The Quest to Slow Ageing through Drug Discovery

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ABSTRACT

Although death is inevitable, individuals have long sought to alter the course of the ageing process. Indeed, ageing has proved to be modifiable; by intervening in biological systems such as nutrient-sensing, cellular senescence, the systemic environment and the gut microbiome, phenotypes of ageing can be slowed sufficiently to mitigate age-related functional decline. These interventions can also delay the onset of many disabling, chronic diseases, including cancer, cardiovascular disease and neurodegeneration in animal models. Here we examine the most promising chemical interventions to slow ageing, and group them into two tiers based on the robustness of the preclinical, and some clinical, results. We then focus on the potential of the interventions and the feasibility of conducting clinical trials with these chemicals, with the overall aim of maintaining health for longer before the end of life.

[H1] Introduction

Research into ageing is still a small field relative to mature areas, including those focused on major age-related diseases. Publications on cancer, cardiovascular disease (CVD) and Alzheimer's Disease (AD) greatly outstrip those on ageing and gerontology. However, advancing age is the major risk factor for all of these diseases, and recent events have conspired to bring the rapidly expanding field of research into ageing to the forefront. First, global demographic changes are dramatically altering the age-structure of humans on Earth. For instance, a combination of longer lives and declining birth rates has resulted in more people over the age of 65 than under 5, and this trend will continue, with many countries facing a deluge of elders (**FIG. 1a**). Healthspan **[G]** has not kept up with increasing lifespan and, since ageing is the predominant risk factor for most chronic diseases[1-3] (**FIG. 1b**), the greying population is increasingly threatening economic growth and sustainability, with the healthcare sector particularly vulnerable[4,5]. Second, the pharmaceutical sector has spent large amounts of time and resource on development of treatments for age-related chronic disease, with only limited success. Some conditions, for instance neurodegenerative diseases[6,7], remain largely refractory to treatment and most others can at best be delayed. Third, research from animal models, including mammals, has demonstrated that delayed ageing and extended longevity are feasible[3,8-11] and, more importantly, are often associated with an extension of healthspan, the disease free and high functioning segment of life[12-17]. Similar success in humans would improve life quality by preserving functional capacity with age, decreasing disease burden across a wide spectrum of age-related conditions, and would result in dramatic savings in healthcare costs[18,19].

Strategies for combating mechanisms of ageing to prevent disease, known as 'geroprotection', are far reaching, and currently include recommendations for exercise, diet and other aspects of lifestyle. However, these alone are not sufficient to prevent the ills of old age, and increasing efforts are directed to tackling the underlying processes of ageing[3]. These include damage to the genetic material and its packaging and expression, cellular senescence, and dysregulated proteostasis, mitochondrial function, nutrient-sensing, intercellular communication and stem cell function[20]. These hallmarks of ageing are casually connected, and they interact with one another to produce ageing-related decline. Currently, the most promising strategies for geroprotection include mild lowering of the activity of the nutrient-sensing network, especially the mechanistic target of rapamycin protein complex 1(mTORC1) **[Au: Edit OK? Yes OK]** , removal of senescent cells, use of natural metabolites from the systemic environment that can rejuvenate stem cells and transfer of the microbiome. Increasing autophagy, probably including mitophagy, and reduction in age-related inflammation are emerging as key mechanisms by which these interventions exert their effects. The private sector has entered the fray in a large way in recent years, with dozens of companies exploring strategies to target these hallmarks of ageing[21]. An important strategy is development of small molecules, both drugs and natural products, that have geroprotective effects by combating the mechanisms of ageing (**FIG. 2**). A major theme here is the prospect for indication expansion, where small molecules and agents that have good safety profiles and were previously identified for other properties are candidates for repurposing as geroprotectors[22,23].

Several hundreds of potential geroprotectors have been reported to modulate ageing in one or more species (see recent reviews[24-33] for more complete lists). Here, we review a select list of agents grouped as ***Tier1*** or ***Tier 2*** that, in our view, are the most developed experimentally and nearest to clinical testing, or intriguing based on information linked to mechanisms of ageing[20]. To select these agents **[Au: Edit OK? Yes OK]** , we used a set of geroprotector criteria modified from a recent review[26] (**BOX 1**). Tier 1 agents meet the Primary and most Secondary criteria and are generally robust in ageing studies. Meanwhile, Tier 2 compounds are comprised of mature agents that either meet fewer criteria or have generated conflicting data as to their effect on ageing, and emerging compounds that show strong promise, but have not reached a high level of developmental maturity, at least for targeting ageing. We highlight the mechanisms of ageing that have so far been shown to be targeted by these compounds and the strength of the evidence for their geroprotective effects (**FIG. 2**). Shedding light on these geroprotective mechanisms will likely implicate further agents, including new chemical entities, from chemical screens (**BOX 2**) and *in silico* approaches (**BOX 3**) that outperform the compounds reviewed here.

Human ageing may be modifiable and research into ageing is entering a new and exciting phase where interventions to extend healthspan will be tested in humans and, if validated, potentially approved for use in humans. In addition to making the case for clinical testing of a select set of agents, we also discuss potential routes to testing the effects of candidates on human ageing and, if these are successful, how they could be employed to enhance human healthspan.

[H1] Tier 1 [Au: Edit OK? Yes OK]

[H2] Rapamycin and mTOR inhibitors

Rapamycin is a macrolide compound, first discovered in 1960 as an antifungal agent isolated from bacteria in an Easter Island (Rapa Nui) soil sample. It was subsequently found to have immunosuppressive and antiproliferative properties in mammalian cells[34,35]. Rapalogs (Sirolimus, and its derivatives) are used as immune modulators to prevent organ transplant rejection, as cancer chemotherapeutics, and to prevent restenosis after cardiac surgery[36,37]. Rapamycin binds to FK-506 binding protein FKBP12, creating a trimolecular complex with mTOR **[Au: Edit OK? Yes OK]**. This rapamycin-FKBP12 binding event leads to destabilization, and thus inhibition, of mTORC1 **[Au: Edit OK? Yes OK]** , a central regulator of cell and organismal physiology. mTORC1 integrates growth factor, nutritional, stress and other inputs to phosphorylate numerous targets, and modulates cellular processes including autophagy, ribosome biogenesis, protein synthesis and turnover, metabolism of lipids, nucleotides and glucose, as well as cell growth. **[Au: Edit OK? Yes OK]**

Genetic and pharmacological inhibition of mTORC1 activity can increase lifespan in budding yeast[38-40], *C. elegans*[41-44] and *Drosophila*[42,45]. An ageing research milestone occurred with publications from the NIA Intervention Testing Program (ITP) (**BOX 4**) showing that rapamycin extended both median and maximum lifespan of genetically heterogeneous mice when administered starting at either 9 or 20 months of age[46,47]. It is striking that rapamycin treatment can affect longevity even when initiated at 20 months, equivalent to about age 65 in humans, and perhaps even more surprising that a three-month treatment between 20 and 23 months is also sufficient to extend lifespan by up to 60%, based on the remaining lifespan of the animals[14]. An even shorter six week treatment initiated at the same age can also delay

ageing[48]. Notably, and unlike several other interventions, rapamycin has been reported to extend lifespan in multiple mouse strains. Finally, genetic modulation of mTOR signaling can ameliorate ageing in many organisms including mice[49].
Rapamycin not only extends lifespan, but healthspan as well. As a potent anti-cancer agent[50-53] it was proposed that rapamycin extends lifespan solely through an anti-tumour mechanism, suppressing a major pathology in mouse strains[54,55]. However, recently, widespread testing of rapamycin's effects has led to the general conclusion that rapamycin has much broader effects on healthspan. Multiple age-related changes in mice have been reported to be slowed or even reversed by rapamycin treatment, including arterial structure and function[56], cognitive defects[57,58], cardiac hypertrophy and diastolic dysfunction[59,60], periodontitis[61], duration of ovarian function[62], immune senescence[48], multifocal macrovesicular lipidosis in the liver, abnormalities of nuclear size and chromatin conformation in the myocardium, endometrial cystic hyperplasia, adrenal tumours, decline in spontaneous activity and loss of elasticity in tendons[55]. However, cataract severity and testicular degeneration are increased[55]. It should be noted that one study, where rapamycin treatment was started in young, middle-aged and old mice, concluded that, while rapamycin treatment extended lifespan and was able to rescue age-related decline in learning and memory and exploratory behavior, many other traits were either unaffected or even worsened[54]. The reasons for the different findings are not clear. In addition to cancer, rapamycin has also proved protective in a wide range of mouse models of age-related disease, including metabolic diseases such as type II diabetes[63]; neurological diseases[64,65] including AD[66-69], Parkinson's disease (PD)[70], Huntington's[71,72], and Leigh Syndrome[73]; lung diseases[74], cardiovascular syndromes[75] and many others[76].

The effects of rapamycin on ageing have also been investigated in two non-standard animal models. The Dog Aging Project[77] conducted a randomized, double-blind, veterinary, clinical trial to assess safety and effects of a 10-week, low-dose, non-immunosuppressive rapamycin treatment in healthy middle aged dogs[78], which were recruited after an initial screen for factors including existing health conditions. Rapamycin was well tolerated, with no significant adverse effects, and led to an improvement in left ventricular systolic and diastolic function, similar to that previously reported in middle aged mice treated with rapamycin[54,59,60]. The improvement was particularly marked in the dogs with the lowest cardiac function before rapamycin treatment. Rapamycin will now be tested in dogs on a larger scale, including measures of cognitive and heart function, immunity and incidence of cancer[77]. Common marmosets (*Callithrix jacchus*) have been used to assess the effects of long-term (14 months) rapamycin treatment in a non-human primate that has similar age-related pathologies to those seen in humans[79-81]. There were no significant effects on body weight, activity, blood lipid concentrations or markers of glucose metabolism, and there were indications of tissue-specific up-regulation of components of the proteostasis network.

The mTORC1 **[Au: If using m as replacement for mechanistic when first defined is this edit OK? Yes OK]** complex is a central cellular sensor and regulator with multiple inputs and downstream targets (**FIG. 3a**). Several molecular and cellular mechanisms may therefore contribute to the extension of lifespan by inhibition of mTORC1[82]. Deletion of the mTORC1 target S6K1 can extend lifespan of female mice[83], and inhibition of S6K1 kinase activity **[Au: Edit OK? Yes OK]** is required for rapamycin to extend *Drosophila* lifespan[45], although in neither animal have the downstream mechanisms been elucidated. Increased macroautophagy (**FIG. 3b**) also plays a role, since blocking its increase in *Drosophila* treated with rapamycin prevents the extension of lifespan[45]. Rapamycin can also reverse the stem cell dysfunction that occurs during ageing in mouse hematopoietic[48], tracheal and muscle stem cells[84], as well as the intestine in mice[85] and *Drosophila*[86]. mTORC1 is also implicated in

enhancing the survival and secretory phenotype of senescent cells, phenotypes that can be reversed by rapamycin[87-90].

The current clinical uses of rapamycin[91,92] are limited by its toxic side-effects, which include hyperglycemia, hyperlipidemia, kidney toxicity, impaired wound healing, lowered blood platelets and immunosuppression. As well as acutely inhibiting mTORC1 **[Au: Edit OK? Yes OK]** , in some cells and tissues depending on FKBP12 levels[93], prolonged treatment with rapamycin can also indirectly inhibit the mTORC2 complex, probably because rapamycin sequesters mTOR, limiting its availability to form mTORC2 complexes[94,95]. mTORC2 **[Au: Edit OK? Yes OK]** regulates cytoskeletal function, cell proliferation and survival, and importantly activates Akt, controlling the insulin signalling network. Inhibition of mTORC2 can thus impair glucose homeostasis in mice by blocking insulin-mediated suppression of hepatic gluconeogenesis[94]. Rapamycin is approved as an immunosuppressant for transplant surgery, because it can inhibit lymphocyte proliferation by blocking T cell activation[96,97]. Other studies indicate that rapamycin is more immunomodulatory in healthy individuals, with complex effects on specific lymphoid populations[98-103].

Both the animal studies and recent trials with humans indicate that pharmacological inhibition of mTORC1 can be geroprotective with much weaker and briefer inhibition than is used clinically, and with few if any side-effects. Immunosenescence **[G]** is a major problem in elderly humans, leading both to increased infections (particularly respiratory)[104] and a reduced response to vaccination, including against influenza[105]. This age-related decline in immune function is in part attributable to a decreasing ability of haematopoetic stem cells (HSCs) to generate naïve lymphocytes. Elderly mice show a similarly lowered response to vaccination against influenza, and a 6-week pre-treatment with rapamycin rejuvenated HSC function, increasing the level of naïve lymphocytes and boosting the response to immunization[48]. Inhibiting age-related immunosenescence in humans is a practical goal in a clinical trial **[Au: Since immunosenescience is not a specific target, is this edit OK? Yes OK]** , because any improvement can be assessed on a relatively short timescale. A double-blind human clinical trial examined the effects of a 6-week treatment with the mTOR inhibitor RAD001, an analog of rapamycin, on the response to influenza vaccination in elderly volunteers[106]. After a 6-week dosing regimen followed by a 2-week treatment-free interval, volunteers were given a seasonal influenza vaccination. RAD001 was generally well tolerated, particularly at lower doses. These treatments also met the primary endpoint of the study, which was a 1.2-fold increase in the geometric mean titres of antibodies to 2 out of 3 of the influenza strains present in the vaccine, an extent of increase previously associated with a decrease in influenza illness. The increase in titres was greatest in subjects with low baseline influenza titres, suggesting that RAD001 was especially protective in individuals at greatest risk. Although no change in the percentage of naïve lymphocytes was detected, the pooled post-immunisation RAD001-treated cohorts showed a lower percentage of PD-1-positive CD4 and CD8 T cells, which accumulate with age and have an impaired response to antigenic stimulation. A more recent study compared everolimus, another rapalog, with BEZ235, a dual PI3K/mTOR inhibitor, and a combination of the two (which proved most effective), finding that six weeks of dosing was sufficient to substantially reduce infections in the following year[107]. However, a Phase 3 clinical trial failed to reach its primary endpoint[108], and it will be important to resolve the reasons for the diverse findings of these clinical trials.

Targeting the mTORC1 pathway currently carries the strongest preclinical and clinical evidence as a strategy to ameliorate ageing. Strategies to reduce risk include improving dosing regimens for current rapalogs, combination of rapalogs with kinase inhibitors and

development of novel rapamycin variants with altered mTORC1/mTORC2 specificity. More human studies are also needed, but the balance of data suggests that reducing mTORC1 signaling may be a viable strategy for extension of human healthspan.

[H2] Senolytics

Cellular senescence is a permanent cell cycle arrest in normally proliferating cells in response to various stresses, including replicative exhaustion and DNA damage. Senescent cells become resistant to apoptosis and secrete an array of pro-inflammatory molecules and proteases, the 'senescence associated secretory phenotype' (SASP)[109]. Cellular senescence participates in tissue remodelling during development[110] and in wound healing[111], after which the senescent cells are normally removed by macrophages. It is also a potent anti-cancer mechanism, since it occurs in response to stresses that make cells vulnerable to malignant transformation[112]. However, increased NF-kB signalling and expression of the pro-inflammatory cytokines IL-6 and IL-8 are the most conserved and robust features of the SASP, and they can promote cell migration, growth and invasion, angiogenesis and eventually metastasis. Senescence can hence promote, as well as prevent, cancer[113-115]. During ageing in mice, senescent cells persist in multiple tissues, and can cause tissue damage, because the SASP recruits inflammatory cells that remodel the extracellular matrix, trigger inappropriate cell death, induce fibrosis and inhibit stem cell function[109,116,117]. Senescent cells are involved in the aetiology of multiple human age-related diseases, including osteoporosis[118,119], atherosclerosis[120-122], hepatic steatosis[123], fibrotic pulmonary disease[124] and osteoarthritis[114,115,125,126]. Characterisation of the SASP proteomes of senescent cells has provided potential plasma markers for human ageing[127].

Genetic ablation of p16-expressing senescent cells in mouse can rescue features of ageing, including in kidney, heart and fat, with preservation of functionality of glomeruli, cardio-protective KATP channels and adipocytes, respectively. Clearance also increased the median lifespan of the mice[128,129]. Ablation of senescent cells in obese mice resulted in improved metabolic function, reduced circulating inflammatory markers and reduced invasion of white adipose tissue by macrophages[130]. Senescent cells are not abundant, even in aged tissues: a maximum of 15% has so far been reported, and genetic ablation only modestly reduced this number. Senescent cells have autocrine and paracrine effects, and can act at a distance on other cell types[131], which may explain why a mild reduction in their number can be beneficial.

Chemical elimination of senescent cells by senolytics (**FIG. 4**), or disruption of the SASP by senostatics **[G]** , are potentially attractive strategies for combating a broad range of age-related conditions. Inhibition of the SASP would require continuous treatment, since the senescent cells persist. The composition of the SASP varies, depending upon both the original cell type and the nature of the stress that induced senescence, giving the potential for targeting specific senescent cell subtypes. Because senolysis eliminates senescent cells, a brief treatment could be used, with the advantage of leaving cell senescence during wound healing unimpaired. Senescent cells express diverse markers and use a variety of mechanisms to resist apoptosis, providing a further basis for specificity of senolytics.

Senescent cells become resistant to apoptosis. Pro-survival pathways in senescent cells include BCL-2 proteins, PI3K/Akt, p53/FOXO4, HSP90 and HIF1α. Pharmacological targeting of BCL-2 proteins can eliminate senescent cells induced by radiation in mouse lungs and in aged mice[132], but with the side-effect of thrombocytopenia. However, combination of BCL-2 inhibition by the favonoid quercetin with dasatinib, which inhibits

multiple tyrosine kinases, reduced senescent cell number in white adipose tissue and liver, increased cardiac ejection fraction and vascular endothelial function in old mice, and reduced senescent cell burden in several tissues, as well as increasing healthspan in progeroid mice[132]. Intermittent administration of the two drugs improved vasomotor function in aged mice[122], which led to improved cardiovascular function and exercise endurance, and reduced osteoporosis and frailty. A combination of dasatinib and the BCL-2 inhibitor quercetin administered orally to >24 month mice led to a 36% increase in remaining lifespan, and did not cause prolonged late-life morbidity[133]. Combination treatment with dasatinib and quercetin also ameliorated uterine ageing in mice[134]. Because dasatinib and quercetin affect the activity of multiple proteins, it will be important to determine their *in vivo* effects on non-senescent cells, as well as their senolytic activity.

Expression of the transcription factor FOXO4 increases during radiation-induced senescence in fibroblasts, and preventing this increase leads to apoptosis. Perturbing the interaction of FOXO4 with p53 with a FOXO4 peptide caused nuclear exclusion of p53 and apoptosis of senescent cells[135](**FIG. 4b yes please do specify each panel of the figure but the order that they appear in the text should be reflected by the order in the figure**). Doxorubicin induces cellular senescence in mouse and human liver, together with increased expression of FOXO4, and preventing the increase reduced doxorubicin-induced senescence and liver toxicity. Preventing the interaction between p53 and FOXO4 also reduced cellular senescence and several phenotypes of ageing in a mouse model of accelerated ageing, and reduced frailty and loss of renal function in naturally aged mice[135], potentially providing another target for senolysis.

A chemical screen in mouse embryonic fibroblasts with reduced DNA repair capacity identified two HSP90 inhibitors as inducers of apoptosis specifically in senescent cells. Treatment of Ercc1 -/Δ mice, a mouse model of a human progeroid syndrome, with the HSP90 inhibitor 17-DMAG extended healthspan, delayed the onset of several age-related symptoms and reduced p16INK4a expression[136]. HSP90 plays roles in protein folding, stabilisation and proteasomal degradation, and in cellular stress responses. It has been targeted in cancer, although so far no licensed drugs exist. HSP90 has multiple isoforms, and these are targeted by different HSP90 inhibitors, potentially allowing specific targeting of senescent cells[137].

In addition to quercetin, another natural product, fisetin, has been shown to have senolytic properties. A recent report found that administration of fisetin to mice late in life was sufficient to reduce age-related pathology and extend both median and maximum lifespan[12]. This approach offers an attractive alternative to other senolytic compounds that may have greater toxicity. However, and as with other natural products, fisetin has a number of activities[138], making it harder to attribute its beneficial effects to ablation of senescent cells.

More recently, two studies[139,140] report cardiac glycosides as powerful and specific senolytics. These compounds, including digoxin, digitoxin and ouabain, target the Na+/K+ ATPase pump, resulting in disruption of the cellular electrochemical gradient and hence cellular acidification (**FIG. 4a**). Senescent cells already have an acidic pH, which may explain their selective vulnerability to apoptosis when treated by cardiac glycosides. The compounds selectively killed cells in which diverse inducers had led to senescence and, in combination with other chemotherapeutics, they also inhibited tumour xenograft growth, killed senescent pre-neoplastic cells, and attenuated some features of ageing in mice. Cardiac glycosides are used to treat congestive heart failure and cardiac arrhythmias, and these observed senolytic

effects were achieved at clinical doses. These are promising findings that warrant further work with these compounds.

Clinical trials are already underway for the treatment of osteoarthritis by the senolytic UBX0101[141] and of idiopathic pulmonary fibrosis by dasatinib and quercetin[142] (**FIG. 4d**). Targeting senescent cells in ageing is also a promising prospect, but there are important outstanding questions[143,144]. So far, most classified senolytics may also affect non-senescent cells, and any such effects need to be evaluated. Timing of senolytic treatment may also be important, because it could result in exhaustion of stem cells. Finally, failure to clear apoptotic, senescent cells could also be problematic. Therefore, precise targeting of senolytics to specific senescent cell types may help circumvent these potential hurdles. **[Au: Edit OK? Yes OK]**

[H2] Metformin

Metformin is a biguanide drug widely prescribed for type II diabetes[145-147]. In 2013, it was estimated that 83.6% of individuals in the UK with type 2 diabetes were prescribed metformin, and in 2012[148] there were 61.6 million prescriptions for metformin in the USA[149,150]. It is derived from a compound isolated from French lilac (goat's rue, *galega officinalis*), used for centuries as a herbal remedy for treatment of frequent urination (a symptom of diabetes[151]). FDA approval came in 1994[145]. Metformin reduces diabetic hyperglycemia by suppressing hepatic gluconeogenesis, inducing glycolysis and increasing insulin sensitivity[152]; it also reduces lipolysis, and lowers levels of circulating free fatty acids.

Preclinical studies of metformin suggest a role for the drug in mitigating ageing. Metformin robustly increases lifespan in *C. elegans* by up to 36% cite ref 155, and this effect has been attributed to AMP kinase activation[153], mitohormesis[154], the lysosomal pathway and metabolic alterations of the microbiome[155]. Recent studies suggest that alterations of the microbiome may also mediate some of the anti-diabetic effects of metformin in humans[156]. In *Drosophil*a, metformin did not increase lifespan though it did activate AMPK and reduce lipid stores[157]. Initial studies showed effects on ageing in mice[158], but in short-lived mouse models in some cases prone to cancer. Two recent studies have been performed in relatively long-lived C57/Bl6 and genetically outbred mice[159,160] in which slightly increased longevity reached statistical significance in some contexts[159]. Metformin did not increase lifespan in the outbred mice in the ITP (**BOX 4**). The more modest effects seen in long-lived mouse strains is a cause for concern, although another possibility is that metformin may be more effective in more stressful situations that shorten lifespan.

Metformin interacts with several known longevity pathways. Its effects resemble those of dietary restriction **[G]** (DR), including increased insulin sensitivity, and metformin treated mice exhibit DR-like mRNA profiles[160,161]. Mechanistically, the strongest evidence is that metformin inhibits complex I of the electron transport chain, leading to reduced ATP levels and activation of AMP kinase; however, many but not all phenotypes associated with metformin administration are AMP kinase-dependent[162,163]. Consistent with observations in *C. elegans*, metformin also alters mouse and human microbiomes in a manner that appears anti-inflammatory[164-167]. More directly, metformin has been reported to repress TNFα-dependent IκB degradation and consequent expression of inflammatory cytokines, in a manner independent of AMP kinase and mitochondrial action[168-172]. This property may underlie its ability to suppress the SASP in senescent cells[173]. Metformin also binds the alarmin HMGB1 and inhibits its proinflammatory activity[174]. More recently, the H3K27me3

demethylase KDM6A/UTX has been proposed as a direct target of metformin, suggesting a role in chromatin modification[175].

Retrospective, epidemiological analyses of data from patients prescribed metformin have concluded that its use is associated with reductions in: CVD incidence and mortality[176-179]; cancer rates[179-187], and of overall mortality[188]; as well as depression and frailty-related diseases[179]. Meta-analyses of metformin in age-related conditions are also encouraging (but not always positive), with a range of studies showing protection from cancer, CVD, chronic kidney and liver disease, and neurodegeneration. One study detected an 18% increase in median all cause survival in metformin-treated diabetics relative to the rest of the population, despite higher levels of morbidity in the former[189], a finding replicated in a more recent study[190] and in a systematic review of clinical studies[191], but not in another large meta-analysis[192]. However, these studies were all conducted in groups with type 2 diabetes, and metformin could be beneficial for other conditions through mitigation of the type 2 diabetes rather than of ageing. It is thus not clear if metformin would have benefits in non-affected individuals. While intriguing, these clinical epidemiological studies have other limitations from the standpoint of ageing: they assess diabetic patients who have enhanced mortality rates compared to the unaffected population, some studies compare metformin to other diabetes drugs, which could have adverse effects, and metformin users have had contact with a clinician, and hence may on average have greater health-seeking behaviours than some control populations.

The Targeting Aging with Metformin (TAME) initiative was proposed to study effects of metformin on 3,000 non-diabetic subjects aged 65-79 at many centers in the U.S. with an estimated cost of US$50 million[193,194]. Effects of metformin are to be examined on multiple markers of age-related health, including CVD, cancer, dementia and mortality, under the premise that a drug extending healthspan would prevent onset of many distinct age-related conditions[195]. As the TAME trial gets underway, a small, short-term intervention in healthy adults was performed, showing that metformin triggers both metabolic and non-metabolic pathways linked to ageing in non-diabetic individuals of average age 70[196]. Metformin has an excellent safety profile, and the TAME initiative will serve as a benchmark in the development of metformin (and possibly other geroprotectors) for use in humans to offset ageing. However, an experimental study has indicated that metformin can blunt the increases in whole-body insulin sensitivity and skeletal muscle mitochondrial respiration in response to aerobic exercise training in older adults, with marked individual differences in the responses[197]. It will therefore be important to understand how metformin affects muscle physiology and function with and without exercise and the individual variability in responses, and to find predictive biomarkers for positive responders.

[H2] Acarbose

Metabolic dysfunction is commonly observed in human ageing, and type 2 diabetes is a risk factor for several other age-related conditions, including CVD, kidney disease, cancer and dementia[198]. Maintenance of glycemic control during ageing could thus induce multiple health benefits. Acarbose is a bacterial product that inhibits α-glucosidases in the intestine, thus slowing the breakdown of starch and disaccharides to glucose. It is used clinically to prevent post-prandial hyperglycemia[199], and generally causes weight loss and improved glycemic control[198]. Acarbose can rescue age-related glucose intolerance in rats[200] and it has been considered as a potential mimic of DR in mice **[Au: Edit OK ?no would prefer to leave it as it was ie vague – it has been speculated as a DR mimetic for humans]** [201].

In the ITP (**BOX 4**), acarbose increased median lifespan in male mice, by 22%, with only a small effect in females (5%), but maximum lifespan was significantly increased in both sexes (females 9%, males 11%). Body weight was reduced, more so in females, fasting blood glucose levels and IGF1 levels in plasma were lower in both sexes, with fasting insulin levels lower only in males. Acarbose increased healthspan in the mice, with reductions in lung tumours in males, liver degeneration in both sexes, glomerulosclerosis in females, blood glucose response to refeeding in males, and improved rotarod performance in ageing females[202]. In mice **[Au: Edit OK Yes OK?]**, acarbose also reduced post-mortem liver degeneration, lipidosis[201] and hypothalamic inflammation[203] in males, and abolished male-specific insulin insensitivity and glucose intolerance[204], all potentially contributing to the greater effect of the drug on male lifespan, which, interestingly, was abolished by castration[204]. Acarbose-treated mice showed alterations in the composition and fermentation products of their microbiome and in the composition of the short chain fatty acids in the gut, although these effects differed between the 3 ITP test sites[205]. It is likely that acarbose and DR increase lifespan by partly different mechanisms, because DR reduced levels of circulating FGF-21 and increased activity levels, while acarbose had opposite effects on these phenotypes[201]. In summary, while acarbose has some undesirable, although not dangerous, digestive side effects[206], there are ample reasons to evaluated this small molecule in the clinical as it may be among the more efficacious geroprotectors to be identified to date.

Spermidine

Spermidine is a naturally occurring polyamine that plays key roles in control of gene expression, apoptosis and autophagy, and is essential for cell growth and proliferation[207]. Levels of spermidine decline during ageing in both model organisms and several human organs[208,209]. Since supplementation of spermidine in the diet can extend lifespan in yeast, *C. elegans*, *Drosophila* and mice, and addition to the culture medium can increase survival of human immune cells[209-211], it is classified as a geroprotector. **[Au: Edit OK? Yes OK]**. In *Drosophila*, increased production of spermidine contributes to extension of lifespan by reduced insulin/Igf signalling[212]. Additionally, a prospective, population-based study in humans found an association between high levels of spermidine in the diet and reduced all-cause mortality[213].

Spermidine may exert its geroprotective effects by more than one mechanism, with increased autophagy and, in mammals, protection of cardiac and immune function appears to be implicated **[Au: Edit OK?Yes OK]**. Feeding spermidine increases the serum level of free thiols in old mice to levels seen in youth, potentially indicative of reduced oxidative stress[209]. In yeast and mammalian cells, spermidine supplementation decreases histone H3 acetylation[209], with possible functional consequences for gene expression. Spermidine inhibits the acetyl transferase activity of EP300, which in turn inhibits autophagy, by acetylating lysine residues in autophagy-related proteins[214,215]. Accordingly, in yeast, *C. elegans*, *Drosophila* and in human cells, spermidine increases markers of autophagy, and mutants blocking the increase in autophagy prevent the increase in survival in response to spermidine, implying a causal connection[209].

In mice, increased lifespan from supplemented dietary spermidine is associated with a delay in the age-related decline in cardiovascular function[210,211]. Increased dietary spermidine upregulates autophagy, mitophagy and mitochondrial biogenesis and function in the heart[216], and it also improves the mechanical properties of cardiomyocytes in vivo, benefits that are lost if the increase in autophagy is blocked. Furthermore, high levels of dietary spermidine in humans correlate with reduced blood pressure and a lower incidence of CVD[210]. Spermidine

can also enhance immunity. Autophagy declines specifically in B and T cells in aged mice, and a 6-week spermidine treatment attenuated this decline and improved B cell function. Spermidine promotes the hypusination of the translation factor eIF5A, which is required for synthesis of the autophagy transcription factor TFEB, so supplementation with spermidine restored this pathway and reversed the senescence of old human B cells[217]. Because spermidine can reverse the reduction of polyamine synthesis and autophagy observed in aged and osteoarthritic cartilage, it is alaos a promising candidate for prevention of osteoarthritis[218],. Finally, spermidine can also improve stem cell function in muscle of old mice[219], and is neuroprotective in *Drosophila*[220] and mice[221,222].

Clinical trials with spermidine could thus be considered [223], although some caution may be warranted given that targeting of polyamine metabolism is being considered for both chemotherapy and chemoprevention in cancer[224]. As increased autophagy is a recurring theme for geroprotectors, it will be important to understand the downstream mechanisms of protection and the most effective means of inducing them.

NAD(+) Enhancers

NAD+ is a coenzyme that catalyzes a wide range of cellular metabolic functions through cellular redox reactions, in which it becomes converted to NADH. These reactions are scattered throughout the glycolytic pathway, the tricarboxylic acid cycle and β-fatty acid oxidation, among other cellular functions. However, NAD+ also acts as a substrate for sirtuins, Poly-ADP-ribose polymerases and CD38, reactions through which it is consumed[225-227]. Levels of the compound decrease with ageing in mammals[228-230], contributing to a reduction in activity of sirtuins. Strategies to supplement NAD+ levels lead to increased healthspan in mice[231,232]; however, the myriad cellular roles of the small molecule have made it difficult to link phenotypes to its specific biochemical actions.

NAD+ is not taken up by cells, making direct supplementation infeasible. It is possible to exploit NAD+ synthesis pathways through addition of precursors to increase NAD+ levels in vivo, the two most commonly tested in vivo being nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN). Both have been tested in invertebrate and murine ageing studies[225-227]. NR is reported to increase yeast replicative lifespan[233], and both NR and NMN to increase worm lifespan[234]. In mice, NR elicits a wide range of beneficial effects, including a modest lifespan extension[231]. NMN administration in mice also leads to a range of beneficial phenotypes during ageing[232,235], including an improvement in oocyte quality[236], although lifespan has not been reported. Both NR and NMN are also reported to be protective in a range of age-associated disease models[225-227].

Given that NR and NMN are natural products, both are being tested in humans and there is extensive debate over which of the two molecules is likely to be most efficacious. Differences in bioavailability and stability have been reported. NMN is being tested in clinical studies, but findings are yet to be reported. Several studies have been completed with NR, although trial sizes are generally quite small. Common conclusions of the trials are that NR is bioavailable[237], increases NAD+ levels[238,239], and can be administered safely[240]. One recent study showed that NR administration in aged men for three weeks was sufficient to reduce inflammatory cytokine levels[241]. In another study, however, no improvement in metabolism was detectable in obese, insulin-resistant men[242]. Further work is needed to determine whether NR or NMN have advantageous properties in humans. Given that NAD+ precursors are natural products that are already on the market, it will be critical to better define their effects on healthspan.

NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat mild-to-moderate pain and, at higher doses, decrease inflammation. The primary target for this class of drugs, which include aspirin and ibuprofen, are cyclooxygenases (COX-1 and COX-2)[243], although NSAIDs also have antithrombotic and anti-oxidant activities that probably occur through at least partially different mechanisms[244]. Aspirin (acetylsalicylic acid) is reported to extend lifespan in *C. elegans*[245], *Drosophila*[246,247] and male mice[248], possibly attributable to a higher ratio in males of aspirin to its metabolite salicylic acid. However, this lifespan extension was not repeatable by the ITP (**BOX 4**). In *C. elegans*, lifespan extension by aspirin requires DAF-16/FOXO, AMPK, and LKB1, but not SIR-2.1, and aspirin does not further extend the lifespan of DR animals[245]. In mammals, both COX-dependent and independent effects of aspirin, which include activation of AMPK and consequent inhibition of mTORC1[249], together with inhibition of IKK-β[250] and Wnt/β-catenin [251], mean that multiple mechanisms could contribute to altered ageing[244].

Interestingly, ibuprofen has been reported to extend lifespan in yeast, worms and flies, through a mechanism (at least in yeast) that involves degradation of the tryptophan transporter, reducing intracellular amino acid pools and consequent mTOR inhibition[252]. A third NSAID, celecoxib, has also been reported to extend *C. elegans* lifespan, through an insulin-IGF dependent mechanism[253]. Ibuprofen, aspirin, and celecoxib have not been directly compared to determine if they function through common mechanisms for lifespan extension. Another NSAID, a nitrosylated variant of flurbiprofen, failed to alter mouse lifespan[248].

Epidemiological evidence links NSAIDs to protection from a range of chronic diseases of ageing. For instance, long-term aspirin users are reported to experience a 33% reduction in colorectal cancer incidence and mortality[254,255], by mechanisms that remain unclear. In a panel of tumour lines, aspirin strongly suppressed cell size and cell growth, with lowered phosphorylation of the mTORC1 targets p70S6K and S6, through both AMPK-dependent and AMPK-independent mechanisms[256]. Aspirin may also reduce metastatic spread, possibly through a platelet-mediated mechanism[257,258]. Multiple epidemiological studies of aspirin in primary prevention of stroke, myocardial infarction/coronary events and cardiovascular death in those without a history of CVD have concluded that aspirin modestly reduces nonfatal myocardial infarction/coronary events and major CVD events, and at daily doses of less than 100 mg reduces the incidence of stroke, with both effects increasing with age[259]. However, aspirin also increases major gastrointestinal bleeding risk, thus complicating conclusions about net benefit[259]. Similarly, ibuprofen has been reported to reduce the risk of both AD and PD[48,260], although these findings remain controversial[261].

Unfortunately, clinical trials with aspirin in primary prevention have largely failed to confirm the promising epidemiological findings. Trials with healthy elders found no evidence of protection against cardiovascular events and there was an observed increase in gastrointestinal bleeds incidences[242,262]. Although there was some protection against cardiovascular events in adults with type 2 diabetes, this protection was counterbalanced by major bleeding events[263]. One trial found no effect of aspirin use on disability-free survival[264] and a significant increase in all-cause mortality, primarily attributable to increased incidence of cancer[265]. Although NSAIDs have some characteristics of geroprotective drugs, the current clinical evidence raises significant doubts regarding ageing studies in humans.

Now that the results of the clinical trials have been published and have not been challenged, I think NSAIDs should go into Tier 2 – thanks for pointing this out

Lithium

By the middle of the 19th century lithium carbonate was used as a medical treatment for a range of disorders, including cancer, where now it is mainly used to treat bipolar disorder. Lithium induces a dose-dependent extension of lifespan in fission yeast[266], *C. elegans*[267-269] and *Drosophila*[270], with higher doses highly toxic to survival. In *C. elegans* and *Drosophila*, locomotor performance during ageing is also maintained by lithium treatment[269,270]. Experimental effects of the drug on mammalian lifespan have not yet been reported. In humans, lithium treatment has been associated with longer leukocyte telomeres[271], and natural levels in the drinking water in parts of Japan have also been linked to lower suicide rates[272,273] and reduced all-cause mortality[268]. Mesenchymal stem cells from ageing humans show impaired myogenic differentiation, a defect associated with impaired Wnt/β-catenin signalling, which can be rescued by lithium[274]. Lithium is neuroprotective[275,276] and can ameliorate pathology in several animal models of disease, including Alzheimer's disease, Huntingtons disease and stroke[277-279].

Lithium has multiple targets, and its mode of action as a drug in humans is incompletely understood. It can induce autophagy in mammalian cell culture, by inhibition of inositol monophosphatase[280]. Consistently, extension of lifespan in *C. elegans* is accompanied by increased autophagy, as well as increased mitochondrial DNA copy number and enhanced energetics[269]. In *Drosophila*, lifespan-extension is mediated by suppression of GSK3 and hence activation of the cap-n-collar transcription factor CncC, the fly orthologue of mammalian NRF2, accompanied by a hormetic response to lithium itself and to xenobiotics[270].

There is yet no evidence for geroprotective effects of lithium in mammals, and its narrow therapeutic range is a problem for widespread, long-term use. If activation of autophagy is a mediator of its benefits, other inducers could be used or it could be combined with, for instance, mTORC1 inhibitors, to allow lower doses with fewer side-effects[281]. Clearer identification of the therapeutic targets of lithium, especially in mammals, may also yield more specific drugs.

[H1] Tier 2

[H2] Reverse Transcriptase Inhibitors

The human genome is littered with a large number of repeated elements, among which long interspersed nuclear elements (LINEs) are the most prevalent, comprising roughly 20% of the mouse and human genomes[282,283]. A subset of the 6 kilobase LINEs are fully functional retrotransposable elements, relying on an encoded reverse transcriptase to excise from the genome and reinsert in other locations, and are hence a source of genome instability[284-286]. LINE1 activation has been linked to age-related diseases[286-288] and is also prevalent in a progeroid model, *Sirt6*$^{-/-}$ mice[289,290]. Consistently, Sirt6 enzyme activity represents one of several mechanisms by which cells suppress LINE1 activation[289].

A number of nucleoside reverse transcriptase inhibitors (NRTIs) have been generated and used in the clinic to inhibit HIV reverse transcriptase and, fortuitously, some of these also impair the RT activity associated with ORF2 of LINE1[291,292]. Two recent studies reported that

NRTIs can ameliorate pathologies linked to ageing in mice[293,294]. In both studies, LINE1 elements, expressed specifically in late-stage senescent cells, are not restricted to the nucleus and accumulate in the cytoplasm and activate the IFN-1 interferon response, which may underlie induction of the SASP and some of the chronic inflammation associated with ageing. In one report, the NRTIs lamivudine and stavudine were found to reduce DNA damage, suppress in vivo pathology and extend the lifespan of *Sirt6*$^{-/-}$ mice[293]. This study also found activation of LINE1 elements with ageing, as previously reported. A contemporaneous report focused on cell senescence, and reported that lamivudine reduced the SASP and inflammation in aged mice[294]. It is yet to be demonstrated that NRTI treatment results in enhanced mouse longevity, although it is reported to reduce DNA methylation age[293], an emerging biomarker of ageing. These findings make NRTIs interesting new geroprotector candidates. However, any strategy to employ NRTIs as a means to enhance human healthspan will have to account for their associated side effects in the clinic[295].

[H1] Systemic Circulating Factors

Dysregulated intercellular communication is a hallmark of ageing, and is characterised *inter alia* by age-related, sterile inflammation, often known as 'inflammaging'[296,297], and a deteriorating systemic environment that impairs the function of multiple tissues[298,299]. Therefore, altering the concentration of select blood metabolites to improve health during ageing is receiving increased attention. **[Au: Edit OK? Yes OK]**

Heterochronic parabiosis, where the circulatory systems of mice of different ages are shared[300], has shown that stem cell regenerative capacity of old mice is improved by a young systemic environment in muscle[301,302], liver[301], spinal cord[303] and brain[304]. Young blood can also reverse the age-related: structural deterioration and molecular changes in mouse kidney[305]; decline in β-cell replication[306]; and decline in bone repair and regenerative capacity[307]. **[Au: Edit OK? Yes OK]** In an extension of the parabiosis concept, human umbilical cord plasma administered to immunocompromised mice induced expression of genes in the hippocampus that suggested increased long-term potentiation and memory, and it also increased long-term potentiation in hippocampal brain slices and improved cognitive function in old mice[308].

Progress has been made in identifying the mechanisms that impair the ageing systemic environment. For instance, exposure to young blood ameliorates declines in cognitive function, and of dendritic spine density and synaptic plasticity in hippocampal neurons of ageing mice, mediated in part by activation of the cyclic AMP response element binding protein CREB[309]. Levels of β2-microglobulin, a component of major histocompatibility complex class 1 (MHC I) molecules, increase during ageing and negatively regulate cognitive function and regenerative capacity in the hippocampus of ageing mice[310]. In addition to these identified components, Tet2 catalyses the production of 5-hydroxymethylcytosine (5hmC), and the levels of both Tet2 and 5hmC **[Au: Edit OK? Yes OK]** decline in mouse hippocampus during ageing. Tet2 expression levels are restored in the hippocampus of old heterochronic parabionts, and inhibition of Tet2 expression in young mice impairs neurogenesis and cognitive function, while Tet2 **[Au: Edit OK? Yes OK]** overexpression restores them and 5hmC levels in old mice[311].

The growth differentiation factor 11 (GDF11) levels in mouse blood have been reported to decline with age. Age-related cardiac hypertrophy in old mice is ameliorated by exposure to young blood, and a proteomic screen identified GDF11 as a mediating factor[312]. Furthermore, supplementation of GDF11 by heterochronic parabiosis or direct delivery restored stem cell

function and structure, increased strength and endurance exercise capacity in ageing mice[313], and increased cerebral blood flow, neural stem cell proliferation and olfactory neurogenesis and function[314]. Other studies have reported that GDF11 levels in rat and human sera instead increase with age, and that administration of GDF11 inhibits muscle regeneration and stem cell division in mice[315]. To this end, the specificity of detection of GDF11 may have played a role in these discrepant findings[316].

In addition to the components described above, young blood reverses the declines in fracture repair and osteoblastic differentiation capacity of old mice by modulating signalling through β-catenin[307]. Combined proteomic analysis of human umbilical cord plasma and of changes in mouse plasma during ageing produced a list of candidate proteins for rejuvenation of the ageing mouse hippocampus. One of these, TIMP2, could improve learning and memory in aged mice when directly administered, while depletion of TIMP2 in cord plasma abolished its rejuvenating effects[308]. The protein VCAM1, a member of the immunoglobulin superfamily, increases in expression in mouse and human plasma during ageing. It is induced in endothelial cells in response to inflammation and facilitates leucocyte tethering. Anti-VCAM1 antibody administration, or ablation of Vcam1 specifically in brain endothelial cells, counteracted the adverse effects of plasma from aged mice on microglial activation, neural progenitor cell activity and cognition in young mice[317].

Identification of blood factors that improve the youthful, or impair the ageing, systemic environment is opening translational opportunities. A preclinical study showed that parabiosis with a young mouse or direct administration of plasma from young mice could ameliorate molecular defects in hippocampus and impaired working memory in a mouse model of AD[318], and a recent, randomised clinical trial concluded that administration of young plasma to patients with mild to moderate AD dementia was safe, tolerable and feasible[319]. Given the ready accessibility of the human circulatory system, modulation of its molecular composition is an approach of considerable promise.

[H2] The Gut Microbiome [Au: Edit OK? Yes OK although in future eg. skin microbiome may turn out to be also of potential interest]

The vast assemblage of microorganisms associated with animals is increasingly recognised as playing a significant biological role. The population size and composition of the gut microbiome both change with age in *C. elegans*, *Drosophila*, mice and humans[320-323], which affects metabolite composition and is likely important for changes in health. A broad spectrum improvement in health in diverse organisms is induced with DR. In mice, transfer of gut microbiome from subjects following DR to a sterile recipient resulted in reduced weight gain and increased glucose tolerance, insulin sensitivity, glucose uptake into white adipose tissue , which also resulted in browning of this white adipose tissue[324]. These results suggest that some of the health benefits of DR may be caused by changes in the composition of the microbiome. Transfers of gut microbiome of young turquoise killifish to older recipients delayed the age-related changes in microbiome composition and improved swimming performance and extended the lifespan of older recipients[325]. It is not yet understood how these health improvements from microbiome transfer are mediated, but they likely involve changes to the overall composition of metabolites. Therefore, it will be important to identify these metabolite changes to understand the downstream biological effects and determine if this can offer a more standardised intervention to improve health during ageing. **[Au: Edit OK? Yes OK]**

[H2] Glucosamine

Glucosamine is an essential amino-monosaccharide component of glycoproteins, proteoglycans and glycosoaminoglycans. It is widely used as a supplement for individuals with osteoarthritis, but recent literature suggests that it has potential benefits for a wide range of chronic diseases, including cancer, skin disorders, and CVD[326]. Glucosamine extends lifespan in *C. elegans* and confers modest extension when administered to old mice[327]. In worms, glucosamine was found to extend lifespan in a manner independent of the hexosamine pathway, through a mechanism that may mimic a low carbohydrate diet. Consistently, AMP kinase was activated and mitochondrial biogenesis was enhanced. Interestingly, glucosamine stimulated reactive oxygen species (ROS) production, a finding consistent with reports that under some circumstances increased ROS production in worms can enhance longevity[328] and trigger AMP kinase activation[329]. Studies in mice correlated with these findings, as mitochondrial biogenesis was also found to be enhanced.

Glucosamine exhibits a wide range of other effects in mammals that could be linked to ageing[326], including acting as an anti-inflammatory agent, inhibiting mTOR and stimulating autophagy, paradoxically acting as an anti-oxidant and, through its conversion to uridine diphosphate (UDP-N-acetylglucosamine (UDP-GlcNAc), as a substrate for O-GlcNAc modification of proteins, which itself is linked to many protective effects against chronic disease[330]. Glucosamine and related molecules need to be evaluated further to determine the mechanisms by which they act and to validate them as geroprotective agents.

Glycine

A recent study by the NIA ITP (**BOX 4**) showed that glycine supplementation leads to both median and maximum lifespan extension in mice of both sexes[331]. This finding supports an earlier report of enhanced longevity in rats[332] and recent studies in *C. elegans*[333,334]. In both of the rodent studies, glycine administration was associated with weight loss, but only in females. Glycine has also been reported to have anti-cancer and anti-inflammatory effects in rodents[335-337]. In limited human clinical studies, glycine supplementation may be protective in the context of metabolic diseases[336,338], although larger studies are needed.

A number of nutritional studies point to reduced amino acids being associated with longer lifespan, making the results with glycine potentially paradoxical[339-342]. However, glycine has a unique property in that it is an acceptor of methyl groups in the catabolism of methionine by glycine N-methyl transferase, and thus serves an important role in hepatic methionine clearance[338]. Methionine restriction is known to enhance longevity in several ageing model organisms[343], but is difficult to implement this restriction practically **[Au: Edit OK? Yes Ok]**, so glycine supplementation could be a preferable alternative.

The mechanisms by which glycine extends lifespan may however be more complex, because glycine supplementation in *C. elegans* may influence one carbon metabolism and the production of S-adenosyl methionine, resulting in transcriptional changes through epigenetic mechanisms[333]. Serine, which also acts in one carbon metabolism, was also found to extend lifespan through a similar mechanism[333]. *C. elegans* may have key differences from mammals regarding amino acid supplementation, as a prior report found that a supplementation of a wide range of amino acids led to lifespan extension[334]. In summary, there is significant promise for glycine in modulating ageing; however efforts to understand its modes of action are essential.

17-alpha Estradiol

17-alpha estradiol (17α-E2) is a nonfeminizing estrogen with reduced affinity for the estrogen receptor. The NIA ITP demonstrated that 17α-E2 extends lifespan preferentially in male mice[201], and a later study replicated this sex difference even at higher doses[344] (**BOX 4**). The male-specific benefits extend to metabolic phenotypes, including increased insulin sensitivity and glucose tolerance [204], and require gonadal hormones, as castrated males were refractory to 17α-E2, while ovariectomized females showed a metabolic response. The metabolic benefits were linked to increased hepatic mTORC2 and AKT signalling, accompanied by FOXO1A phosphorylation. In young mice, 17α-E2 reduced overall body mass and increased the lean-to-fat mass ratio[345]. In older mice, however, the hormone preserves body weight and muscle strength in males[346].

Several findings link the beneficial effects of 17α-E2 to effects on brain function. First, 17α-E2 is the predominant form of estradiol expressed in the rodent brain, and has been postulated to have neuroprotective roles in humans. It has also been reported to confer protection from oxidative stress, as well as amyloid toxicity associated with AD and PD in animal models[347-350]. Also, the metabolic and longevity benefits of 17α-E2 may be attributable to effects on the hypothalamus, as treated mice have reduced food intake, probably due to activation of hypothalamic anorexogenic pathways[345,351]. 17α-E2 is also highly anti-inflammatory, both in adipose tissue and in the hypothalamus[203,345]. In summary, 17α-E2 reduces food intake, possibly mimicking DR, leading to improved metabolic function and reduced age-associated inflammation, but the mechanisms by which 17α-E2 confers these effects requires further studies.

[H1] THE PATH TO HUMAN INTERVENTION

Animal models have been highly successful in generating candidate healthspan interventions, many of which work in mammals, which raises a question in many researchers' minds. How do we test these interventions in humans and accelerate their widespread use to extend our healthspan? The long lifespan of humans makes direct testing barely feasible. Instead, three major approaches have been implemented, of which two have been illustrated in prior sections.

A first approach, used most widely, has been to test longevity interventions in the context of disease indications, including the evaluation of sirtuin activating compounds in clinical studies with psoriasis and ulcerative colitis as indications[352]. Interestingly, these diseases are not naturally linked to ageing and sirtuin activating compounds have yet to be clinically approved. While this is perhaps the most direct approach, ameliorating ageing is not the same as treating a disease process, and most indications are that geropotective drugs will act preventatively, rather than as treatments for age-related conditions. More recent approaches have chosen diseases or processes more closely linked to ageing, including senolytic approaches to treat osteoarthritis and idiopathic pulmonary fibrosis as well as evaluating the use of rapalogs to reverse immunosenescence. These studies show more promise as they progress toward clinical use. However, whether this approach is an effective avenue to move interventions toward primary prevention in healthy people to keep them disease free and functional for longer has yet to be determined.

A second approach is embodied by the TAME trial with metformin and more directly addresses the promise of interventions that slow ageing: to prevent multiple chronic diseases simultaneously. Clinical testing based on ageing itself as an indication is now permitted by the FDA, and in 2018 the World Health Organization ICD-11 for the first time included an extension code "Ageing-Related" (XT9T) for ageing-related diseases, thus recognising aging as a major risk factor for these diseases. The upside of this approach is that, if successful, a path toward widespread use in at risk populations can be readily imagined. The downside is the cost and duration of the study, which will follow over 3000 people for up to three years. Therefore, the approach is cost prohibitive for studies of a large number of interventions and, given that it is only conjecture to know which will work best at this stage, other approaches are warranted. The TAME trial is potentially groundbreaking in ageing research; however, multiple types of interventions should be used at the beginning of human intervention studies as we learn the best paths forward.

A third, and promising, approach is only now becoming feasible. Until recently, measurements of ageing have been limited largely to physiological or functional measures, including walking speed, pulse wave velocity, VO_2 max, and measures of organ function. However, using artificial intelligence strategies to analyze deep datasets, several molecular biomarkers that can be generated using non-invasive or minimally invasive strategies have been proposed to measure biological age. Among these is the epigenetic clock[353,354], which integrates DNA methylation data from over 300 sites in the genome and can be assessed in multiple tissues, including peripheral blood mononuclear cells. In mice, where a similar clock has also been elucidated, anti-ageing interventions can delay clock progression[355]. Other biomarkers have been proposed, including transcriptomic and metabolomic profiles of blood, complete blood counts, accelerometry data on iPhones and even facial pattern recognition[356]. None of these biomarkers have been fully validated, but they offer great promise. However, there are major questions: How will these biomarkers respond to longevity interventions? Are they dynamic? Will interventions slow the rate of clock progression or reverse the clock? How do the different clocks relate to each other? Do different biomarkers inform on different aspects of the ageing process? Will it be possible to detect individual differences in the progress of the different mechanisms of ageing and thus tailor geroprotective interventions? Despite the many unanswered questions, the discovery of biomarkers and clocks is a major breakthrough that opens the possibility of using them as primary endpoints in the clinic, if they can be tied to changes in clinical outcomes. These discoveries may open the way to relatively short-term, smaller studies determining which interventions alter which clocks. Human studies using these biomarkers are only just beginning in earnest. Given that none of them may be validated by regulatory agencies in the near future, these studies may only be an entry point to identify interventions with the largest possible impact on ageing, leading to studies like the TAME trial as a step to clinical approval.

A final approach is to avoid drugs altogether and develop natural products as supplements to slow ageing. These compounds are less tightly regulated than are drugs, and many are already quite legally marketed as treatments for a wide range of conditions, often without clear clinical evidence to support their use. In the context of geroprotection, a combination of two compounds to modulate NAD(+) and sirtuin activity is being marketed and has undergone limited human testing. Other reagents to enhance NAD(+) levels are also available. This approach has the advantage of rapidly reaching a large population, but raises important questions about how marketed products can be safety-tested and experimentally validated. The natural product market is thus a double-edged sword – quicker to market but less regulated. These compounds should be tested in scientifically rigorous, placebo-controlled

trials, to demonstrate that the benefits of supplements outweigh any risks and the costs to the consumer. Can public studies be performed using non-invasive biomarkers? Again, although many unvalidated products are sold as "anti-ageing," we are at an early stage in terms of generated, validated products.

Ageing is a complex process, and no geroprotective intervention has ameliorated all of its features, with DR so far coming the closest. Genetic studies in model organisms have indicated that combinatorial interventions targeting different pathways can be the most effective in ameliorating ageing[357-359]. The same is likely to be true of pharmacological interventions, and indeed combinatorial treatments in yeast[360], *C. elegans*[361] and *Drosophila*[362] have proved more effective than administration of single agents. The evidence from animal studies, and our understanding of human ageing, indicate that multiple approaches to ameliorating the effects of ageing should be pursued in parallel.

While human translational studies in ageing are at an early stage, they represent a major step forward in ageing research. We have yet to understand how achievable or difficult **[Au: Edit OK? Yes OK]** it will be to lessen the effects of human ageing and what will be the best methods to validate success. Nevertheless, it is now possible to envision geroprotective strategies to delay the onset of many debilitating diseases and maintain function later in life.

[H1] CONCLUSIONS

After long and laborious studies on the fundamental drivers of the ageing process, numerous small molecules have emerged as candidates to delay human ageing, prevent disease onset and/or progression and maintain human functional capacity later in life. While individual scientists certainly have their favorite candidates, there is an emerging consensus on what the best approaches will be. Mild inhibition of the activity of the nutrient-sensing network, particularly of mTORC1, is a promising strategy and is currently furthest down the road to clinical validation and delivery. A major challenge will be to identify the most effective targets for health improvement, which may be tissue-specific and hence require further drug development, combined with the fewest side-effects, which will require fine-tuning of dose and timing of drug administration. Senostasis and senolysis are also promising strategies, and further experimental work in animals and clinical trials are needed to determine the safety and efficacy of these approaches in humans, and also any potential negative side-effects, especially on the longer term. Localised, compartment-specific treatment, for instance of arthritic knees, may be safer and more effective than systemic administration. Although our understanding of potential geroprotective effects of systemically circulating molecules is in its infancy, the experimental results with mice strongly encourage further research to understand the rejuvenating effects. Experimental work on ageing with the microbiome is also in its infancy, but holds great promise. It is also highly likely that new interventions, better than the ones we know about today, will emerge **[Au: Edit OK? Yes OK]** . Nevertheless, excitement is rising as interventions begin to be tested in the clinic, and there is a general expectation that at least some are likely to prove efficacious on the reasonably short term.

While a number of challenges remain, including regulatory hurdles, clinical design questions, incompletely validated biomarkers of human ageing, and commercial challenges to bring the

new interventions to market, it is likely that strong evidence will emerge in the near future for feasible strategies to delay human ageing. While it will be incumbent to administer these interventions in a safe manner that is inclusive of everyone regardless of financial capacity, this approach has the promise to tilt medical treatment away from “sick” care and more towards broad spectrum prevention, a major advance that can revolutionize medicine, maximizing improvement of life quality and mitigating the soaring costs of age-associated chronic diseases.

Box 1. Geroprotector inclusion criteria

A number of agents have been reported to affect ageing in animal models, and in a few cases, some data exists in humans. Rather than provide a complete list, we have chosen to focus on a smaller subset, classified as Tier 1 or Tier 2 based on published geroprotector inclusion criteria, which we have modified as described.

Primary Inclusion Criteria:

- Increased lifespan in animal models
- Amelioration of Human Biomarkers of Ageing
- Minimal side effects at therapeutic dose
- Reproducibility in multiple species and/or different strains of a mammalian species
- Acceptable toxicity

Secondary Inclusion Criteria

- Evidence for target pathway in ageing, ideally in humans
- Increased stress resistance
- Protection from multiple age-related diseases

Box 2. *In vivo* screening to identify geroprotectors

Given that ageing studies are long in duration and costly, direct screening for geroprotectors has been of limited feasibility. Nevertheless, several approaches have led to interesting candidates. A relatively direct approach performed screens of over 80,000 small molecules for extension of lifespan in *C. elegans* when administered in early adulthood[363,364]. A range of molecules was identified, including those: resembling serotonin or dopamine; increasing oxidative stress resistance; and affecting several signaling pathways.

In *C. elegans*, a separate approach screened for molecules that induce multiple forms of stress resistance and then tested their effects on lifespan[365]. Using surrogate phenotypes, such as stress resistance, can make the screening technique easier, but restricts the classes of molecules identified. A similar approach was used in yeast, where a correlation was found between properties of G1 cell cycle progression and replicative lifespan. FDA approved compounds were identified first for the cell cycle effect and then tested for longevity, leading to the identification of ibuprofen and other molecules[252,366]. The labour-intensive nature of the yeast replicative ageing assay has precluded large compound screens, but high-throughput ageing analysis has recently been developed, opening the way for more comprehensive screens[367,368].

Screens in mammalian cell culture have also been performed in a recent approach identifying compounds that reduce senescent markers[369]. Interestingly, the two most potent compounds identified also robustly extended *C. elegans* lifespan, pointing once again to the conserved nature of longevity pathways across species **[Au: Edit OK? Yes OK]**. Despite the difficulty of chemical screens to identify geroprotectors, this approach has proven fruitful and, with more high throughput analysis and better surrogate phenotypes to assess, the approach may grow in significance in the near future.

Box 3. *In silico* approaches to identify geroprotectors

In silico approaches have in general used structural and genetic information, or a combination of both, to identify candidate geroprotectors[370]. Databases such as Digital Aging Atlas[371,372], Human Aging Genomic Resources (HAGR)[373,374] and Aging Clusters[375], are powerful repositories of diverse ageing-relevant data.

Structural approaches
Structural information can be used to find chemical similarities between compounds and hence candidate geroprotectors.

Compounds that increase lifespan in *C. elegans* were identified from DrugAge in HAGR and in an experimental screen[364]. Their structures were found using PubChem[376,377], ChemSpider[377,378] and the literature. Resulting molecular descriptors were combined with drug-protein interactions from STITCH[379] to identify other candidates for increasing worm lifespan, one of which, 2-bromo-4'-nitroacetophenone, was experimentally validated[380].

In a different structural approach, 2054 putative ageing genes from 9 model organisms were identified in GenAge, and 94 prioritized based on their effect on lifespan. These were screened against all DrugBank compounds for similarities in ligand-binding structures, yielding 31 candidates. Several of these were validated as extending lifespan or healthspan in a rotifer[381]. A related study associated the gene ontology terms and chemical descriptors for the protein targets of compounds in DrugAge that extended worm lifespan, to identify related drugs as candidate geroprotectors, but these were not further validated[382].

Genetic approaches
Changes in gene expression with age, in response to genetic and environmental interventions that ameliorate ageing, and in response to treatment with drugs or small molecules, have been used to identify candidate geroprotectors.

Transcriptional profiles of bone marrow cells from young and old humans were compared with the profiles from 70 drugs that extended lifespan in *C. elegans*. Candidates from the overlap were analysed for their effects on phenotypes of late passage human embryonic lung fibroblasts, and were enriched for compounds that restored their phenotypes to a younger state and increased their long-term survival[383].

Several studies have been based upon age-related changes in gene expression in human tissues, from the GTEx database. Age-related changes in gene expression in ageing human brain were combined with data from the Connectivity Map to identify 24 small molecule candidates, significantly enriched for compounds that had already been shown to extend lifespan in worms or fruit flies[384]. In a closely related approach, changes in gene expression with age in multiple tissues were combined and used to identify small molecules in Connectivity Map that shifted the transcriptional profile towards a 'young' one, and identified 31 candidates that were significantly enriched for known geroprotectors and for novel compounds that proved to extend lifespan in *C. elegans*[385]. Similarly, expression profiles from young and old human adipose tissue were used to calculate gene co-expression networks, and the Connectivity Map then interrogated for small molecules that reversed the age-associated changes[386].

In a broader use of genetic information, ageing-related gene products in humans from Aging Clusters were combined with their interactions with compounds in STITCH and DrugBank. 19 compounds were enriched for ageing-related targets, 6 of which were already shown to have pro-longevity properties in animal models, a significant enrichment. Tanespimycin, an inhibitor of HSP-90 was the top-ranked novel candidate, and was shown to increase lifespan in *C. elegans* through its HSP-90 target[387].

Gene expression profiles from rat cells exposed to sera from DR rats or rhesus monkeys were used to identify 39 genes that had human orthologs, and these were compared to gene expression changes in Connectivity Map[388], a database of expression profiles from a panel of human cell lines responding to treatment by drugs and unlicensed small molecules[389,390]. Profiles from 11 of the 39 candidate drugs mimicked those of DR, and 3 of these, rapamycin, LY-294002 and trichostatin A, had already been show to increase lifespan in *C. elegans* (see also section on rapamycin and mTOR inhibition). These 3 candidates and allantoin increased normal worm lifespan and rescued the age-related decline in pharyngeal pumping[391].

Box 4. The National Institute on Aging Interventions Testing Program (ITP)

The ITP[392] tests the potential of interventions delivered in the diet to promote healthy ageing. Both sexes of a genetically variable population of mice, the result of a 4-way cross among inbred strains, are evaluated in 3 different centres (Jackson Laboratory, University of Texas Health Science Center, University of Michigan), at numbers sufficient to detect a 10% increase in lifespan with 80% power. The three testing sites use standardized operating procedures, including diets, caging, bedding and mouse handling. Interventions for testing are proposed by the research community through an annual call-for-proposals, and tested compounds have ranged from drugs and dietary supplements to micronutrients and metabolic intermediates[47].

Positive Findings from the ITP

Acarbose (see text) – Increased lifespan in both males and females, but the effects were greater in males, when initiated at 4 months of age[201]. When initiated at 16 months of age overall lifespan was extended only in males, but maximum lifespan in both sexes[344].
Aspirin (see text) – Increased lifespan in males but not females[248]. A later study failed to replicate lifespan extension with higher doses[331].
Glycine (see text) – Increased median and maximum lifespan in males and females[331]
Nordihydroguaiaretic acid – Increased mean lifespan in males but not females[248], even at doses that gave equivalent blood levels in males and females[201].
Protandim® – Increased lifespan in males but not females[344].
Rapamycin (see text) – Increased mean and maximal lifespan in both males and females when initiated at 20 months of age[46] and when initiated at 9 months of age[47]. Females responded more robustly than males at equivalent doses and blood levels of rapamycin were greater in females; when ~ equal blood levels were achieved, the response of lifespan was about equivalent in females and males[47].
17α Estradiol (see text) – Increased lifespan in males but not females, at 4.8 ppm dose[201] and 14.4 ppm dose[344].

Negative findings from the ITP
Curcumin
Fish oil
Green tea extract
HBX (2-(2-hydroxyphenyl) benzothiazole)
INT-767 (FXR,TGR5 agonist)
Inulin
Medium-chain triglyceride oil
Metformin
Methylene blue
Nitroflurbiprofen
Oxaloacetic acid
Resveratrol
Simvastatin
TM441, pan-inhibitor of PAI-1
Ursodeoxycholic acid
Ursolic acid
4-OH-α-phenyl-N-tert-butyl nitrone

Figure 1. Age composition of the population and incidence of major age-related diseases. Changes in the age composition of the global human population over time, showing the decline in 0-15-year-olds and the increase in 65+year-olds. Plotted from data in https://population.un.org/wpp/DataQuery (panel a)**.** The incidence of three major age-related diseases, dementias, cardiovascular disease and neoplasms, in two low (Afghanistan and Ethiopia), two middle (India and Brazil) and two high (Japan and Switzerland) income countries. Rates are normalized to incidence at age 20 (cardiovascular disease and neoplasms) or age 40 (dementias) for each country because of the strong relationship between overall incidence rate and average income, indicating variation in rates of diagnosis. Plotted from data in the Global burden of Disease Study 2017 http://ghdx.healthdata.org/gbd-2017. (panel b).

Figure 2. Agents and their influence on different hallmarks of ageing. Geroprotective agents, small molecules and metabolites ameliorate one or more of the hallmarks of ageing to prevent ageing-related decline in function and ageing-related diseases. *Impaired protein homeostasis also includes autophagy.

Figure 3. Effects of rapamycin and inhibition of mTORC1. (a) Inputs to and outputs from mTORC1. (b) In the process of macroautophagy, damaged organelles and other cellular components are accumulated in a double-membrane-enclosed autophagosome, which fuses with a lysosome and releases its contents for degradation and recycling.

Figure 4. Some of the modes of action for senolytics. (a) Cardiac glycosides disrupt the Na+/K+ ATPase pumps in the plasma membrane, leading to lowering of pH in senescent cells, which already have a low pH, thus rendering them vulnerable to apoptosis. (b) A FOXO4 peptide disrupts the association of FOXO4 with p53 leading to p53 nuclear exclusion and cellular apoptosis. (c) the molecular chaperone HSP90 stabilizes phosphorylated AKT (pAKT), which is elevated in senescent cells and protects them against apoptosis. Inhibition of HSP90 destabilizes pAKT resulting in selective apoptosis of the senescent cells. (d) BCL2 proteins, which inhibit mitochondrial activation of apopotosis, are elevated in senescent cells and their inhibition selectively induces apoptosis in these cells. This needs changing in the light of the changed order of panels in the Figure to correspond to the order in which they are mentioned in the text

Glossary terms

Healthspan: the time in someone's life when they are in general good health.

Immunosenescence: Decline in function of the immune system with age.

Senostatics: Chemicals that prevent senescent cells from producing the senescence associated secretory phenotype (SASP), which can damage surrounding tissue and cause systemic inflammation.

Dietary Restriction (DR): Reduced food intake from its voluntary level while avoiding malnutrition.

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Author Contributions

LP and BK discussed content and wrote the article, LP and MF revised the manuscript before submission, MF developed Figure 1.

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Competing Interests

B.K.K. is board chair of Torcept Therapeutics, a board member and scientific adviser for PDL Pharma, a scientific adviser for Affirmativ Health, and a board member of L-Nutra. B.K.K. is named on patents held by PDL Pharma related to aging interventions. B.K.K. performs corporate-sponsored research for Gero LLC.