faeces, and selected tissues (i.e. heart, kidneys, liver, lungs, muscle, subcutaneous adipose tissue, and testicular adipose tissue) was measured at various intervals up to 72 hours after exposure. Blood and urine samples were analysed by HPLC for parent compound and metabolites.

After gavage exposure, low levels of radioactivity were present in the blood, and no parent ^{14}C-labelled isoeugenol was present at any of the sampling intervals (LOQ, 1.5 ng/mL). However, 40% of the administered radioactivity was detected in the urine within 6 hours and 85% after 24 hours. Low levels of radioactivity were detected in the urine between 24 and 72 hours

after exposure. Approximately 10% of the administered radioactivity was detected in the faeces, < 0.1% was recovered in the expired air, and < 0.2% was observed in tissue samples 72 hours after dosing. Similar patterns were observed after intravenous exposure. For example, within 24 hours, 85% and 10% of the administered radioactivity was observed in the urine and faeces, respectively; and < 0.1% and 0.2% of the administered radioactivity was detected in expired air and tissue samples, respectively. Analysis of the blood samples showed that parent ^{14}C-labelled isoeugenol disappeared rapidly with a half-life of approximately 12.1 minutes and clearance of 1.9 L/min/kg bw after intravenous administration.

The National Toxicology Program (NTP) (NTP, 2010; also summarized in Hong et al., 2013) further explored the dose-, sex-, and species-dependent effects of isoeugenol (as mixture of *cis*- and *trans*-isomers) on various toxicokinetic parameters in male and female rats and mice after gavage and intravenous exposures. Isoeugenol was administered to male and female F344 rats (groups of 21 males and 21 females per dose; age, ~13 weeks) as a single intravenous dose of 17 mg/kg bw or a single gavage dose of 17, 70, or 140 mg/kg bw. In male and female B6C3F$_1$ mice (age, ~13 weeks), isoeugenol was administered as a single intravenous dose of 35 mg/kg bw (41 males and 41 females) or a single gavage dose of 35, 70, or 140 mg/kg bw (groups of 42 males and 42 females per dose). Plasma isoeugenol concentrations were determined at various time intervals in rats and mice up to 6 hours after dosing by intravenous injection and up to 10 hours after dosing by gavage.

After intravenous administration, the following observations were made: (i) female mice showed higher values for the area under the curve $(AUC)_\infty$ and lower values for clearance, compared with male mice; (ii) AUC_∞ and clearance values in male and female rats were not significantly different; and (iii) the apparent volume of distribution at steady state appeared to be higher in mice than in rats.

The apparent volume of distribution at steady state was high in both mice and rats, suggesting distribution to extravascular tissues in both species.

After gavage dosing, isoeugenol was rapidly absorbed in rats and mice; measurable amounts in the plasma were detected within 2 minutes and T_{max} values ranged from 10 to 20 minutes. The reported mean C_{max} values in female rats (i.e. 0.364 ± 0.103, 1.82 ± 0.88, and 5.91 ± 2.28, respectively), were almost twice as high as those reported in male rats (i.e. 0.192 ± 0.022, 1.02 ± 0.41, and 2.06 ± 0.73, respectively) after gavage exposure to isoeugenol as a single dose of 17, 70 or 140 bw. Higher C_{max} values were also reported in female mice (1.94 ± 0.17, 2.54 ± 0.17, and 3.99 ± 2.10, respectively) than in male mice (1.13 ± 0.18, 1.27 ± 0.13, and 1.91 ± 0.14, respectively). However, the absolute bioavailability was greater in mice (~30% for males and females) than in rats (~10% for males; 19% for females). [The Working Group noted that the low absolute bioavailability estimates reported by NTP (2010) and Hong et al. (2013) for mice and rats probably reflected extensive first-pass metabolism.] As the dose increased, clearance decreased in male and female rats, suggesting saturation of isoeugenol metabolism. In contrast, clearance values appeared to increase in male mice as the dose increased. In female mice, clearance appeared to be unaffected by increasing dose. Based on the AUC_T values observed after exposure by gavage, systemic exposure in females appeared to be greater than in males. Notably, secondary peak plasma concentrations of isoeugenol were observed in both species after exposure by gavage. Because of the presence of these "large" secondary peaks, some toxicokinetic parameters were not reported, while others were estimated because they could not be directly calculated. [The Working Group noted that it was speculated that the source of the secondary peaks in the

plasma concentration-versus-time curve, after gavage exposure, was the vehicle used (Hong et al., 2013) or enterohepatic recirculation (NTP, 2010). According to Hong et al. (2013), corn oil may markedly delay, but not diminish, the overall extent of absorption from the gut compared with aqueous vehicles.]

More recently, Zhou et al. (2022) detected isoeugenol in the blood of rats 5 hours after oral exposure to a dried ethanol extract of tsantan sumtang (a traditional Tibetan medicine formula, consisting of *Choerospondias axillaris* (Roxb.) Burtt et Hill, *Santalum album* L., and *Myristica fragrans* Houtt). Sprague-Dawley rats (body weight, 200–250 g) were exposed orally to the tsantan sumtang extract at 2000 mg/kg bw and blood samples were taken 30, 60, 120, 180, 240, and 300 minutes after dosing. The low-molecular-weight compounds of blood serum were analysed using ultra HPLC-MS/MS methods. [The Working Group noted that although the quantification of isoeugenol in the tsantan sumtang preparation and in the blood serum of treated rats was not reported by Zhou et al. (2022), isoeugenol and 10 other tsantan sumtang-related compounds were detected in the serum of exposed rats up to 5 hours after exposure. It was also noted that the isomerism of the test material was not provided in the publication.]

(ii) Dermal route

Liu and Hotchkiss (1998, 1997) described the disposition of isoeugenol in rat skin. In three male F344 rats, Liu and Hotchkiss (1998) applied ^{14}C-labelled isoeugenol at 2.6 mg/cm^2 to the dorsal skin under occluded conditions for 24 hours. Urine samples were collected up until the rats were killed, 24 hours after the dermal exposure. Excised skin and urine were then analysed for the presence of radioactivity. After 24 hours of topical occluded exposure, low levels of radioactivity were detected in the skin (0.8 ± 0.2% of the applied dose); 25.0 ± 1.0% of the applied radioactivity was recovered in the urine as metabolites; and no parent ^{14}C-labelled isoeugenol was recovered in the urine. Liu and Hotchkiss (1997) also described the results of an in vitro study investigating the percutaneous absorption of ^{14}C-labelled isoeugenol in rat skin and in human skin (see Section 4.1(a)). Using a flow-through diffusion cell model, 46.3 ± 4.8% and 15.7 ± 3.5% of the radioactivity was detected in the skin and receptor fluid, respectively, 72 hours after the application of ^{14}C-labelled isoeugenol at 15.5 mg/cm^2 to the skin (Liu and Hotchkiss (1997).] [The Working Group noted that the study was of low informativeness since it did not report sufficient details, including the identity of the test material.]

4.1.2 Metabolism

(a) Humans

(i) Exposed humans

No studies relevant to the metabolism of isoeugenol in exposed humans were available to the Working Group.

(ii) Human cells in vitro

de Sousa et al. (2016) reported that when isoeugenol was administered at concentrations of 2.5, 5.0, and 10.0 μM, > 80% was metabolized by cryopreserved human hepatocyte primary cultures within 60 minutes of incubation. Quantification of "overall metabolism" was determined by quantifying unchanged isoeugenol in the cells using spectroscopy (e.g. absorbance at 270 nm) at various time intervals. [The Working Group noted that the identity of the test material was not provided in the publication.]

(b) Experimental systems

HPLC analysis of pooled urine samples from male rats exposed to ^{14}C-labelled isoeugenol at 156 mg/kg bw showed five discernible peaks (Badger et al., 2002; the study methods are briefly summarized in Section 4.1.1(b)). Each of these peaks was characterized using MS and MS/MS

analysis after treatment with β-glucuronidase and sulfatase. Three of the five peaks were identified as glucuronide (20%) and sulfate (53%) conjugates. The other two peaks were resistant to sulfatase and β-glucuronidase treatment and were not consistent with the peak for parent ^{14}C-labelled isoeugenol. On the basis of the mass spectral data, Badger et al. (2002) proposed a metabolic scheme for orally administered isoeugenol, which is partially accounted for in Fig. 4.1.

The NTP (2010) investigated the effects of repeated oral exposure to isoeugenol (as a mixture of *cis*- and *trans*-isomers) on the activities of 7-ethoxyresorufin-*O*-deethylase (cytochrome P450 family 1 subfamily A member 1, CYP1A1), acetanilide-4-hydroxylase (CYP1A2), and 7-pentoxyresorufin-*O*-deethylase (CYP2B) in rats. Male and female F344 rats (10 of each sex per dose) were exposed to isoeugenol in corn oil at 0, 37.5, 75, 150, 300, or 600 mg/kg bw per day for 31 days (5 days/week). After 31 days of exposure, the rats were killed, and homogenates of the liver were prepared for analysis of enzyme activities. No significant effect on the activity of these enzymes was observed in female rats. However, compared with the controls, treated male rats showed a dose-related and statistically significant decrease in CYP1A1 and CYP2B activities at doses of ≥ 75 mg/kg bw per day. Male rats at 600 mg/kg bw per day also showed a statistically significant decrease in CYP1A2 activity compared with controls. Zhao and O'Brien (1996) also showed that isoeugenol inhibited CYP1A1 activity in 3-methylcholanthrene-induced mouse hepatic microsomes. [The Working Group noted that the magnitude of the CYP inhibition observed was relatively small (e.g. ~70% to 80% of control levels at the highest dose) and may be of limited physiological relevance.]

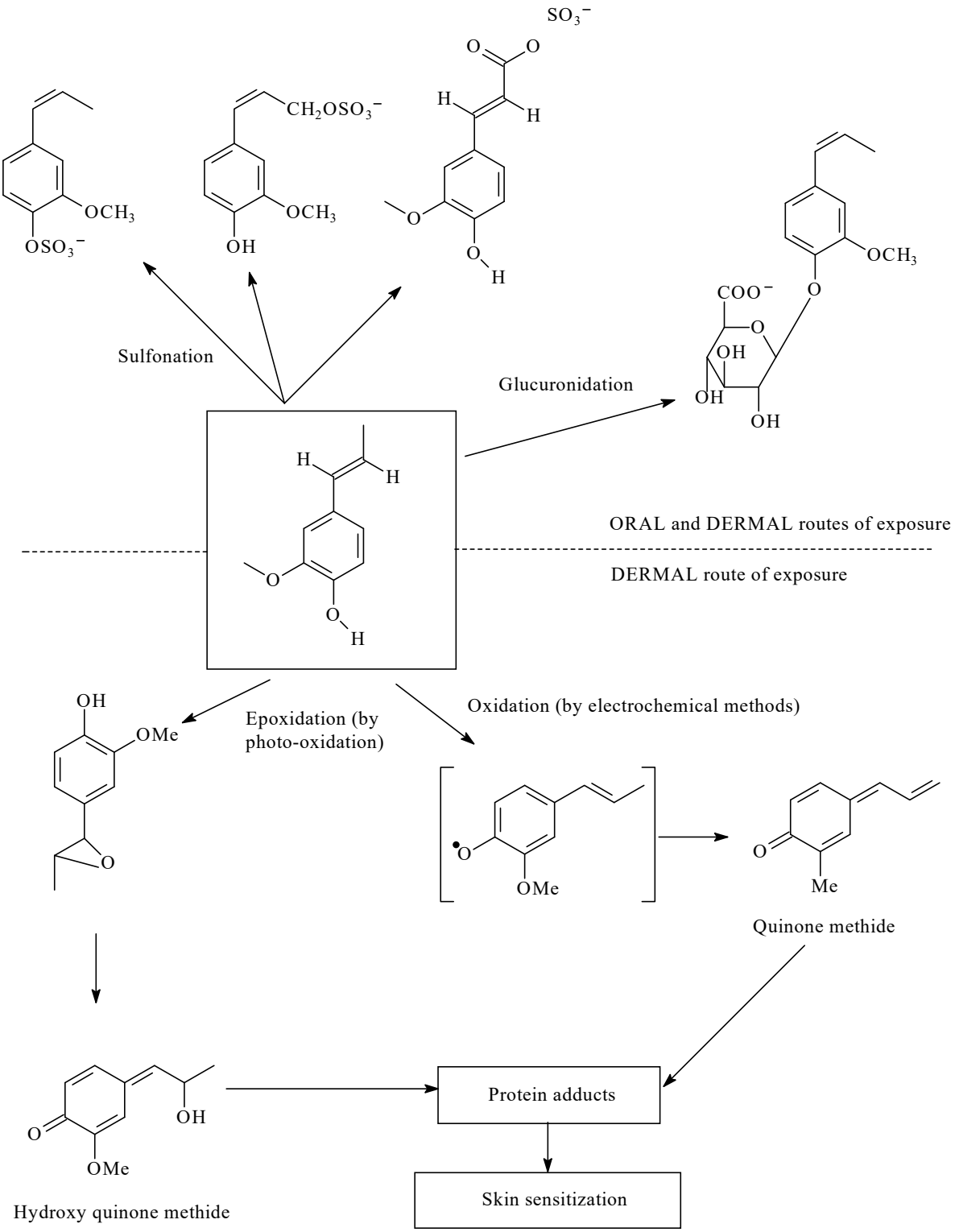
Liu and Hotchkiss (1998; see summary in Section 4.1.1(b) above for study details) used HPLC and GC-MS methods to analyse the urine of male F344 rats after occlusive dermal exposure to ^{14}C-labelled isoeugenol. All urinary metabolites were identified as sulfate or glucuronide conjugates; the sulfate ester of 4-hydroxy-3-methoxy-cinnamic acid (Fig. 4.1) was the predominant urinary metabolite. Of the limited amounts of radioactivity detected in the skin (e.g. 0.8 ± 0.2% of the applied dose), 33.2 ± 4.7% was attributable to the parent ^{14}C-labelled isoeugenol and 58.4 ± 1.9% to metabolites (none specifically identified).

Scholes et al. (1994) examined the effects of CYP modulation on the skin sensitization potency of isoeugenol in a series of studies using the mouse local lymph node assays (LLNA). In the LLNA studies, isoeugenol was co-administered with five CYP1A modulators (i.e. benzo[*a*]pyrene, β-naphthoflavone, and 3-methylcholanthrene were used as inducers, and α-naphthoflavone and clotrimazole were used as inhibitors). However, only the results of co-exposure with clotrimazole could be interpreted, since the other modulators were found to be potent skin sensitizers when administered independently. When co-administered with clotrimazole (an inhibitor of CYP1A activity), the sensitization potency of isoeugenol was increased substantially (~5-fold), suggesting that CYP1A metabolism of isoeugenol decreases its reactivity. [The Working Group noted that the identity of the test material was not provided in the publication.]

4.2 Evidence relevant to key characteristics of carcinogens

This section reviews the mechanistic data for the key characteristics of carcinogens (Smith et al., 2016) encompassed by isoeugenol. Evidence, mostly from studies in experimental systems, was available on whether isoeugenol exhibits the key characteristics "is electrophilic or can be metabolically activated to an electrophile", "is genotoxic", "induces oxidative stress", "induces chronic inflammation", "modulates

Fig. 4.1 Proposed metabolic pathway for isoeugenol after oral and dermal exposure



Adapted from Badger et al. (2002) and Ahn et al. (2023).

receptor-mediated effects", and "alters cell proliferation, cell death, or nutrient supply". The evidence for "alters DNA repair and/or causes genomic instability" was sparse. No data were available for the evaluation of other key characteristics of carcinogens. The exposure assessment for the mechanistic study by Sieben et al. (2001) is reported in Section 1.6 and in Supplementary Table S1.4 (Annex 1, Supplementary material for Section 1, Exposure Characterization, online only, available from: https://publications.iarc.who.int/627).

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

No studies relevant to electrophilicity in humans exposed to isoeugenol were available to the Working Group.

(a) Experimental systems

(i) Formation of DNA adducts

Ishii et al. (2023) investigated the formation of DNA adducts in the liver in male and female B6C3F$_1$ *gpt* delta mice treated with isoeugenol by gavage at doses of 0 (corn oil vehicle only), 150, 300, or 600 mg/kg bw per day for 13 weeks. Another group received estragole as a positive control. Using an adductome approach, the authors analysed DNA by LC-MS (Table 4.1). No specific adducts were detected in isoeugenol-treated mice, and the adductome maps were indistinguishable from those of control mice, whereas the estragole-treated mice showed distinct adducts that were attributable to treatment with this genotoxic carcinogen.

In a turkey egg genotoxicity assay, isoeugenol did not form DNA adducts (as measured by ^{32}P-postlabelling analysis) in the liver of fetuses harvested from White turkey eggs (containing fetuses aged 22–24 days) injected with isoeugenol at doses of 1 or 4 mg/egg per day for 3 days (Kobets et al., 2016) (see also Table 4.2 in Section 4.2.2(b)). [The Working Group noted that, in the same study, methyleugenol gave rise to DNA adducts (see the monograph on methyleugenol in the present volume).]

(ii) Metabolic activation in the skin

Melles et al. (2013) used electrochemical methods, LC, and MS to detect reactive species that may be responsible for the skin sensitization properties (i.e. haptenation) of isoeugenol. The authors used an amperometric thin-layer cell with boron-doped diamond working electrodes and palladium/hydrogen (Pd/H$_2$) as a reference electrode to generate oxidation products from isoeugenol. The oxidation products were then mixed with and without glutathione (GSH) before LC-MS detection. Similarly, to detect protein adducts, β-lactoglobulin A was mixed with the oxidation products of isoeugenol before LC-MS detection. The most abundant oxidation products detected were probably formed via hydroxylation at the aromatic moiety or at the double bond in the side chain. Other products that probably involved demethylation were also detected. After the introduction of GSH, multiple adducts were formed between GSH and isoeugenol oxidation products, including an O-demethylation product. Similarly, three different protein adducts were observed after the introduction of β-lactoglobulin A. [The Working Group noted that these reactive intermediates may also react with other biological matrices, including DNA.] On the basis of the mass spectral data, Melles et al. (2013) proposed an oxidation pathway for dermally administered isoeugenol that included generation of reactive intermediates such as quinone or quinone methide.

Ahn et al. (2020) proposed that isoeugenol is susceptible to abiotic activation (e.g. via photoinduced oxidative conditions) through rapid oxidation to form dimeric 7,4′-oxyneolignan, which is capable of adduct formation with proteins. Subsequently, using kinetic nuclear magnetic resonance (NMR) experiments and in vitro methods (i.e. direct peptide reactivity assay,

Table 4.1 End-points relevant to genotoxicity and related effects in non-human mammals in vivo exposed to isoeugenol

End-point	Species, strain (sex)	Tissue, cell	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA adducts, LC-MS/MS	Mouse, B6C3F$_1$ *gpt* delta (M, F),	Liver	–	600 mg/kg bw	Gavage for 13 wk: dose range: 150–600 mg/kg bw per day		Ishii et al. (2023)
gpt mutation frequency	Mouse, B6C3F$_1$ *gpt* delta (M, F)	Liver	–	600 mg/kg bw			
Micronucleus formation	Mouse, B6C3F$_1$ (M)	Peripheral blood; normochromatic erythrocytes	–	600 mg/kg bw	Gavage, for 3 mo; dose range, 37.5–600 mg/kg bw per day		NTP (2010)
	Mouse, B6C3F$_1$ (F)	Peripheral blood; normochromatic erythrocytes	+	600 mg/kg bw		Increase of 3.2-fold, with a significant trend, at the highest dose only	

F, female; HID, highest ineffective dose; LED, lowest effective dose; LC-MS/MS, liquid chromatography-tandem mass spectrometry; M, male; mo, month(s); wk, week(s).
[a] +, positive; –, negative.

Table 4.2 End-points relevant to genotoxicity and related effects in non-human mammalian cells in vitro exposed to isoeugenol

End-point	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Unscheduled DNA synthesis	Rat, F344, primary hepatocytes	–	NT	Up to nearly 1 mM	Exact concentrations not stated (displayed graphically with a log scale)	Burkey et al. (2000)
	Mouse, B6C3F$_1$, primary hepatocytes	–	NT			
Chromosomal aberration	Chinese hamster, ovary cells	–	–	200 µg/mL –S9 170 µg/mL +S9		NTP (2010)

HIC, highest ineffective concentration; LEC, lowest effective concentration, NT, not tested; S9, 9000 × *g* supernatant.
[a] –, negative.

KeratinoSens, and a human cell line activation assay), Ahn et al. (2023) further characterized the haptenation pathway of isoeugenol, proposing that a short-lived isoeugenol epoxide might be formed abiotically or enzymatically, followed by isomerization to a hydroxy quinone methide.

(iii) QSAR models

Dimitrov et al. (2005) developed a quantitative structure–activity relationship (QSAR) that incorporates a metabolic simulator designed to mimic enzyme activation (phase I and II reactions) in the skin. On the basis of QSAR predictions, Dimitrov et al. (2005) showed that that isoeugenol undergoes demethylation, followed by oxidation to *o*-quinone, or formation of semiquinone free radicals, or sulfate or glucuronide conjugation. [The Working Group noted that the reactive metabolites of isoeugenol may form quinone methide–protein adducts, which are ultimately responsible for dermal sensitization (Bertrand et al., 1997) and that DNA could similarly be susceptible to electrophilic attack.]

The action of isoeugenol as a skin sensitizer involves covalent modification of skin proteins in the form of isoeugenol–protein adducts, and this reaction in KeratinoSens reporter cells is not dependent on metabolic activation by microsomes from S9 (9000 × *g* supernatant) but is probably the result of spontaneous oxidation (Natsch and Haupt, 2013). Melles et al. (2013) showed that eugenol and isoeugenol form quinones and quinone methides electrochemically and that they are electrophilic and react in an acellular system with thiol groups in proteins. Isoeugenol formed a larger variety of adducts with GSH and proteins than did eugenol. In the acellular study, Ahn et al. (2020) demonstrated that an electrophilic species, a dimeric 7,4′-oxyneolignan, resulted from photo-oxidation of isoeugenol and that it bound to thiol groups. [The Working Group noted that although these studies demonstrated that electrophiles are derived from isoeugenol, these are mainly linked to the skin-sensitizing properties of isoeugenol, resulting in covalent reaction with proteins and subsequent protein modification. Electrophiles were generated either photochemically or electrochemically in acellular systems. In one study in turkey embryos and another in mouse liver, isoeugenol–DNA adducts were not detected.]

4.2.2 Is genotoxic

(a) Humans

(i) Exposed humans

No studies relevant to genotoxicity in humans exposed to isoeugenol were available to the Working Group.

(ii) Human cells in vitro

One study in humans cells in vitro was available to the Working Group. Isoeugenol was identified as a component of the semi-volatile particulate fraction of cigarette smoke and tested for the induction of sister-chromatid exchange (SCE) in human lymphocytes (Jansson et al., 1986). Isoeugenol was found to induce SCE ($P < 0.05$) at concentrations of 0.25 and 0.5 mM. Similar findings were reported in a follow-up report investigating a larger number of cigarette-smoke condensate components (Jansson et al., 1988). [The Working Group noted that the purity of the material tested in these studies ranged between 93.4% and 95%.] [The Working Group also noted that the genotoxicity research community now considers results from the SCE assay to be of less relevance than other currently available genotoxicity tests.]

(b) Experimental systems

See Table 4.1 and Table 4.2.

(i) Non-human mammals in vivo

The frequency of micronucleated erythrocytes did not increase in peripheral blood samples from male B6C3F$_1$ mice after exposure to isoeugenol by gavage at doses ranging from

37.5 to 600 mg/kg bw for 3 months. However, an increase of 3.2-fold in the frequency of micronucleated normochromatic erythrocytes and a significant trend was observed in female mice exposed to isoeugenol at 600 mg/kg bw (NTP, 2010). [The Working Group noted that the highest frequency of micronucleated normochromatic erythrocytes in female mice was similar to that in male mice at the lowest dose.]

Ishii et al. (2023) investigated mutation frequency in the liver of male and female B6C3F$_1$ *gpt* delta mice treated with isoeugenol by gavage at doses of 150, 300, or 600 mg/kg bw per day for 13 weeks. Corn oil was used as the vehicle control. Another group received estragole as a positive control. The *gpt* mutation frequency in isoeugenol-treated mice was not increased above the levels in control mice, whereas there was a significant increase in mutation frequency with the positive control, estragole.

(ii) Non-human mammalian cells in vitro

Isoeugenol at concentrations of up to 200 µg/mL in the cell medium did not induce chromosomal aberrations in cultured Chinese hamster ovary cells, with or without S9 activation (NTP, 2010).]

(iii) Non-mammalian experimental systems

See Table 4.3.

In a turkey egg genotoxicity assay, isoeugenol did not cause DNA strand breaks (measured by comet assay) in the liver of fetuses harvested from White turkey eggs (containing fetuses aged 22–24 days) injected with isoeugenol at doses of 1 or 4 mg/egg per day for 3 days (Kobets et al., 2016) (Kobets et al., 2016). [The Working Group noted that, in the same study, methyleugenol (see the monograph on methyleugenol in the present volume) induced DNA strand breakage.]

Isoeugenol gave negative results in the *Drosophila melanogaster* wing spot somatic mutation and recombination test (SMART) at concentrations of up to 25 mM (Munerato et al., 2005).

In an early study, isoeugenol did not induce an increase in mutation frequency when tested at a dose range of up to 600 µg/plate in *E. coli* WP2 *uvrA* and in *S. typhimurium* strains TA100, TA1535, TA98, TA1537, and TA1538 in the presence or absence of exogenous (S9) metabolic activation (Sekizawa and Shibamoto, 1982).

Similarly, isoeugenol gave negative results for mutagenicity when tested at higher dose range (3–2000 µg/plate) in two independent assays in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 *uvrA* in the presence or absence of exogenous (S9) metabolic activation (NTP, 2010). [The Working Group noted that the highest doses tested were limited by the cytoxicity of isoeugenol.]

When tested in the *Bacillus subtilis* DNA repair test (rec assay) in the absence of S9, isoeugenol gave positive results (concentration, 0.8 mg/disk), with preferential killing of rec$^-$ cells over rec$^+$ cells (Sekizawa and Shibamoto, 1982).

4.2.3 Alters DNA repair or causes genomic instability

(a) Humans

No studies relevant to alteration of DNA repair and/or genomic instability in humans or human cells exposed to isoeugenol were available to the Working Group.

(b) Experimental systems

Isoeugenol did not induce unscheduled DNA synthesis at concentrations of nearly 1 mM in rat or mice hepatocytes (Burkey et al., 2000). [The Working Group noted that the highest concentration was reported inexactly because the graph showing results had a log scale on the *x*-axis.]

Isoeugenol was reported to enhance the cytotoxicity of camptothecin (an inhibitor of topoisomerase 1, TOP1) and etoposide (an inhibitor of TOP2) through a mechanism involving

Table 4.3 End-points relevant to genotoxicity and related effects in non-mammalian experimental systems exposed to isoeugenol

Test system (species, strain)	End-point (assay)	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Drosophila melanogaster	Somatic mutation and recombination test (SMART)	–	NA	25 mM		Munerato et al. (2005)
Salmonella typhimurium, TA98, TA100, TA1535, TA1537, TA1538	Mutation	–	–	600 µg/plate		Sekizawa and Shibamoto (1982)
Salmonella typhimurium, TA98, TA100, TA1535, TA1537	Mutation	–	–	333 µg/plate: TA1535, TA1537. 1500 µg/plate: TA98, TA100	Isoeugenol was toxic at higher doses	NTP (2010)
Escherichia coli, WP2 *uvrA*	Mutation	–	–	600 µg/plate	Isoeugenol was toxic at higher doses	Sekizawa and Shibamoto (1982)
Escherichia coli, WP2 *uvrA*/pKM101	Mutation	–	–	1000 µg/plate	Isoeugenol was toxic at higher doses	NTP (2010)
Bacillus subtilis, rec H17 and M45 strains	DNA damage, (Rec assay)	+	NR	0.8 mg/disk	Preferential killing of rec^- cells over rec^+	Sekizawa and Shibamoto (1982)
Turkey fetus	DNA adducts (^{32}P-postlabelling)	–	NR	4 mg/egg	Fertilized eggs treated on days 22–24	Kobets et al. (2016)
Turkey fetus	DNA strand breaks (comet assay)	–	NR	4 mg/egg	Fertilized eggs treated on days 22–24	Kobets et al. (2016)

HIC, highest ineffective concentration; LEC, lowest effective concentration, NR, not reported.

[a] +, positive; –, negative.

inhibition of tyrosyl DNA phosphodiesterase 2 (TDP2) catalytic activity. Isoeugenol itself displayed inhibitory activity towards TDP2 but not TDP1 (Elsayed et al., 2016). [The Working Group considered that this study was not particularly informative.]

4.2.4 Induces oxidative stress

See Table 4.4.

(a) Humans

(i) Exposed humans

No studies relevant to oxidative damage in humans exposed to isoeugenol were available to the Working Group.

(ii) Human cells in vitro

In a series of experiments in primary human gingival fibroblasts and in a cell line from a human submandibular adenocarcinoma, an increase in 5-(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFH-DA) fluorescence, assumed to be caused by reaction with intracellular reactive oxygen species (ROS), was detected with isoeugenol only at concentrations of 500 and 1000 µM. Extensive cytotoxicity (measured as a reduction in metabolic activity) was observed at concentrations greater than 100 µM. No increase in ROS was observed at isoeugenol concentrations of 100 µM or lower. The fluorescence observed with isoeugenol at 1000 µM was comparable to that observed after a parallel exposure to 10 µM H_2O_2. Exposure to isoeugenol at 1000 µM also induced a significant depletion in intracellular GSH (Atsumi et al., 2000, 2005, 2006; Fujisawa et al., 2004). Although no increase in ROS was observed after exposure to isoeugenol at 10 µM, isoeugenol at 5 µM had a synergistic effect on the induction of ROS by visible light and H_2O_2 (Atsumi et al., 2005).

[The Working Group noted that although no cytotoxicity was observed when the cells were treated with visible light, H_2O_2, or 5 µM isoeugenol alone, the cytotoxicity of the combined treatments was not evaluated. Nonetheless, a synergistic ROS induction by isoeugenol and visible light, if properly documented, might be relevant to the skin sensitization findings discussed in Section 4.2.1.]

(b) Experimental systems

Non-human mammals in vivo

See Table 4.5.

Exposure of male Sprague-Dawley rats to isoeugenol at an intraperitoneal dose of 10 mg/kg bw per day for 14 days did not induce consistent membrane lipid peroxidation or cellular responses to oxidative damage as measured by enzyme activities of superoxide dismutase or catalase or glutathione peroxidase in brain, liver, kidney, or heart tissues isolated 5 days after the end of the exposure. Levels of lipid peroxidation and the oxidized form of GSH were decreased in the liver only (Rauscher et al., 2001).

(c) Antioxidant effects

Regarding the role of isoeugenol in oxidative stress, the Working Group identified several studies investigating the potential of isoeugenol to act as an antioxidant.

Rajakumar and Rao reported that the incubation of rat brain homogenates (ex vivo) with isoeugenol reduced the baseline level of membrane lipid peroxidation. The effect was significantly less potent than that reported for equimolar amounts of butylated hydroxytoluene (Rajakumar and Rao, 1993). Isoeugenol solutions were shown to have antioxidant activity comparable to that of well-characterized antioxidants such as α-tocopherol, butylated hydroxytoluene, and butylated hydroxyanisol in the few experiments in which they were used as positive controls (Tominaga et al., 2005; Kadoma et al., 2006; Fındık et al., 2011).

Exposure to lipopolysaccharide (LPS) induced nitric oxide release in the rodent

Table 4.4 End-points relevant to oxidative stress in human cells in vitro exposed to isoeugenol

End-points	Assays	Tissue, cell line	Results[a]	Concentration (LEC or HIC)	Comments	Reference
ROS	CDFH-DA	Human submandibular gland adenocarcinoma cell line (HSG)	No change	1000 µM	Not clear how the authors went from a concentration–response curve (1, 10, 100, 1000 µM) to a single number	Fujisawa et al. (2004)
ROS	ESR	Human submandibular gland adenocarcinoma cell line (HSG)	No change	100 000 µM	Measurement of phenoxyl radicals; dose range, 10 µM to 100 mM	Atsumi et al. (2000)
ROS	CDFH-DA	Human submandibular gland adenocarcinoma cell line (HSG)	↑	500 µM	Substantial cytotoxicity observed with isoeugenol at 100 and 1000 µM	Atsumi et al. (2005)
ROS			↑	5 µM isoeugenol + H_2O_2 (100 µM)/HRP (1 µg/mL)		
ROS			↑	5 µM isoeugenol + visible light		
GSH	Thiols by fluorimetry		↓	1000 µM		
ROS	CDFH-DA	Primary human gingival fibroblasts (HGF) and submandibular gland adenocarcinoma cell line (HSG)	No change	20 µM	Single dose at non-toxic concentration	Atsumi et al. (2006)

CDFH-DA, 5- (and 6-)-carboxy-2′,7′-dichlorofluorescein diacetate; ESR, electron spin resonance spectroscopy; H_2O_2, hydrogen peroxide; HIC, highest ineffective concentration; HRP, horseradish peroxidase; LEC, lowest effective concentration; ROS, reactive oxygen species.

[a] ↓, decrease; ↑, increase.

Table 4.5 End-points relevant to oxidative stress in non-human mammals in vivo exposed to isoeugenol

End-point	Assay	Species, strain (sex) Tissue, cells	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Reference
Lipid peroxidation	TBARS assay	Rats, Sprague-Dawley (M) Liver, kidney, brain, and heart	↓ TBARS in heart and liver	10 mg/kg bw per day	Intraperitoneal, for 14 days	Rauscher et al. (2001)
CAT, SOD, GPX or GSR	Enzyme activity		No change in CAT, SOD, GPX and GSR in all tissues			
GSH/GSSG	Protein level		↓ GSSG in liver No change in GSH in all tissues			

bw, body weight; CAT, catalase; GPX, glutathione peroxidase; GSR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; HID, highest ineffective dose; LED, lowest effective dose (units as reported); M, male; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

[a] ↑, increase; ↓, decrease;.

macrophage-like cell line RAW 264.7 through increases in the transcription and expression of nitric oxide synthase (iNOS). In cells co-exposed to LPS and isoeugenol, the transcription and expression of iNOS was significantly reduced, although there were inconsistent results from mechanistic experiments examining the time-course of the responses and the role of transcription factors (e.g. nuclear factor kappa B, NF-κB) known to regulate the response (Choi et al., 2007; Yeh et al., 2011; Murakami et al., 2017) (see Section 4.2.5).

Co-exposure of rat brain tissue (ex vivo) to isoeugenol and acrylamide (Prasad and Muralidhara, 2013), or of rat kidney tissue to isoeugenol and cisplatin (Rao et al., 1999), reduced the lipid peroxidation associated with those agents. In addition, isoeugenol exposure was reported to inhibit lipid peroxidation induced by hydroxy radicals generated by a Fenton reaction system in rat liver microsomes (Taira et al., 1992); however, the mechanism by which isoeugenol interfered with those agents was not established.

Isoeugenol did not protect zebrafish larvae from H_2O_2 toxicity. Isoeugenol protected zebrafish from arsenate toxicity (Endo et al., 2020). [The Working Group noted that it was not clear whether this effect was due to antioxidant activity since arsenate has multiple toxicological modes of action.]

Several groups studied the antioxidant potential of isoeugenol in aqueous and non-aqueous solutions. Although most of the experiments in solution used nonspecific radical sources like 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) or 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Fındık et al., 2011; Prasad and Muralidhara, 2013; Zuo et al., 2018), others had more specific antioxidant activities, such as trapping superoxide or hydroxyl radical or reduction of ferric compounds (Fındık et al., 2011; Zuo et al., 2018). A series of experiments examined the free radical-scavenging activity of isoeugenol by evaluating the kinetics of radical-initiated polymerization of methylmethacrylate in a non-aqueous system (Fujisawa et al., 2004; Fujisawa and Kadoma, 2006; Kadoma et al., 2006).

[The Working Group considered that the data available for review did not provide evidence that the antioxidant activity of isoeugenol ameliorated cellular or tissue oxidative damage.]

4.2.5 Induces chronic inflammation

(a) Humans

(i) Exposed humans

One study investigated the T-cell response to a mixture of eight fragrances, including isoeugenol, or to isoeugenol only using a skin patch test. Thirty-two fragrance-allergic patients were tested with all eight individual constituents of the mixture, including isoeugenol, using the patch test 3–4 weeks after an initial positive skin test. Positive allergic responses were observed after exposure to isoeugenol in 28.6% of patients (Sieben et al., 2001). After a second skin patch test, the peripheral blood from patients who had a positive allergic response to isoeugenol was taken within 1–2 weeks, and the peripheral blood mononuclear cells were isolated for other tests in vitro. [The Working Group considered this finding to be not highly informative because it examined allergic reactions other than T-cell responses in exposed humans. In addition, the isoeugenol concentration was not reported.]

(ii) Human primary cells

In the same study described above, Sieben et al. investigated the responses of peripheral blood mononuclear cells and T cells from skin lesions of fragrance-allergic patients to isoeugenol exposure. Consistent with findings from the patch test, Sieben et al. (2001) reported a significant increase in lymphocyte transformation, measured as incorporation of [^{3}H]thymidine in the lymphocyte transformation test (LTT), and

the LTT simulation index ranged from 3.2 to 6.4 after exposure to isoeugenol (concentration range, 0.1–100 μg/mL). The authors further investigated the ability of antigen-modified human liver microsomes to stimulate T lymphocytes in the presence of the metabolizing enzyme recombinant CYP1A1 (20 μg/mL) and reported that the T-cell response to isoeugenol was increased in the presence of this enzyme (Sieben et al., 2001) (see Section 4.2.6). [The Working Group considered this finding not very informative to the key characteristic of chronic inflammation since skin sensitization is a local inflammatory process.]

Banerjee et al. measured allergen-induced T-cell proliferation. Concentration-dependent increases in interleukins IL-1α and IL-1β were observed in mononuclear cells derived from human peripheral blood samples exposed to isoeugenol at 0.1–10 ppm (Banerjee et al., 2003). IL-8 release was also stimulated by exposure to isoeugenol (500 μM) in human primary neutrophils, but not in monocytic leukaemia THP-1 cells, in a study that aimed to compare the metabolic activity of the two cell types (Kiorpelidou et al., 2012).

(iii) Human cells in vitro

Levels of IL-8 mRNA levels in the skin were transiently elevated in in vitro models of reconstructed human epidermis exposed to isoeugenol at 3 mg/mL for 4 hours (Frankart et al., 2012) and in human monocyte-derived dendritic cells exposed to isoeugenol at 200 μM for 30 hours (Skazik et al., 2011). In human monocytic leukaemia THP-1 cells, exposure to isoeugenol at 75 μg/mL induced a significant increase in IL-8 mRNA expression (Mitjans et al., 2010); however, the increase in IL-8 secretion observed with isoeugenol at 100 μg/mL was not statistically significant (Mitjans et al., 2010; Galbiati et al., 2012) [The Working Group noted that these models are commonly employed to assess the sensitizing potential of contact allergens.]

Potential proliferative effects and the immune-mediated response were evaluated in normal human keratinocytes (NSK) from neonatal foreskin after treatment with various skin sensitizers and allergens, including isoeugenol. Levels of IL-8 increased after treatment with isoeugenol at concentrations of up to 2 μM (test range, 0.4, 2, 10, or 50 μM) for 24 hours (Bae et al., 2015).

A concentration-dependent increase in IL-18 mRNA was reported in a human keratinocyte cell line (NCTC254) exposed to isoeugenol at 37.5–150 μg/mL for 24 hours (Galbiati et al., 2011).

Furthermore, the p38MAPK pathway, a key regulator of pro-inflammatory cytokine biosynthesis, was shown to be activated in THP-1 cells 15 minutes after isoeugenol treatment at concentrations ranging from 50 to 100 μg/mL (Mitjans et al., 2010). CD1-mediated T-cell activation, indicated by increases in interferon gamma (IFNγ), a pro-inflammatory cytokine, was demonstrated in THP-1 cells treated with isoeugenol at 250 μM (Betts et al., 2017).

Other studies showed an increase in the expression of the cell surface markers cyclins CD86 and CD54 in co-cultures of THP-1 cells and keratinocytes exposed to isoeugenol, as detected by flow cytometry (Hennen et al., 2011; Cao et al., 2012). Consistent upregulation of the expression of cyclins CD86, CD54, and CD40 was also observed in THP-1 cells exposed to isoeugenol in the presence of Aroclor-induced rat liver microsomes (S9) (Chipinda et al., 2011). The expression of CD86 was also confirmed in another study in THP-1 cells, without effects on IL-8 cytokine release (Galbiati et al., 2012). A slight upregulation in the expression of CD83 and elevated expression of CCL27 and TLR2 (toll-like receptor 2) were observed in immature dendritic cells (iDC), and a concentration-dependent upregulation of CD86 was observed in dendritic cell-related cells (DCrc) (Cluzel-Tailhardat et al., 2007; Schreiner et al., 2008).

Some studies reported different or negative results. Lee et al. (2014) did not observe any significant induction of IL-6 or IL-8 release in reconstructed human full-thickness skin with incorporated in vitro-generated immature MUTZ-3-derived Langerhans-like cells (MUTZ-LCs) after exposure to isoeugenol (Lee et al., 2014). No effects were observed in monocyte-derived LC-like cells (Bock et al., 2018).

The reconstructed human full-thickness skin model was employed in another study in which the expression of cell surface markers CD54 and CD86 was evaluated for dendritic cell maturation. Isoeugenol induced a very mild non-significant increase in CD86 and CD54 (Lee et al., 2014).

Guironnet et al. (2000) reported that exposure to isoeugenol did not induce an allergen-specific T-cell response in monocyte-derived dendritic cells (Guironnet et al., 2000).

While assessing the accuracy of a novel in vitro sensitization test, the IL-8 Luc assay, Takahashi et al. (2011) reported inconsistent results when testing isoeugenol, which was ultimately classified as a non-sensitizer.

[The Working Group considered that it was difficult to assess the evidence for chronic inflammation on the basis of results from the above in vitro systems, given the short duration of such assays.]

(b) Experimental systems

(i) Non-human mammals in vivo

Isoeugenol (purity, > 99%) was administered by gavage at doses ranging from 75 to 300 mg/kg bw in a 3-month study and a 2-year study in F344/N rats and B6C3F$_1$ mice exposed under similar conditions (NTP, 2010) (see also Section 3.1). In rats, the incidence of minimal to mild olfactory epithelial degeneration was observed in both sexes at both times of exposure. In mice, a dose-dependent increase in atrophy of the olfactory epithelium was also observed. Additionally, the incidence of forestomach inflammation and ulceration (males only) was significantly increased at the highest dose in the 2-year study. [The Working Group noted that the atrophy observed in the olfactory epithelium could be the result of chronic olfactory inflammation (LaFever et al., 2022).]

In another study, oral exposure of adjuvant-induced arthritic male Wistar rats to isoeugenol at a dose of 10 or 25 mg/kg bw per day for 14 days induced a significant and dose-dependent reduction in the production of prostaglandin E$_2$ (PGE2) and nitric oxide, as well as histological evidence of joint inflammation and leukocyte infiltration, observed 10 days after the end of the exposure (Kaur and Sultana, 2012).

In a series of assays for skin irritation and skin sensitization (the mouse ear swelling test, MEST; in vivo and ex vivo LNNA), repeated dermal exposure to isoeugenol at doses ranging from 0.4% to 50% w/v in acetone:olive oil (4:1) solution applied to the dorsum of the ears of CBA or Balb/c mice induced a consistent significant increase in ear thickness (Garrigue et al., 1994; NTP, 2010, 2020; Bonefeld et al., 2011; Arancioglu et al., 2015).

Topical exposure to isoeugenol (at repeated doses ranging from 1% to 10% w/v) applied to the dorsum of both ears of female CBA/J mice for 3 days in the LLNA induced a proliferative response in the draining lymph node cells (Gerberick et al., 2002) and an increase in the percentage of cells expressing the B-cell marker B220 (Jung et al., 2012). [The Working Group noted that the B220 cell marker is considered to be a secondary end-point for cell proliferation in the LLNA.]

In the same study, Jung et al. (2012) also observed a significant increase in cytokine production (IL-2, IL-6, IFNγ, and TNFα) and an increase, although not significant, in the production of two other proteins involved in the inflammatory response – granulocyte-macrophage colony-stimulating factor (GM-CSF) and monocyte

chemoattractant protein-1 (MCP-1) (Jung et al., 2012). The increase in the production of IFNγ (as previously observed in isolated lymph node cells of mice exposed to various allergens, including isoeugenol at 10%, 25%, and 50% w/v), was considered to be a characteristic profile of the stimulation of a subpopulation of T-helper (Th1) cells (Dearman et al., 1997).

(ii) Non-human mammalian systems in vitro

The effects of exposure to isoeugenol were also investigated in various cell types in vitro. Isoeugenol induced a decrease in iNOS protein expression, as well as significant attenuation of cytokines IL-1b and TNFα in LPS-stimulated murine macrophages (Yeh et al., 2011). Isoeugenol was also shown to suppress, in a concentration-dependent manner, the concanavalin A (ConA)-initiated-lymphoproliferation of $B6C3F_1$ mouse splenocyte cultures and to reduce the production of IL-2 in mice splenocytes and ELA4.IL-2 mouse T cells activated with phorbol 12-myristate 13-acetate (PMA) or ionomycin. The authors also suggested that the effect was mediated by indirect downregulation of the transcription factors nuclear factor of activated T cells (NFAT) and NF-κB (Park et al., 2007). Similar results were obtained in a more recent study in which exposure to isoeugenol inhibited NF-κB-dependent transcriptional activity and DNA-binding activity, as well as signalling upstream of NF-κB activation (e.g. degradation of I-κBα, NF-κB inhibitor alpha) in LPS-stimulated RAW 264.7 cells (Choi et al., 2017). Increases in IL-1α and macrophage inflammatory protein-2 (MIP-2) were observed in murine epidermal keratinocyte cells (HEL-30) exposed to isoeugenol at a concentration range of 1 mL of 0.01, 0.1, 0.5, and 1 × CV_{75} concentration (CV_{75}, concentration that induces 75% of cell viability, which was 318.1 ± 161.2 μg/mL), although only the lowest dose of isoeugenol elicited a significant response in MIP-2 levels relative to the controls (Son et al., 2013).

Murakami et al. (2017) reported that exposure of RAW 264.7 murine macrophages to isoeugenol at concentrations of 0.1 or 1000 μM for 3.5 hours upregulated the expression of nitric oxide synthase 2 (Nos2) and cyclooxygenase 2 (Cox-2) mRNA at the higher concentration, but not the mRNA expression of haem oxygenase 1 (HO-1, encoding an oxidative stress-responsive protein with a key role in tissue resolution). In LPS-stimulated RAW 264.7 cells, isoeugenol at 40 μM (but not at 10 μM) was shown to inhibit Cox-2 mRNA expression (Murakami et al., 2017), although the same authors previously reported that isoeugenol did not inhibit Cox-2 expression or NF-κβ activation in LPS-stimulated macrophages (Murakami et al., 2005). [The Working Group noted that this study was of limited relevance because the measurement of mRNA expression alone is not sufficiently informative to determine whether isoeugenol elicited an inflammatory response in this cell system.]

4.2.6 Modulates receptor-mediated effects

(a) Humans

(i) Exposed humans

No studies relevant to the modulation of receptor-mediated effects in humans exposed to isoeugenol were available to the Working Group.

(ii) Human cells in vitro

Three studies in human cell lines investigated the aryl hydrocarbon receptor (AhR)-mediated effects of isoeugenol exposure and reported positive results (Kalmes et al., 2006; McKim et al., 2010; Kalmes and Blömeke, 2012). Using human immortalized keratinocytes (HaCaT) and a HaCaT-AhR-knock-down cell variant (siAhR HaCaT), Kalmes et al. (2006) demonstrated that isoeugenol (at 60–300 μM) may induce cell cycle arrest (a concentration-dependent increase in arrested cells) by transporting AhR into the nucleus. In a subsequent study, Kalmes and Blömeke (2012) provided corroborative evidence

that isoeugenol exposure (at 300 µM) caused the rapid transport of AhR into the nucleus, induced the expression of AhR-target genes *CYP1A1* and *AHRR* (aryl hydrocarbon receptor repressor) and inhibited the proliferation of HaCaT cells. Isoeugenol also reduced levels of the G1-phase cell cycle-related proteins retinoblastoma (RB, known to interact with AhR) and cyclin-dependent kinase 6 (CDK6) (Kalmes and Blömeke, 2012). In both studies by Kalmes and colleagues, these effects were seen only with isoeugenol at doses up to 300 µM because cytotoxicity (observed as reduced cell viability) was observed at the higher dose (600 µM) tested. Moreover, McKim et al. (2010) reported that isoeugenol (100 and 250 µM) increased CYP1A1 induction and gene expression in HaCaT keratinocytes in a concentration-dependent manner (McKim et al., 2010).

One study employed two regulatory in vitro tests for effects on the androgen receptor (AR): the androgen receptor AR-EcoScreen™ Androgen Receptor TransActivation Assay (AR TA) and the 22Rv1/MMTV_GRKO AR TA assay (described in Organisation for Economic Co-operation and Development, OECD Test Guideline No. 458).

In the AR-EcoScreen™ AR TA assay, the Dual-Glo luciferase assay system was used to detect AR agonist and antagonist activity, including cytoxicity. 5α-Dihydrotestosterone (DHT) at 10 nM and 500 pM was used as the positive standard for the assessment of agonist and antagonist activity, respectively. In the 22Rv1/MMTV_GR-KO AR TA assay, cells were incubated in hormone-deprived conditions, followed by incubation with isoeugenol in the absence (AR agonist screening) or presence (AR antagonist screening) of DHT. The Steady-Glo luciferase assay system was used to measure luciferase activity, using as a positive control 10 nM DHT in the AR agonist assay format, and 800 pM DHT + bicalutamide in the AR antagonist assay format. Isoeugenol exhibited AR antagonism in both assays, although this antagonist activity was decreased significantly in the presence of phase I and II enzymes from pooled rat liver S9 fraction (Park et al., 2021).

(b) Experimental systems

(i) Non-human mammals in vivo

Ishii et al. (2023) recently conducted a study of global gene expression in the liver of male and female B6C3F$_1$ *gpt* delta mice treated with isoeugenol at oral doses of 0, 150, 300, and 600 mg/kg bw per day for 13 weeks. Pathway analyses indicated that the peroxisome proliferator-activated receptor (PPAR) signalling pathway (both PPARα and PPARγ) was activated in the livers of male mice treated with isoeugenol. [The Working Group noted that only global expression data were presented in the paper, and the evidence was less clear in female mice.] This study also used quantitative polymerase chain reaction (qPCR) to confirm mRNA expression of some genes involved in the PPAR pathway, such as *Cd36, Cyp4a10, Cyp4a14*, and *Acadm*, in male mice at 150 mg/kg bw. In female mice, only *Cd36* mRNA expression was significantly increased. *Cth* gene expression significantly increased in male mice treated with isoeugenol at 600 mg/kg bw (Ishii et al., 2023).

(ii) Non-human mammalian cells in vitro

In an AR competitive binding assay performed in vitro with rat AR protein, isoeugenol was found to be a weak agonist on the basis of its reported relative binding affinity (RBA = 0.0015) relative to the median inhibitory concentration (IC_{50}) of the synthetic androgen R1881 (RBA = 1.00) (Fang et al., 2003). [The Working Group noted that the results from the binding affinity assay were unclear.] The capacity of isoeugenol to interact with the mouse olfactory receptor (mOR-EG) in mOR-EG-expressing HEK293 cells was reported in another study that documented different types of interaction according to the chemical state of the molecule (freshly purified versus stored isoeugenol)

(Oka et al., 2004). [The Working Group noted that mOR-EG is not a nuclear receptor, and no evidence was available on its relevance to carcinogenicity.]

4.2.7 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

(i) Exposed humans

No studies relevant to alterations in cell proliferation, cell death, or nutrient supply in humans exposed to isoeugenol were available to the Working Group.

(ii) Human cells in vitro

Ghosh et al. (2005) assessed the ability of isoeugenol to inhibit the proliferation of melanoma cells. Different cell types, each representing a different stage of disease progression, were tested: Sbcl2 cells, primary melanoma; WM3211 cells, primary radial growth phase; WM98-1, primary vertical growth phase; and WM1205Lu, metastatic melanoma. Cell growth was not inhibited by treatment with isoeugenol at 0.5, 2.5 or 5 μM for 72 hours. [The Working Group noted that no clear information on the origin of the isoeugenol used in the study was provided.]

Similarly, Pisano et al. (2007) evaluated the effects on cell growth in the melanoma cell lines WM266-4, SK-Mel-28, LCP-Mel, LCM-Mel, PNP-Mel, CN-MelA, 13443, and GR-Mel, which were incubated for up to 6 days in the presence of 100 μM isoeugenol. The cells were established as primary short-term cell cultures starting from tumour samples from donor patients diagnosed with melanoma. Treatment with isoeugenol did not inhibit cell growth [%] when compared with untreated cultures, as measured by colorimetric assay.

In a study conducted by Atsumi et al. (2000) in a human submandibular gland tumour cell line and primary human gingival fibroblast cells incubated for 48 hours with serial dilutions of isoeugenol in the medium (10^{-7} to 10^{-3} M), a concentration-dependent decrease in cell viability, significant at 10^{-4} M, was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. IC_{50} values were 26.8 μM and 72.6 μM in the submandibular tumour cell line and fibroblast cells, respectively. In addition, similar concentrations of isoeugenol induced DNA-synthesis inhibition, measured by CyQUANT cell proliferation assay kit, after 24 hours incubation in submandibular gland tumour cells.

Bae et al. (2015) investigated potential proliferative effects and the immune-mediated response in normal human keratinocytes (NSK) from neonatal foreskin after treatment with various skin sensitizers and allergens, including isoeugenol. However, isoeugenol treatment at 0.4, 2, 10, and 50 μM for 24 hours did not induce an increase in cell proliferation but significantly decreased cell viability to 7 ± 6% at 10 μM, as measured by the WST-1 assay. In addition, isoeugenol did not modify the production of vascular endothelium growth factor (VEGF) at any tested concentration, as measured by immunoassay in the supernatants from treated NSK cells compared with those from untreated control cells.

Frankart et al. (2012) investigated the effect of isoeugenol on mRNA expression of IL-8 and its release in a reconstructed human epidermis model. Topical exposure to isoeugenol at 3 mg/mL for 24 hours caused a transient expression of IL-8 mRNA associated with IL-8 release, which also correlated with transient activation of epidermal growth factor receptor (EGFR).

As described in Section 4.2.6(a)(ii), Kalmes et al. (2006) investigated whether AhR mediates the cell cycle arrest induced by isoeugenol in human keratinocytes (HaCat cell line). Cells were incubated with isoeugenol (300 μM) in the presence and absence of an AhR antagonist (3′-methoxy-4′-nitroflavone, MNF). After

exposure to isoeugenol, 32–34% of cells were in G_0/G_1 phase, as assessed by fluorescence-activated cell sorting (FACS) analysis, whereas the induced G_0/G_1 arrest was reduced in the presence of the AhR antagonist MNF. In a follow-up study, the authors confirmed the mediation of AhR in cell cycle regulation. They also observed a reduction in levels of the G_1-phase cell cycle-related proteins retinoblastoma (RB), and cyclin-dependent kinase 6 (CDK6), but not CDK2 and CDK4, and an increase in protein levels of the CDK inhibitor p27Kip1 (CDKN1B) (Kalmes and Blömeke, 2012).

Scozzafava et al. (2015) investigated the capacity of isoeugenol and other phenolic compounds (e.g. vanillin, eugenol, and guaiacol) to inhibit catalytically active human isozymes of the Zn^{2+}-containing carbonic anhydrases. Phenol red was used as an indicator, the hydration of carbon dioxide by carbonic anhydrases was followed for 10–100 seconds, and results were expressed as inhibition constants. Isoeugenol showed inhibition constants of 10.29 µM, 6.73 µM, 9.32 µM, and 9.13 µM with human carbonic anhydrase isozymes hCAI, hCAII, hCAIX, and hCAXII, respectively. [The Working Group noted that this study was not particularly relevant. In this acellular system, isoeugenol showed inhibitory effects on carbonic anhydrases that were similar to those shown by other similar phenolic compounds, including catechol. This metalloenzyme family is involved in numerous pathological and physiological processes in different tissues and organs, including biosynthetic reactions such as gluconeogenesis, lipid and urea synthesis, calcification, lipogenesis, ureagenesis, tumorigenicity, and the growth and virulence of various pathogens.]

(b) Experimental systems

(i) Non-human mammals in vivo

Isoeugenol (purity, > 99%) was administered at doses of 75–300 mg/kg by gavage in a 2-year study in F344/N rats and $B6C3F_1$ mice (NTP, 2010) (see Section 3.1). In male mice, the incidence of hyperplasia of the Bowman glands (olfactory glands) was increased significantly in all exposed groups in the 2-year gavage study. [The Working Group noted that lesions of the olfactory epithelium of the nose are unusual, considering that exposure was not by the inhalation route.] The incidence of forestomach squamous hyperplasia increased in a dose-dependent manner and was significant at the highest dose.

Arancioglu et al. (2015), also described in Section 4.2.5(b)(i), reported a threefold increase in lymphocyte proliferation (measured by LLNA) in the ears of female Balb/c mice after exposure to isoeugenol. Similarly, T-lymphocyte proliferation (measured by LNNA) was reported in the lymph nodes of CBA/Ca mice exposed to isoeugenol (2.5%, 5%, or 10%) dissolved in acetone:olive oil (4:1) daily for 3 days (Kimber et al., 1991). The authors also observed that pre-treatment with a 25% isoeugenol ointment solution applied on the shaved flank for 48 hours, 5 days before the three consecutive days of isoeugenol exposure (5%) on the dorsum of both ears, induced an enhancement in cell proliferation. ter Burg et al. (2014) studied the effects of isoeugenol on cell proliferation in the mandibular lymph nodes by LLNA in male BALB/c mice after daily inhalation exposure for three consecutive days. Isoeugenol was nebulized in acetone to produce an aerosol of liquid droplets at a target concentration of 75 mg/m^3. Isoeugenol increased cell proliferation starting from the first exposure, 45 minutes/day, and induced a threefold increase in cell proliferation at the maximum exposure of 360 minutes/day.

[The Working Group considered that the end-point of cell proliferation from the LLNA was not particularly relevant to the key characteristic

of "alters cell proliferation, cell death, or nutrient supply", because it focused on a localized topical effect.]

(ii) Non-human mammalian cells in vitro

Kim et al. (2016) studied the effects of isoeugenol in cultures of primary myoblasts from mice (C2C12) and rats (L6). Cells were treated with isoeugenol at 30 μM for 3 hours. Glucose uptake was analysed by measuring the uptake of 2-deoxy-D(H^3)-glucose. Intracellular calcium concentration was measured by detecting the fluorescence of cells treated with a calcium-sensitive indicator, fluo-3 AM. The cells were transiently transfected with small interfering RNAs (siRNAs) against genes encoding AMP-activated protein kinases AMPKa2 and AMPKa1 and Akt substrate 160 (AS160). Cell surface expression of Myc-tagged glucose transporter type 4 (Myc-GLUT4) was quantified by an antibody-coupled colorimetric absorbance assay. Isoeugenol stimulated glucose uptake via AMPK phosphorylation in myoblasts of mouse C2C12 cells and, more effectively, in rat L6 cells, with the involvement of both AMPKa2 and AMPKa1. Isoeugenol increased glucose uptake through the calcium-mediated calcium/calmodulin-dependent protein kinase (CaMKK)–AMPK and the protein kinase C-alpha (PKCα) pathways and stimulated GLUT4 translocation through the AMPK–AS160 pathway. [The Working Group noted some limitations of the study because of lack of clarity in the description of the cells' source.]

Liu et al. (2008) reported that the exposure of vascular smooth muscle cells derived from Sprague-Dawley rats to isoeugenol at 50 μM did not inhibit the stimulation of phosphorylation of platelet-derived growth factor receptor beta (PDGFRβ) by PDGF or H_2O_2, thus showing no effect on growth factor signalling that could lead to cell proliferation or migration.

4.2.8 Data relevant to multiple key characteristics

(a) Microarray and omics data

There were 12 papers describing the development of predictive methods for identifying skin sensitizers (Cluzel-Tailhardat et al., 2007; McKim et al., 2010; Vandebriel et al., 2010; Williams et al., 2010; Andreas et al., 2011; Takahashi et al., 2011; Jung et al., 2012; Corsini et al., 2013; Neves et al., 2013; Saito et al., 2013, 2017; Bae et al., 2015). Given that isoeugenol is an OECD reference chemical for skin sensitization, these studies included isoeugenol among the training set of chemicals to develop methods for toxicity prediction. [The Working Group considered that although most of these studies used microarrays to identify predictive gene sets, none provided any mechanistic information.] Isoeugenol was used as a negative training compound to identify gene classifiers for the prediction of hepatocarcinogens in the mouse liver, but no data were provided to show changes in gene expression related to carcinogenesis (Auerbach et al., 2010). [The Working Group noted that this study did not provide any mechanistic insights.]

Ishii et al. (2023) recently examined gene expression changes in the livers of *gpt* delta mice treated with isoeugenol at 150 or 600 mg/kg bw per day for 13 weeks. Genes that were identified as altered in expression included those known to be regulated by PPAR subtypes and involved in fatty acid metabolism. More genes were altered in the livers of male mice than in those of female mice, consistent with males being more susceptible than females to hepatocellular adenoma and carcinoma. [The Working Group noted that since PPARα is the major subtype expressed in the mouse liver, the gene expression changes are likely due to PPARα and not PPARγ.]

(b) *Evaluation of high-throughput in vitro toxicity screening data*

The analysis of the in vitro bioactivity of the agents reviewed in *IARC Monographs* Volume 134 was informed by data from high-throughput screening assays generated by the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes of the government of the USA (Thomas et al., 2018). Isoeugenol was one of thousands of chemicals tested across the large assay battery of the Tox21 and ToxCast research programmes. Detailed information about the chemicals tested, assays used, and associated procedures for data analysis is publicly available (US EPA, 2024).

The ToxCast/Tox21 high-throughput screening results are presented according to the assays that have been mapped to the key characteristics of carcinogens (Reisfeld et al., 2022). The detailed results are available in the supplementary material for this volume (Annex 4, Supplementary material for Section 4, Mechanistic Evidence, online only, available from: https://publications.iarc.who.int/627). Here, for brevity, assays for which there is a positive "hit call" are referred to as "active" assays. A summary of these results is given below as the number of active assays (without any caution flags) out of the total number of key characteristic-related assays for the chemical.

Among the 290 assays in which isoeugenol was tested, it was found to be active and without caution flags in three assays relevant to key characteristics of carcinogens. Isoeugenol was active in one assay mapped to key characteristic 5, "induces oxidative stress". This assay, ATG_NRF2_ARE_CIS_up, is a cell-based, multiplexed-readout assay in HepG2 (a human liver cell line), with measurements taken 24 hours after chemical dosing in a 24-well plate. The assay measures the activation of a reporter gene under the control of the antioxidant responsive element regulated by the oxidant-induced transcription factor NRF2 (NFE2 like bZIP transcription factor 2). The AC_{50} (50% of maximum activity) was 17.11. There are two high-throughput assays that measure activation of Nrf2 (TOX21_ARE_BLA_agonist_ratio, ATG_NRF2_ARE_CIS_up). [The Working Group noted that, given that only one out of the two NRF2 assays gave positive results, the evidence is weak that isoeugenol activates NRF2.]

Isoeugenol gave positive results in two assays mapped to key characteristic 8, "modulates receptor-mediated effects". The two assays are called OT_ER_ERaERb_1440 and ATG_PPARg_TRANS_up. OT_ER_ERaERb_1440 is a cell-based assay that uses HEK293T (a human kidney cell line), with measurements taken 24 hours after chemical dosing in a 384-well plate. The assay measures the interaction of green fluorescent protein (GFP)-tagged estrogen receptor alpha and beta (ERα and ERβ) with nuclear DNA and is one of 17 assays that assess the activity of compounds for the ability to modulate ER activity (Judson et al., 2015). [The Working Group noted that, given that only one out of the 17 ER assays gave positive results, the evidence was very weak that isoeugenol activates ER.]

Isoeugenol gave positive results for the activation of PPARγ. The ATG_TRANS assays are cell-based, multiplexed-readout assays that use HepG2 (a human liver cell line), with measurements taken 24 hours after chemical dosing in a 24-well plate. The assay measures the ability of the compound to activate a reporter gene through the ligand-binding domain of PPARγ. The AC_{50} was 60.36 μM. There are six assays that measure modulation of PPARγ (ATG_PPARg_TRANS_up, ATG_PPRE_CIS_up, OT_PPARg_PPARgSRC1_0480, OT_PPARg_PPARgSRC1_1440, TOX21_PPARg_BLA_Agonist_ratio, and TOX21_PPARg_BLA_antagonist_ratio). [The Working Group noted that, given that only one out of the six PPARγ assays (ATG_PPARg_TRANS_up) gave

positive results, the evidence was very weak that isoeugenol activates PPARγ.]

5. Summary of Data Reported

5.1 Exposure characterization

Isoeugenol is a flavour compound that can be synthesized from eugenol and that also occurs naturally in more than 500 plant species such as cloves, Ceylon cinnamon, sweet flag, nutmeg, basil, perilla, and ylang-ylang. It has been identified as a pyrolysis product of lignin and has been detected in wood smoke, smoked foods, and the smoke particulate fraction of commercial cigarette brands.

In many countries around the world, isoeugenol is approved for use in food, cosmetics, animal feed, and veterinary medicines. It is widely used as a fragrance or flavouring agent in perfumes, cosmetics, personal care products, household cleaners, and various food products. Its sweet and spicy floral aroma adds a distinctive fragrance and flavour to soft drinks, bakery products, confectionery, and chewing gum. Isoeugenol is a skin sensitizer and is commonly used in allergen patch testing. It is also the active ingredient in anaesthetics used in aquaculture.

People in a variety of occupations, including workers involved in the synthesis of isoeugenol, hairdressers, beauticians, janitors, cleaners, and firefighters, may be exposed to isoeugenol by dermal and/or inhalation routes. Exposure of the general population to isoeugenol occurs by several routes, and daily intake through consumption of foods containing isoeugenol is estimated to be in the low-micrograms range. Inhalation and dermal contact contribute to exposure, mainly through the use of personal care products; the presence of isoeugenol as a fragrance in household products and cosmetics is an important source of exposure.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

Treatment with isoeugenol caused an increase in the incidence of malignant neoplasms or an appropriate combination of benign and malignant neoplasms in one species (mouse) and a single rare result in three organs, the occurrence of histiocytic sarcoma at multiple sites in female mice and the presence of thymoma and mammary gland carcinoma in male rats.

Isoeugenol was administered by oral administration (gavage) in one study that complied with Good Laboratory Practice (GLP) in male and female B6C3F$_1$ mice. In male mice, isoeugenol caused an increased incidence of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined). In female mice, there was a significant positive trend in the incidence of histiocytic sarcoma (multiple sites).

Isoeugenol was administered by oral administration (gavage) in one study that complied with GLP in male and female F344 rats. In male rats, isoeugenol caused a significant positive trend in the incidence of mammary gland carcinoma and benign or malignant thymoma.

5.4 Mechanistic evidence

Data on the absorption, distribution, metabolism, and excretion of isoeugenol in humans were sparse. Isoeugenol is capable of both permeation and penetration of human skin after dermal exposures. No information on oral and inhalation exposure in humans was available. After oral and dermal exposure in rodents, isoeugenol is rapidly absorbed and excreted, predominantly in the urine as glucuronide or sulfate conjugates, with very little retention in tissues. Lower absorption was observed after dermal exposure

than after oral exposure. Isoeugenol has been shown to inhibit the activity of cytochrome P450 (CYP) enzymes in experimental systems.

Overall, the mechanistic evidence considered for the key characteristics of carcinogens "is electrophilic or can be metabolic activated to an electrophile", "is genotoxic", "alters DNA repair and/or genomic instability", "induces oxidative stress", "induces chronic inflammation", "modulates receptor-mediated effects", and "alters cell proliferation, cell death, or nutrient supply" was sparse or the available results were negative in experimental systems. There was only one study evaluating the allergic reaction to isoeugenol in exposed humans, and this was considered uninformative for the key characteristics of carcinogens.

Regarding the key characteristic "is electrophilic or can be metabolically activated to an electrophile", isoeugenol is a skin sensitizer that can be converted photochemically to electrophiles that form protein adducts. However, in two studies in vivo, one in mouse liver and another in turkey fetuses, isoeugenol–DNA adducts were not detected.

Regarding the key characteristic "is genotoxic", isoeugenol induced sister-chromatid exchange in human lymphocytes and caused an increase in the frequency of micronucleated normochromatic erythrocytes in female mice but not in male mice in one study at the highest dose tested. Isoeugenol gave negative results for *gpt* mutations in the liver of transgenic mice and did not induce unscheduled DNA synthesis in rodent hepatocytes or DNA strand breaks in turkey fetuses. It gave negative results for mutagenicity in two studies in bacteria and in one study in *Drosophila melanogaster*.

Regarding the key characteristic "induces oxidative stress", two in vitro studies, one in human primary cells and one in a human cell line found evidence that isoeugenol exposure increased intracellular ROS and depleted intracellular glutathione, but only at concentrations associated with significant cytotoxicity. There were no studies examining whether ROS associated with isoeugenol exposure caused any type of oxidative stress or damage.

Regarding the key characteristic "induces chronic inflammation", three studies in human primary cells showed that isoeugenol stimulated inflammatory markers, including T-cell responses, releasing interleukins IL-8, IL-1α, and IL-1β. Several studies in human cell lines observed increases in inflammatory cytokines such as IL-8 and interferon gamma (IFNγ). However, a few studies in human cells did not observe any effects on inflammatory cytokines. Additionally, isoeugenol-induced atrophy, attributable to chronic inflammation, was observed in the olfactory epithelium of treated rats and mice of both sexes at two exposure durations (3 months and 2 years). The same study also reported that the incidence of forestomach inflammation and of ulceration (in males only), was significantly increased at the highest dose in the 2-year study.

For the key characteristic "modulates receptor-mediated effects", a few studies in human keratinocytes in vitro indicated that isoeugenol activates the aryl hydrocarbon receptor (AhR), as assessed by increases in CYP1A1 expression. In another study, isoeugenol exhibited androgen receptor (AR) antagonism in in vitro transactivation assays. One microarray study showed that isoeugenol induced the expression of a set of genes known to be under the control of peroxisome proliferator-activated receptor alpha (PPARα) in mouse liver.

Regarding the key characteristic "alters cell proliferation, cell death, or nutrient supply", in human cell lines isoeugenol did not cause cell proliferation. In long-term studies in mice treated by (oral) gavage, hyperplasia was observed in Bowman glands and in the forestomach.

For the other key characteristics, "alters DNA repair or causes genomic instability" and "is immunosuppressive", there was a paucity of data.

Isoeugenol was essentially without effects in the assay battery of the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of isoeugenol.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of isoeugenol.

6.3 Mechanistic evidence

There is *inadequate* mechanistic evidence.

6.4 Overall evaluation

Isoeugenol is *possibly carcinogenic to humans* (Group 2B).

6.5 Rationale

The Group 2B classification for isoeugenol is based on *sufficient evidence* for cancer in experimental animals. The *sufficient evidence* for cancer in experimental animals is based on an increase in the incidence of malignant neoplasms (liver) and a combination of benign and malignant neoplasms (liver), in one species (mouse) and one sex (males) in one study that complied with Good Laboratory Practice. In addition to the liver malignant neoplasms in male mice, there were significant positive trends (based on the poly-3 trend test) in mammary gland carcinoma and benign or malignant thymoma in male rats and histiocytic sarcomas (multiple sites) in female mice. A minority of the Working Group considered that the evidence for carcinogenicity in experimental animals was *limited*, given that the pairwise comparison with the controls did not reach statistical significance in any of the treated groups, and thus that isoeugenol should be classified as Group 3.

The evidence regarding cancer in humans was *inadequate* because no studies were available. The mechanistic evidence was *inadequate* because the available data were sparse or largely negative.

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LIST OF ABBREVIATIONS

acesulfame-K	acesulfame potassium
ADI	acceptable daily intake
AhR	aryl hydrocarbon receptor
AHRR	aryl hydrocarbon receptor repressor
AIF	apoptosis-inducing factor
ANOVA	analysis of variance
APF	aminophenyl fluorescein
AR	androgen receptor
ASB	artificially sweetened beverage
ATP	adenosine triphosphate
AUC	area under the curve
BBN	*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine
BMI	body mass index
BUN	blood urea nitrogen
bw	body weight
CAM	chorioallantoic membrane
cAMP	cyclic adenosine monophosphate
CAR	constitutive androstane receptor
CAS	Chemical Abstracts Service
CAT	catalase
CDK	cyclin-dependent kinase
CI	confidence interval
CLL	chronic lymphocytic leukaemia
C_{max}	maximun concentration
CPID	Consumer Product Information Database
CPS	Cancer Prevention Study
CPS-II	Cancer Prevention Study II
CRP	C-reactive protein
CYP	cytochrome P450
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
DAG	directed acyclic graph
DCFDA	2′,7′-dichlorodifluorescein diacetate

DKP	diketopiperazine
DPPH	1,1-diphenyl-2-picrylhydrazyl
DRIFTS	diffuse reflectance infrared Fourier transform spectroscopy
DSPE	dispersive solid-phase extraction
dw	dry weight
EC_{50}	half-maximal effective concentration
EFSA	European Food Safety Authority
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial–mesenchymal transition
EPIC	European Prospective Investigation into Cancer and Nutrition
ERE	estrogen receptor binding element
ERK	extracellular signal-regulated kinase
FAO	Food and Agriculture Organization of the United Nations
FFQ	food frequency questionnaire
FLD	fluorescence detection
FSH	follicle-stimulating hormone
GABA	gamma-aminobutyric acid
$GABA_A$ receptor	gamma-aminobutyric acid type A receptor
GC	gas chromatography
GCG	proglucagon
GC-MS	gas chromatography-mass spectrometry
GC-MS/MS	gas chromatography-tandem mass spectrometry
GLI1	GLI family zinc finger 1
GLP	Good Laboratory Practice
GLP-1	glucagon-like peptide-1
GLP-2	glucagon-like peptide-2
GLP-2R	glucagon-like peptide-2 receptor
GMVEC	glomerular microvascular endothelial cells
GPx	glutathione peroxidase
GR	glucocorticoid receptor
GRADE	Grading of Recommendations, Assessment, Development and Evaluation
GRAS	generally recognized as safe
GSH	glutathione
GSR	glutathione reductase
GSSG	glutathione disulfide
GST	glutathione *S*-transferase
GTT	glucose tolerance test
HBV	hepatitis B virus
HCV	hepatitis C virus
HDL	high-density lipoprotein
HFS	high-fat/sucrose
HIV	human immunodeficiency virus
HOMA-B	homeostatic model assessment for insulin secretion
HOMA-IR	homeostatic model assessment for insulin resistance
HPFS	Health Professionals Follow-up Study
HPLC	high-performance liquid chromatography
HPLC-UV	high-performance liquid chromatography-ultraviolet detection
HR	hazard ratio
hsCRP	high-sensitivity C-reactive protein

HS-PTV-fast GC-MS	headspace-programmed temperature vapourization-fast gas chromatography-quadrupole mass spectrometry
HS-SPME	headspace solid-phase microextraction
hTRPA1	human transient receptor potential ankyrin 1
HUVEC	human umbilical vein endothelial cells
IARC	International Agency for Research on Cancer
IC_{50}	half-maximal inhibitory concentration
ICAM-1	intracellular adhesion molecule 1
IFNγ	interferon gamma
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor
IL	interleukin
iNOS	inducible isoform of nitric oxide synthase
ISO	International Organization for Standardization
ITT	insulin tolerance test
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	liquid chromatography
LC-ELSD	liquid chromatography-evaporative light-scattering detection
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LH	luteinizing hormone
LIRI	liver ischaemia reperfusion injury
LLN	local lymph node
LLNA	local lymph node assay
LOD	limit of detection
LOQ	limit of quantification
LOOH	lipid hydroperoxides
Lp-PLA2	lipoprotein-associated phospholipase A2
LTC_4	leukotriene C_4
LTT	lymphocyte transformation test
MAPK	mitogen-activated protein kinase
MCCS	Melbourne Collaborative Cohort Study
MCC-Spain	Spanish Multicase–Control Study
MDA	malondialdehyde
MDCK	Madin–Darby canine kidney
N^2-MIE-dG	N^2-(methylisoeugenol-3′-yl)-2′-deoxyguanosine
N^6-MIE-dA	N^6-(methylisoeugenol-3′-yl)-2′-deoxyadenosine
MoLC	monocyte-derived LC-like cells
MRL	maximum residue level
MS	mass spectrometry
MSG	monosodium glutamate
MSDI	maximized survey-derived daily intake
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	*N*-acetyl cysteine
ND	not detected
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NHANES	National Health and Nutrition Examination Survey
NHL	non-Hodgkin lymphoma
NHS	Nurses' Health Study

NHS-II	Nurses' Health Study II
NIH-AARP	National Institutes of Health-American Association of Retired Persons Diet and Health Study
NIOSH	National Institute for Occupational Safety and Health
NICD	notch intracellular domain
NLRP3	NLR family pyrin domain containing 3
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
NMR	nuclear magnetic resonance
NO	nitric oxide
NOES	National Occupational Exposure Survey
NPSH	non-protein thiol
NR	not reported
NTP	National Toxicology Program
OCT	octameric transcription factor
OECD	Organisation for Economic Co-operation and Development
OGTT	oral glucose tolerance test
OR	odds ratio
oxLDL	oxidized low-density lipoprotein
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
Phe/LNAA	phenylalanine/large neutral amino acids
PKU	phenylketonuria
PLCO	Prostate, Lung, Colorectal and Ovarian
PLE	pressurized liquid extraction
PMRA	Pest Management Regulatory Agency
PPAR	peroxisome proliferator-activated receptor
PPARγ	peroxisome proliferator-activated receptor gamma
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PXR	pregnane X receptor
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RHS	reconstructed human skin
ROS	reactive oxygen species
RR	relative risk
RT-PCR	reverse transcription-polymerase chain reaction
SCE	sister-chromatid exchange
SD	Sprague-Dawley
SFE	supercritical fluid extraction
SGOT	serum glutamic-oxaloacetic transaminase (aspartate aminotransaminase)
SGPT	serum glutamic pyruvic transaminase (alanine aminotransaminase)
sHR	subdistribution hazard ratio
sIL-6r	soluble interleukin-6 receptor
SIM-GC-MS	selected ion monitoring-gas chromatography-mass spectrometry
siRNA	small interfering RNA
SMART	somatic mutation and recombination by wing spot test
SOD	superoxide dismutase
SPE	solid-phase extraction
SREBP	sterol regulatory element-binding protein
SV40	simian virus 40
T3	triiodothyronine
T4	thyroxine

TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances
T1R	sweet taste receptor 1
T_{max}	the time taken to reach maximum concentration
TNFα	tumour necrosis factor alpha
TOS	total antioxidant status
TRAP	thrombin receptor-activating protein
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UAE	ultrasound-assisted solvent extraction
UDS	unscheduled DNA synthesis
UK	United Kingdom
UPLC	ultra-performance liquid chromatography
UPLC-MS/MS	ultraperformance liquid chromatography-tandem mass spectrometry
US	United States
USA	United States of America
US FDA	United States Food and Drug Administration
UV	ultraviolet
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WWTP	wastewater treatment plant
XO	xanthine oxidase

ANNEX 1. SUPPLEMENTARY MATERIAL FOR SECTION 1, EXPOSURE CHARACTERIZATION

These supplementary online-only tables are available from: https://publications.iarc.who.int/627.

Please report any errors to imo@iarc.who.int.

Aspartame

Table S1.1 Reported occurrence of aspartame in food

The following tables were produced in draft form by the Working Group and were subsequently fact-checked but not edited:

Table S1.2 Exposure assessment review and critique for epidemiological studies on cancer in humans exposed to aspartame

Table S1.3 Exposure assessment review and critique for mechanistic studies in humans exposed to aspartame

Isoeugenol

The following table was produced in draft form by the Working Group and was subsequently fact-checked but not edited:

Table S1.4 Exposure assessment review and critique for mechanistic studies in humans exposed to isoeugenol

ANNEX 2. SCIENTIFIC AND OTHER PUBLICLY AVAILABLE DATA ON ASPARTAME USE IN ARTIFICIALLY SWEETENED BEVERAGES

1. Introduction

The Working Group considered whether the intake of artificially sweetened beverages (ASBs) could be used as a proxy for aspartame exposure in a given population in a given time period. [The Working Group noted that the main questions were: (1) during which period was a substantial proportion of aspartame intake via ASBs; and (2) during which period were ASBs primarily sweetened with aspartame, thus limiting co-exposure to other artificial sweeteners. The following document is a non-exhaustive collection of scientific data and other publicly available information pertinent to these questions which complements the data reported in Section 1 of the monograph on aspartame in the present volume.]

2. Aspartame approval as a food additive in selected countries

Table 1 provides information on the year of approval of aspartame as a food additive in beverages in different countries.

3. Aspartame use in different countries

USA

Aspartame has been used in solid foods since 1981 and in beverages since 1983. The first soft drink that was sweetened entirely with aspartame entered the market in August 1983, and others followed in early 1984 (Hollie, 1984). In 1984, the major soft-drink makers were sweetening their diet drinks with a blend of four to five parts saccharin to one part aspartame. The major diet and "light" cola brands became 100% sweetened by aspartame at the beginning of 1985 (Anonymous, 1984; Pott and Schrage, 1984; Yoshihara, 1985). As a result, in the USA between 1984 and 1987, the per capita consumption of aspartame increased from [2.6 kg] to [6.4 kg], and the per capita consumption of saccharin decreased from [4.5 kg] to [2.7 kg] (HSPA, 1987). In 1984, 3% of all soft drinks were sweetened by aspartame only versus 17% in 1985. In 1984, 13% of all soft drinks were sweetened with the combination of aspartame plus saccharin versus 4% in 1985 (Stellman, 1988). In 1987, most diet soft drinks were sweetened by aspartame, or a blend of saccharin and aspartame (USDA, 1987).

Table 1. Year of approval of aspartame as a food additive in beverages

Country	Year	Permitted use	Reference
Canada	1981	Soft drinks, desserts, breakfast cereals, chewing gum, tabletop sweetener	Health Canada (2023)
South Africa	1982	Carbonated beverages	Reuters (1982)
USA	1983	Carbonated beverages and carbonated beverage syrup bases	Office of the Federal Register (1983)
Denmark	1983	Tabletop sweetener, foods, beverages	Taylor (1985)
Ireland	1983	Tabletop sweetener, foods, beverages	Taylor (1985)
Sweden	1982	Carbonated beverages; chewing gum, ice cream, and vitamin C with aspartame came onto the market in 1984	Pettersson (1982); Johansson (1983); Taylor (1985)
United Kingdom	1983	Tabletop sweetener, foods, beverages	Government of the United Kingdom (1983)
Switzerland	1983	Tabletop sweetener, foods, beverages	Taylor (1985)
France	1988	Food products, including beverages, chewing gum, etc.	Boussard (1991)
European Union[a]	1994	Foodstuffs intended for human consumption (including beverages)	European Parliament and Council (1994)
China	1986	Foods and beverages	ReportLinker (2023)
Australia	1982 1986	Tabletop sweetener Soft drinks	Shoebridge (1991); Australian Beverages Council (2019); The NutraSweet Company (1988)
USA	1986	Other types of beverages than carbonated	USDA (1987)
USA	1996	General purpose sweetener	Office of the Federal Register (1996)

[a] 1994 is the date of the European Union harmonization. [The Working Group noted that it is expected that national legislation in almost all European Union countries approved aspartame before that date (see examples in the list above).]

It was reported in 2006 that aspartame was found in more than 6000 products (European Commission, 2006). In the USA, 85–90% of all aspartame was used in beverages, mainly diet carbonated soft drinks (USDA, 2012). The most popular aspartame-containing soft drinks in the USA were the diet cola brands of the major soft-drink makers (USDA, 2012).The annual amount of aspartame used in diet soda in the USA in 2006 was 4500 metric tonnes (Schernhammer et al., 2012). With the amount of aspartame used annually across all applications in the USA estimated at 5000 to 5500 metric tonnes (Heinzinger, 2006), diet soda thus accounted for the large majority (~86%) of all aspartame in foods (Schernhammer et al., 2012).

Oceania and Africa

In 1986 and 1987, diet versions of major cola brands containing aspartame were launched in Australia (Shoebridge, 1991). Aspartame demand in Africa and Oceania has not increased since the peak in 1998 (IASR, 2004).

Europe

In the European Union (EU), the surge in aspartame consumption emerged later and more steadily than in the USA. In the mid-1980s, aspartame accounted for little more than 2% of the European market for intense sweeteners. European legislation harmonizing the use of low-calorie sweeteners, including aspartame and acesulfame potassium (acesulfame-K), in foodstuffs and drinks was introduced in 1994

(European Parliament and Council, 1994), and by 1996, the market share for aspartame had risen to 20% in volume terms and stayed approximately on that level at least until 2001. The United Kingdom (UK) was the biggest consumer of intense sweeteners in the EU: in 1987, 31% of the general population used aspartame regularly (i.e. weekly) (IASR, 2004).

In an analysis of 95 aspartame-containing soft drinks from 10 European countries, aspartame concentrations ranged from 30 to 527 mg/L and were highly variable among similar soft drinks bought in different countries (van Vliet et al., 2020).

Denmark

In 1999, the Regional Veterinary and Food Control Authority analysed a representative sample of 81 non-alcoholic light drinks of Danish production. Carbonated and non-carbonated soft drinks were sweetened with a mixture of aspartame and acesulfame-K, with or without cyclamate and/or saccharin. Only 1 carbonated drink out of 21, and 3 non-carbonated drinks out of 60, were sweetened with aspartame only (Leth et al., 2007). In a similar study in 2005, 37% of the non-carbonated flavoured beverages and 50% of the carbonated beverages contained aspartame. No beverage was sweetened with aspartame only (Jensen, 2007). In similar studies carried out in 2008, 2010, 2014, and 2016, no beverage was sweetened by aspartame only (Villadsen and Jakobsen, 2012; Jensen, 2014, 2016), with the exception of 1 out of 10 cola brands (Nielsen and Zederkopff Ballin, 2009).

Ireland

In the 2011 National Adult Nutrition Survey, aspartame was the second most frequently added sweetener; as the only sweetener, it was found in 35 products, most of which were energy-reduced or no added sugar (NAS) dairy products. Among various combinations of sweetener, the most common was aspartame with acesulfame-K, which occurred in 115 products – mainly still and carbonated flavoured drinks (57%), and energy-reduced or NAS dairy products (20%). Aspartame plus saccharin was the second most commonly used combination and was found in 49 products, 98% of which were still and carbonated energy-reduced or NAS flavoured drinks. Aspartame was the sweetener consumed in the highest amount in the total population (1.05 mg/kg bw per day). Aspartame only was used to sweeten 6% of the energy-reduced or NAS carbonated flavoured drinks, and aspartame in combination with other sweeteners was used in 65% (Buffini et al., 2018).

Italy

Products containing aspartame and acesulfame-K consumed by teenagers in Rome in 2000–2001 were found in all the product categories, frequently in combination (Arcella et al., 2004). Among a nationally representative sample of the Italian population, non-alcoholic beverages accounted for 48% of aspartame use and tabletop sweeteners accounted for 43% (LeDonne et al., 2017).

Portugal

In 2006–2007, 25 light soft drinks, 13 mineral water-based soft drinks, and 10 light nectars were analysed. Aspartame and acesulfame-K were detected in 92% and 72%; 62% and 77%, and 80% and 100%, respectively. About [80%] of the drinks were sweetened with both aspartame and acesulfame-K (Lino et al., 2008). In 2015–2016, soft drinks were the main source of exposure to aspartame (48%). Of the non-nutritive intense sweeteners, acesulfame-K and aspartame were

consumed in the highest quantities (Carvalho et al., 2022).

Japan

A major light cola brand, sweetened with aspartame, came onto the Japanese market in 1984 but did not appeal to Japanese consumers and was renewed by adding fructose (12 kcal/100 mL) and renaming as "low-calorie cola". A light cola sweetened with aspartame reappeared on the Japanese market in 1999 (Nakamoto and Nakahashi, 1999). In 1996, aspartame accounted for 25% of the Japanese intense sweetener market (IASR, 2004).

4. Aspartame use in artificially sweetened beverages over time

In the USA, the two most popular diet colas were sweetened with saccharin until 1984, when saccharin was replaced with aspartame (Hollie, 1984; Anonymous, 1984; Pott and Schrage, 1984). Beverages were a major area of aspartame use (USDA, 1985), and aspartame replaced saccharin mainly in the soft-drink market, since many manufacturers switched from a mixture of saccharin and aspartame to a 100% aspartame-sweetened product (USDA, 1986). The advances of aspartame on the USA market were spurred largely by growth in demand for diet soft drinks (USDA, 1995).

In 1993, acesulfame-K had broad approval for use in beverages in the EU, Canada, and Australia, and the first diet colas sweetened with a combination of aspartame and acesulfame-K were launched. In Europe, the blend of acesulfame-K and aspartame became increasingly popular (USDA, 1995). In the USA, acesulfame-K was approved for use in non-alcoholic beverages in 1998 (Office of the Federal Register, 1998), and the two major diet cola manufacturers launched diet brands sweetened with a combination of aspartame and acesulfame-K in 1998 and 1999 (Hays, 1998; Hegenbart, 2000). Many diet soft-drink bottlers in the EU, Canada, and the USA switched from 100% aspartame to blends of aspartame and acesulfame-K for their second-ranking brands and some top-line brands (IASR, 2004; Weihrauch and Diehl, 2004). Also, saccharin in combination with aspartame was widely used in fountain syrups, or about 20–25% of the diet carbonated beverage market in the USA. Between 2002 and 2009, the share of aspartame among high-intensity sweeteners in beverage use in the USA decreased from about 80% to about 70% (USDA, 2012).

Table 2 provides additional information on aspartame use in ASBs found in different types of sources (e.g. scientific publications, journals, newspapers, and websites from specific brands, or social media channels).

Table 2. Timeline of aspartame use in artificially sweetened beverages in selected countries

Country	Beverage	Used as the unique sweetener (period in years)	Used in combination with other sweeteners (period in years)	Reference
USA	Coke Zero		2005, aspartame + acesulfame-K 2010, aspartame + acesulfame-K	NBC News (2005); Franz (2010)
USA	Diet 7-Up	1985	1983–1984 (about 20% aspartame + 80% saccharin)	Hollie (1984); Anonymous (1984); Pott and Schrage (1984)
USA	Diet Cherry Coke	Before 1999	1999, aspartame + acesulfame-K	Hegenbart (2000)
USA	Diet Coke	1985	1983–1984 (20% aspartame + 80% saccharin)	Hollie (1984); Anonymous (1984); Pott and Schrage (1984)
USA	Diet Coke	2010		Franz (2010)
USA	Diet Dr Pepper	2010		Franz (2010)
USA	Diet Pepsi	1985	1983–1984 (about 20% aspartame + 80% saccharin)	Hollie (1984); Anonymous (1984); Pott and Schrage (1984)
USA	Diet Pepsi	1983–2015	2015, sucralose + acesulfame-K (no aspartame)	Roberts (2015)
USA	Diet Pepsi	2010		Franz (2010)
USA	Diet Sprite	Before 1999	1999, aspartame + acesulfame-K	Hegenbart (2000)
USA	Fresca	Before 1999	1999, aspartame + acesulfame-K	Hegenbart (2000)
USA	Pepsi One		1999–2015, aspartame + acesulfame-K	Hegenbart (2000)
USA	Pepsi XL		1995, 50% aspartame + 50% fructose	Collins (1995)
Canada	Pepsi Max		1994–2002, aspartame + fructose	Collins (1995)
Germany	Coke Light		2005, 2006, 2010, 2011, 2012, aspartame + acesulfame-K	Stephen Morris Marketing (2023)
United Kingdom	Diet Pepsi	1994–2015 and after		Roberts (2015)
France	Coca-Cola Light		2014, aspartame + acesulfame-K	Tricoulet (2014)
France	Coca-Cola Zero	1988	2007, aspartame + acesulfame-K	Coca-Cola Web (2023); Quelle Difference? (2015); Tricoulet (2014)
France	Coca-Cola Zero Cherry		2014, aspartame + acesulfame-K	Tricoulet (2014)
France	Pepsi Max		1994, aspartame + acesulfame-K	Tricoulet (2014)
France	Pepsi Light		2014, aspartame + acesulfame-K	Tricoulet (2014)
Denmark	Various beverages		1999, all diet sodas tested (n = 21) except one were sweetened with a mixture of aspartame and acesulfame-K, with or without cyclamate and/or saccharin	Leth et al. (2007)

Table 2. (continued)

Country	Beverage	Used as the unique sweetener (period in years)	Used in combination with other sweeteners (period in years)	Reference
Denmark	Pepsi Max	2008		Nielsen and Zederkopff Ballin (2009)
Denmark	Pepsi Max	2008	2010, aspartame + acesulfame-K	Villadsen and Jakobsen (2012)
Denmark	Coca-Cola Light, Coca-Cola Zero, other diet colas and diet sodas		2014, aspartame + acesulfame-K, with or without cyclamate and/or saccharin	Jensen (2014)
Denmark	All diet sodas tested		2010, aspartame + acesulfame-K, with or without cyclamate and/or saccharin	Jensen (2016)
Sweden	Coca-Cola Light		1983, aspartame + saccharin	Johansson (1983)
Japan	Coca-Cola Light		1995, or earlier with aspartame + fructose	Collins (1995).
Europe	Pepsi Max		1993, aspartame + acesulfame-K	Collins (1995)

acesulfame-K, acesulfame potassium.

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ANNEX 3. SUPPLEMENTARY MATERIAL FOR SECTION 2, CANCER IN HUMANS

Fig. S2.1 illustrates a directed acyclic graph (DAG) created by the Working Group to identify potential confounders of the association between aspartame intake and liver cancer risk. The objective of this exercise was to infer the quality of control for confounding in the available studies. This DAG is a conceptual model of the most influential causal relations for which data are typically available in epidemiological studies and is not intended to be exhaustive. The Daggity web application was used to create the DAG (Textor et al., 2016).

Known risk factors for liver cancer (identified as those with *sufficient* evidence in humans according to the *IARC Monographs* classification; IARC, 2024) that are of relevance for aspartame exposure were added to the DAG. These include:

(i) Aflatoxins (IARC, 2012a)

No arrow was drawn connecting aflatoxins to aspartame exposure, because these two exposures seem unlikely to be associated. Hence, aflatoxin exposure is probably not a confounder.

(ii) Alcoholic beverages (IARC, 2012b)

Consumption of alcoholic beverages may be linked to aspartame exposure through socioeconomic status. Socioeconomic status is known to influence body mass index (BMI) status, which might be associated with aspartame exposure, through consumption of artificially sweetened beverages.

(iii) Estrogen–progestogen oral contraceptives (combined) (IARC, 2012c)

No arrow was drawn that connected contraceptive use and aspartame consumption, since these factors were deemed unlikely to be associated. Hence, exposure to estrogen–progestogen oral contraceptives (combined) is probably not a confounder.

(iv) Chronic infection with hepatitis B virus or hepatitis C virus (strong risk factors for liver cancer; IARC, 2012d) is captured in this DAG as "hepatitis infection".

The potential connection between hepatitis infection and aspartame consumption may be through socioeconomic status, which is connected to BMI status.

(v) Tobacco smoking (in smokers and in smokers' children) (IARC, 2012b)

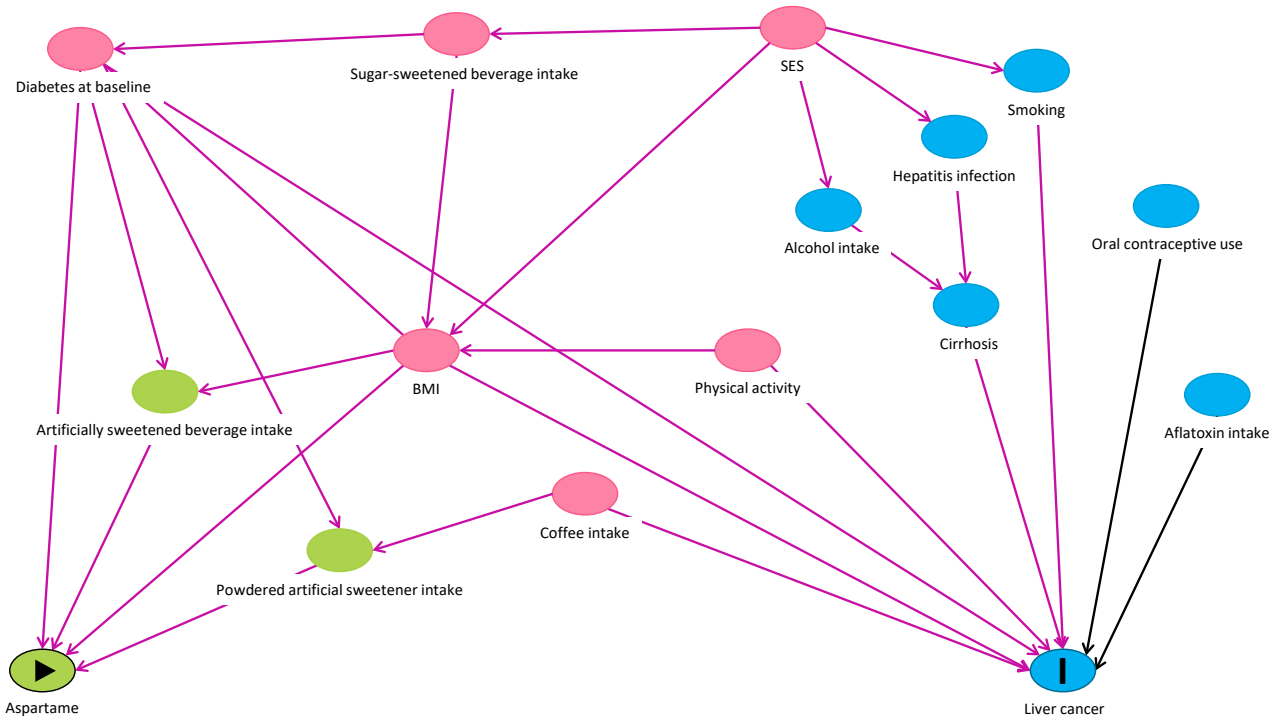
The potential connection between tobacco smoking and aspartame consumption may be through socioeconomic status, which is connected to BMI status.

The following potential risk factors for liver cancer were added:

(i) Higher BMI

This is a recognized risk factor for cancer, given the evidence for the protective effect

Fig. S2.1 Directed acyclic graph for the association between aspartame intake and liver cancer in studies of cancer in humans



BMI, body mass index; SES, socioeconomic status.

of absence of excess body fatness on risk of cancer (with *sufficient* evidence for liver cancer, evidence summarized in the *IARC Handbooks of Cancer Prevention*; Lauby-Secretan et al., 2016). BMI is connected to aspartame exposure via consumption of artificially sweetened beverages. Adjustment for BMI would control for this confounding and, because of the connection between BMI and socioeconomic status, would control for potential confounding by hepatitis, alcohol, and smoking behaviour.

(ii) Coffee intake

This was added because of the determination of *evidence suggesting lack of carcinogenicity* for liver cancer (with evidence of an inverse association) (IARC, 2018) and because of the potential link between coffee consumption and possible use of powdered artificial sweeteners. Because of the inverse association, lack of adjustment for coffee consumption would bias results towards the null.

(iii) Diabetes at baseline

This factor was added because of the emerging evidence that diabetes is a risk factor for liver cancer (Giovannucci et al., 2010).

Overall, the Working Group concluded that age, sex, BMI, socioeconomic status, diabetes, and consumption of sugar and/or sugar-sweetened beverages represented the minimal sufficient adjustment sets for estimating the effect of aspartame on the risk of certain cancers.

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ANNEX 4. SUPPLEMENTARY MATERIAL FOR SECTION 4, EVALUATION OF HIGH-THROUGHPUT IN VITRO TOXICITY SCREENING DATA

These supplementary online-only tables (available from: https://publications.iarc.who.int/627) contain summaries of the findings (including the assay name, the corresponding key characteristic, the resulting "hit calls" both positive and negative, and any reported caution flags) for those chemicals evaluated in the present volume that have been tested in high-throughput screening assays performed by the United States Environmental Protection Agency (US EPA) and the United States National Institutes of Health. The results were generated by the Working Group using the software "kc-hits" (key characteristics of carcinogens – high-throughput screening discovery tool) available from https://gitlab.com/i1650/kc-hits.git (Reisfeld et al., 2022), using the US EPA Toxicity Forecaster (ToxCast) assay data and the curated mapping of key characteristics to assays available at the time of the evaluations performed for *IARC Monographs* Volume 134. Data were available for aspartame, methyleugenol, and isoeugenol.

Please report any errors to imo@iarc.who.int.

1. Aspartame: ToxCast/Tox21 assay results mapped to the key characteristics of carcinogens
2. Methyleugenol: ToxCast/Tox21 assay results mapped to the key characteristics of carcinogens
3. Isoeugenol: ToxCast/Tox21 assay results mapped to the key characteristics of carcinogens

Reference

Reisfeld B, de Conti A, El Ghissassi F, Benbrahim-Tallaa L, Gwinn W, Grosse Y, et al. (2022). kc-hits: a tool to aid in the evaluation and classification of chemical carcinogens. *Bioinformatics*. 38(10):2961–2. doi:10.1093/bioinformatics/btac189 PMID:35561175

SUMMARY OF FINAL EVALUATIONS

Summary of final evaluations for Volume 134

Agent	Evidence stream			Overall evaluation
	Cancer in humans	Cancer in experimental animals	Mechanistic evidence	
Aspartame	*Limited*	*Limited*	*Limited*	Group 2B
Methyleugenol	*Inadequate*	*Sufficient*	*Strong*[a]	Group 2A
Isoeugenol	*Inadequate*	*Sufficient*	*Inadequate*	Group 2B

[a] *Strong* in experimental systems, including studies in humanized mice and supported by mechanistic studies in exposed humans.

IARC MONOGRAPHS

This volume of the *IARC Monographs* provides evaluations of the carcinogenicity of three agents: aspartame, methyleugenol, and isoeugenol.

Aspartame is a low-calorie artificial sweetener that has been widely used in foods and beverages since the 1980s. Historically, artificially sweetened beverages have been the major source of exposure to aspartame, but to a lesser extent at present since aspartame is typically used in mixtures with other sweeteners. The highest concentrations of aspartame are found in tabletop sweeteners, chewing gums, and food supplements. Other sources include cosmetics and medicines.

Methyleugenol is a flavour and fragrance compound that occurs naturally in essential oils of various plants. It is used in cosmetics and personal care products and as an insect attractant. Although its use as a flavouring agent is prohibited in the European Union and the USA, it is still present in various foods and consumer products due to its natural occurrence in herbs and spices. The general population is ubiquitously exposed through the ingestion of food or use of personal care products.

Isoeugenol is a fragrance and flavour compound that occurs in many plant species and in wood smoke. It is used in food, cosmetics, household products, animal feed, and veterinary medicines. Firefighters and workers involved in isoeugenol synthesis or handling isoeugenol-containing products may be exposed.

An *IARC Monographs* Working Group reviewed evidence from epidemiological studies, cancer bioassays in experimental animals, and mechanistic studies to assess the carcinogenic hazard to humans of exposure to these agents and concluded that:

- Aspartame and isoeugenol are *possibly carcinogenic to humans (Group 2B)*;
- Methyleugenol is *probably carcinogenic to humans (Group 2A)*.