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ABSTRACT:

Background: In conjunctival melanoma little is known about the tumour biology and protein-expression patterns. In this study we analysed the expression of the antiapoptotic oncoprotein Bcl-2, the tumour-suppressor PTEN, and the heat-shock-protein HSP-90 in conjunctival melanoma (CoM) and conjunctival nevi (CoN) by immunohistochemistry (IHC).

Material and Methods: IHC was performed on 70 samples of CoM and 12 samples of CoN. Expression-patterns between the diagnosis-groups were compared. ROC-analysis was performed to reveal diagnostic value of the antigens.

Results: HSP-90 ($p=<0.0001$) and PTEN ($p=0.001$) showed potential to differentiate between CoM and CoN. Bcl-2 expression was higher in CoM than in CoN ($p=0.04$) . The loss of nuclear PTEN expression was more pronounced in the malignant melanomas than in CoN ($p=0.02$). Tumours located at unfavourable sites (fornix, palpebral conjunctiva, caruncle) that had developed recurrences expressed almost twice as much HSP-90 than recurrence-free tumours.

Conclusions: Conjunctival melanocytes differentially express Bcl-2, HSP-90 and PTEN depending on their entity. HSP-90- and PTEN-expression may add relevant information for the differentiation between conjunctival melanoma and nevi.

BACKGROUND:

In the differential diagnosis of conjunctival melanocytic lesions, HMB45, S100 and Melan-A (MART-1) became the most important immunohistological antigens that help to distinguish melanomas from benign melanocytic lesions of the conjunctiva.[1] S100, an acidic protein, and Melan-A, a melanosomal antigen, both mark the extension of a melanocytic lesion.[2, 3] Jakobiec et al. showed S100 A1 improves diagnostic assessment of conjunctival nevi and melanomas.[2] HMB45, a cytoplasmic oncofetoprotein, is a marker for a low grade of differentiation.[4] Its expression is increased in conjunctival melanoma and primary acquired melanosis (PAM) with atypia.[1] HMB 45, Melan-A, S100, and Ki67 as proliferation index became part of the standard procedure in the assessment of conjunctival lesions.[3, 4] However, each antigen has its limitations and diagnosis is often a matter of combination.

The differentiation between conjunctival melanoma (CoM), nevus (CoN) and melanosis remains difficult. Primary acquired melanosis (PAM) with atypia has recently been referred to as “melanoma in situ” because of its high risk to progress to invasive melanoma.[5] Local chemotherapy has an increasing relevance in planning a therapeutic approach. The distinction between melanoma in situ and invasive melanoma is essential but the clinical experience proves it to be rather difficult. In this study we have focussed on the differentiation of CoM and CoN because this has a major impact on the therapy as nevi rarely progress to melanoma and do not need adjuvant treatment. Further diagnostic tools that help differentiating melanocytic conjunctival lesions can be of great benefit. We chose the antigens for this study with respect to these two challenges: clarify diagnostic pitfalls and possibly define therapeutic targets.

The tumour suppressor gene phosphatase and tensin homologue deleted on chromosome ten (PTEN) is mutated in a variety of human cancers.[6] PTEN is closely related to the RAS signal-pathway.[7, 8] A loss of the lipid posphatase activity of PTEN leads to cell proliferation and to cell survival by up-regulating antiapoptotic proteins as Bcl-2.[9, 10] The loss of PTEN activity can be caused by mutation, deletion, or reduced expression.[11] A loss of PTEN expression was found in about 65% of cutaneous melanoma and only 8% of melanocytic nevi.[12] This made it an interesting target for our study where we were looking for differential expression of antigens in conjunctival melanoma and nevi.

The B-cell leukaemia / lymphoma-2 protein (Bcl-2) family members control the mitochondrial response to apoptotic stimuli.[13, 14] The antiapoptotic oncoprotein Bcl-2 preserves the integrity of the mitochondrial membrane. Bcl-2 has been suggested to be a sensitive marker for melanocytic tumours in the conjunctiva.[15] Therapeutic strategies that overcome the antiapoptotic effect of Bcl-2 family members are an issue of current research.[16] Studies have shown that Bcl-2 is regulated by PTEN on the transcriptional level.[17] We chose Bcl-2 because it is well established in melanoma research and because of its link to the PTEN-pathway.

When protein-damaging stress acts upon a cell, heat-shock-proteins (HSP`s) are expressed to support cell survival. HSP-90 is an ubiquitously expressed cytoplasmic chaperone with predominantly antiapoptotic characteristics. It has been found to be over-expressed in cutaneous melanomas and metastases compared to nevi.[18, 19] HSP-90 has become an important new target in cancer therapy.[20] We included HSP-90 into the study because it is an anti-apoptotic antigen that is interesting for tumour characterisation with respect to new therapeutic options.

In this study we analysed the immunohistological characteristics of conjunctival

melanoma and benign conjunctival nevi. The selection of antigens was focussed on antigens that might be future targets for diagnostic differentiation or therapy. Further criteria were the experiences with the antigens in melanoma research, a pathway-related connection between two of them (Bcl-2 and PTEN), and an independent anti-apoptotic antigen as HSP-90.

MATERIAL AND METHODS:

Demographic data:

We analysed the clinical data of 70 cases with histologically confirmed malignant melanoma of the conjunctiva (CoM) and performed immunohistochemistry. The patients were treated between 1974 and 2006 in the Department of Ophthalmology of the University Hospital Essen or Tübingen and the samples were acquired from these departments and the Department of Pathology and Neuropathology of the University Hospital Essen. The tumours were staged following the 6th edition of the TNM-staging-system for conjunctival melanoma (American Joint Committee on Cancer 2002; table 1). The patients that had the conjunctival nevi excised (n=12) had no malignant or pre-malignant lesion on the ocular surface at the time of diagnosis.

All experiments and procedures were conducted in accordance with the Declaration of Helsinki. An informed consent was obtained from the patients for the analysis of collected samples. The local Ethics Committee of the Medical Faculty of the University Hospital Essen, University of Duisburg-Essen, Germany approved the study.

Immunohistochemistry:

Diagnosis of CoM was confirmed by hematoxylin-eosin staining and S-100-, HMB-45- and Melan-A immunostaining. Tissue preparation and immunohistological staining have been performed as described before (Westekemper et al.; Br J Ophthalmol (2010) doi:10.1136/bjo.2009.167445). As pre-treatment for Bcl-2-staining, EDTA-buffer (Zytomed Systems, Germany, ZUC029-500) was applied. For PTEN and HSP-90 the pre-treatment was performed with citrate buffer (pH 6.0)

(ZUC028-500). We applied specific antibodies for Bcl-2, (Dako, DK, mouse IgG_1, clone 124, dilution 1:300), HSP90 (Biozol, Germany, mouse IgG_1, clone AC88, dilution 1:800), and PTEN (Novocastra, UK, mouse IgG_1, clone 28H6, dilution 1:800). Endogenous melanin-pigment was identified in HE-stained samples and was appreciated for the estimation of the immuno-staining. For negative controls specific primary antibodies were replaced by normal serum of the same species. As positive controls for HSP-90 we used tissue of a strongly positive GIST (gastrointestinal stromal tumour), and for Bcl-2 and PTEN tonsil or lymph node tissue.

The samples were graded by two independent examiners (HW, FG) using the immuno-reactive score (IRS or Remmele score).[21] It is used to grade the immunoreactivity (staining intensity and percentage of stained cells) and results in a score from 0-12 (table 2).[22] The samples of conjunctival melanoma were arranged as tissue microarrays and we were therefore not able to assess the staining pattern in their whole thickness. Interobserver agreement was assessed by the Kappa-test.[23] In cases of interobserver differences, a third examiner re-estimated the sections and the results were discussed to find a congruent result. For PTEN, we generated the total IRS and additionally counted the percentage of cells with positive staining of the cytoplasm and/or of the nuclei.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2000, SPSS (SPSS for Windows, version 17.0; SPSS Chicago, IL, USA) and StatView for Windows (Abacus Concepts Inc. Version 4.55). A p-value of $p \leq 0.05$ was considered statistically significant. Risks were assessed using univariate Cox-regression analysis.

We analysed clinical or histopathological characteristics to identify risk factors for local recurrence or metastatic disease. These were the status at first presentation, tumour origin, pigmentation, recurrence during follow up, predominant celltype, pTNM-stage, cTNM-stage and occurrence of metastases (table 1).

Mann-Whitney-U-test was used for the comparison of antigen expression and nominal variables (2-variables); Kruskall-Wallis-test was performed for nominal variables with 3 and more variables. Bonferroni-correction was applied to multiple testing with an alpha level set to 0.05. Cases with unknown parameters were treated as missing values.

ROC-analysis (Receiver Operating Characteristic) was performed to analyse the diagnostic potential of the antigens (sensitivity and specificity). In general, the area under the curve is an indicator for the accuracy of a tool. All points above the diagonal reference line are better than a random guess. The asymptomatic significance of $p<0.05$ indicates that a tool is better than a guess in predicting an event (null-hypothesis: true area=0.5).

RESULTS:

Demographic data and therapy

The mean age was 63.7 ± 16.0 years at the time of diagnosis. The mean follow up was 69.6 months (min. 0.33, max 317.16 months, median 38.83). In our cohort, we could not identify clinical or histopathological risk factors for local recurrence or metastatic disease (data not shown).

Antigen-expression in CoM and CoN:

The Kappa-test revealed an overall interobserver reproducibility of 64.8%, which is a substantial agreement. [23]

Bcl-2: Bcl-2 was predominantly expressed in the cytoplasm. Bcl-2 stained homogenously within the TMA-sections. In nevi, conjunctival epithelium was negative for Bcl-2. In the stroma, the staining was intense when present but a significantly higher amount of cells were negative for Bcl-2. Bcl-2 expression was higher in CoM (8.1 ± 3.8 pts. IRS) than in CoN (5.55 ± 3.46 pts. IRS, $p=0.04$) (figure 1A, figure 2 A,B).

HSP-90: HSP-90 was expressed in the cytoplasm as well as in the nuclei of the cells. HSP-90 expression was significantly higher in CoM (7.39 ± 2.87 pts. IRS) than in CoN (1.9; min. 0; max. 6 pts. IRS, $p<0.0001$) (figure 1B). In CoM a high percentage of cells was positive for HSP-90, but the intensity of staining was moderate. In CoN only few cells stained positive and the intensity was low as well (figure 2 C,D).

PTEN and loss of PTEN: The staining of the cells was predominantly nuclear. Cytoplasmic staining was low in intensity and number of stained cells. The IRS showed a significantly higher score for total PTEN in CoM (7.32 ± 2.8pts. IRS) than in

CoN (3.91 ± 2.4 pts. IRS; p=0.0004 figure 2 E,F, figure 3). In CoN the IRS represents a low staining intensity in a high number of cells. In CoM, PTEN-expression was intense when present, but more cells showed a lack of PTEN or a weak staining.

Weak staining (i.e. granular staining pattern or membranous staining) or a lack of staining for PTEN in the nuclear fraction was significantly higher in CoM-cells (55.0% of cells) than in CoN-cells (33.3% of cells; p=0.006).

ROC-analysis of BCl-2, HSP-90 and PTEN:

Comparing CoM and CoN, the ROC-analysis revealed an area under the curve of 93.6% for HSP-90 (figure 4 A). The asymptomatic significance was p<0.001.

For PTEN, the area under the curve was 82.0% with an asymptomatic significance of p=0.001 (figure 4 B). The loss of nuclear PTEN revealed a significant asymptomatic significance of p=0.007 with an area under the curve of 75.6% (figure 4 B).

BCL-2 had no diagnostic relevance, as the area under the curve decreased to non-significant 68% (asymptomatic p-value=0.07; figure 4 A).

Clinico-pathological correlation for CoM:

Bcl-2: No statistically significant correlation was found between Bcl-2 expression and clinical features or histopathological features of the conjunctival melanomas (data not shown).

HSP-90: HSP-90 expression was significantly lower in cases with a cTNM-classification 3 of the primary tumour (p=0.007 T1, T3; p=0.04 T2, T3; p=0.006 T3, T4). T3 tumours represent those located at unfavourable sites (expansion to the fornix, palpebral conjunctiva or caruncle). Within this group (T3), the tumours that developed a local recurrence during follow-up expressed more HSP-90 (IRS: 5.9)

than tumours without recurrences (IRS: 3.0). This analysis did not reach statistical significance (p=0.08) but represents a trend.

PTEN and loss of PTEN: The nuclear PTEN-expression was significantly higher in amelanotic tumours compared to pigmented tumours (p=0.04). As the pigmentation had no impact of the prognosis of a tumour in our cohort, we could not draw further conclusions out of this trend.

Correlation between Bcl2-expression and loss of nuclear PTEN:

In both groups, a trend could be observed towards a correlation between Bcl-2-expression and nuclear PTEN-expression with an elevated Bcl-2 in tumours with less nuclear PTEN. We could not confirm the results with statistical significant p-values in each of the groups.

DISCUSSION:

In this study we found that HSP-90 and PTEN have a potential to differentiate between benign conjunctival nevi and conjunctival melanoma. Although they cannot replace the established tools as conventional histopathology in combination with standard immunohistochemistry (IHC), HSP-90 and PTEN IHC may be used as ancillary technique for the work-up of problematic conjunctival melanocytic proliferations.

The over-expression of HSP-90 in CoM is in analogy to other studies on cutaneous melanoma that showed a higher HSP-90 expression in primary melanoma than in benign nevi.[18, 19] We see a diagnostic benefit, because ROC-analysis revealed an almost ideal profile for the differentiation between nevi and melanoma of the conjunctiva. Besides, other authors have presumed that HSP-90 expression may in future be of therapeutic relevance, as HSP-90 is one target for new target-specific therapies.[18] So, the diagnostic usage of HSP-90 could be combined with the individual testing for a reasonable therapeutic approach.

The localisation of PTEN within the cell and the expression pattern in melanoma and nevi (mostly cutaneous) has not been consistent in former studies. Slipicevic et al. found no immunoreactivity for PTEN in cutaneous nevi and solely cytoplasmic expression in melanoma samples.[24] Other studies described a higher loss of PTEN expression in cutaneous melanoma compared to nevi and a uniformly strong cytoplasmic expression in benign nevi.[12] Today, this effect is not exclusively explained with the use of different antibodies, but the function of PTEN is seen in connection to its cellular localisation. PTEN seems to shuttle between the cytosol and the nucleus. Following this hypothesis, the nuclear PTEN would be mainly

responsible for the tumour-suppressor features of its functional panel: normal, quiescent cells express more nuclear PTEN than neoplastic tissue that shows a shift to cytosolic PTEN.[25] Loss of nuclear PTEN leads to chromosomal alterations and uncontrolled cell cycling.[26] Although we could not observe a clear shift to cytosolic expression of PTEN in neoplastic tissue, we noted a clear decrease of PTEN in the nuclear fraction of the neoplastic cells. It has to be examined in further studies whether this effect, seen on the protein level, correlates with somatic mutations of PTEN in conjunctival melanoma cells.

Zhuang et al. found a significant correlation between Bcl-2 expression and tumour thickness in cutaneous melanoma with a higher expression in thinner tumours (<1mm).[27] Conjunctival melanomas originating from PAM with atypia often tend to be rather flat than nodular. This could be an explanation for our finding of relatively high expression of Bcl-2 in CoM. In line with Furusato et al. we found, that Bcl-2 is expressed in CoM and CoN [15]. However, it is not useful for the differentiation of the entities.

An additional feature of interest was the association of elevated Bcl-2-expression and loss of PTEN-expression. We found no significant association between Bcl-2 expression and the nuclear PTEN. Mikhail et al. showed a weak association for cutaneous melanoma.[28] It has become evident that the cytoplasmic portion of PTEN harbours the lipid-phosphatase activity that regulates the Akt-PI3K pathway and thus rather influences Bcl-2 levels than nuclear PTEN.[29, 30] That could explain, why in our cohort the association was rather weak.

We found a significantly lower HSP-90 expression in T3-tumours (cTNM). That was unexpected because T3 tumours are those located at unfavourable sites and are related to a worse prognosis.[31] In our cohort they had a higher recurrence-rate

(62.5%) than the whole cohort (51.4%). T3-tumours that developed local recurrences during the follow-up had an almost twice as high IRS for HSP-90 than recurrence-free cases. Thus, HSP-90 expression might predispose T3-tumours for the development of local recurrences. It is unlikely that this is an exclusive influence but it may in future be reasonable to add immunohistological features to the risk assessment.

In summary, we have shown that Bcl-2, HSP-90 and PTEN are differentially expressed in conjunctival melanoma and nevi. ROC-analysis revealed that both HSP-90 and PTEN could support the immunohistological differentiation between conjunctival melanoma and nevi. On the other hand, ROC-analysis showed that Bcl-2 was not applicable for diagnostic purposes. With respect to novel developments in immunotherapy and target-specific chemotherapy, we have shown first steps to individual tumour characterisation in conjunctival melanoma.

Competing Interest: None to declare.

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Figure 1: Boxplot of results of Bcl-2- (A) and HSP-90- (B) expression in the two diagnosis groups. For Bcl-2, a statistically significant difference exists in expression between conjunctival melanoma (CoM) and conjunctival nevi (CoN). Also for HSP-90, we see a statistically significant difference in the expression between the groups. The horizontal line within the box denotes the median. The box itself spans from the 25^{th} percentile (lower border) to the 75^{th} percentile (upper border). The error bars extending the box below and above mark the 10^{th} percentile (below) and the 90^{th} percentile (above).

Figure 2: Immunohistology of samples of the two diagnosis-groups and each antigen (Bcl-2: A-B; HSP-90: C-D and PTEN: E-F). Magnification x200.

A-B: Bcl-2 was expressed predominantly in the cytoplasm. Note the intense staining in the CoM (A). In figure 2B the lack of staining in the conjunctival epithelium is evident in CoN.

C-D: HSP-90 was expressed in the cytoplasm and nucleus. In CoM (C) the staining was homogeneous with a moderate to strong intensity. CoN stained very little for HSP-90 (D).

E-F: PTEN was expressed predominantly in the nuclei. The loss of PTEN is partly defined as lack of staining and partly as a qualitatively different staining. E: In this case of CoM the staining is homogeneous where present. About 50-60% of nuclei show no staining for PTEN.

Figure 3: A: Total PTEN-expression in the two diagnosis groups. Statistically significant difference in expression between the malignant melanomas and CoN. Boxplot: The horizontal line within the box denotes the median. The box itself spans

from the 25th percentile (lower border) to the 75th percentile (upper border). The error bars extending the box below and above mark the 10th percentile (below) and the 90th percentile (above). B: % cytoplasmic PTEN-expression. The cytoplasmic expression of PTEN was low in all samples. The light grey part of the column represents the fraction with homogenous cytoplasmic staining. The dark grey part of the column represents the fraction with a loss of staining. C: % nuclear PTEN-expression. The light grey part of the column represents the fraction with homogenous nuclear staining. The dark grey part of the column represents the fraction of nuclei with a lack of staining or weak staining (e.g. granular staining, membranous staining). We assume that a loss of nuclear staining is accompanied by a loss of expression of nuclear PTEN [32].

Figure 4: ROC-analysis (Receiver Operating Characteristic) to analyse the diagnostic potential of the antigens (sensitivity and specificity) in the distinction of conjunctival melanoma and conjunctival nevi. The area under the curve is an indicator for the accuracy of a tool (antigen), aiming for 100%. All points above the diagonal line are better than a random guess. A: ROC-curve of HSP-90 and Bcl-2. For HSP-90 the area under the curve is 93.6%, for Bcl-2 68.0%. B: ROC-curve of PTEN and the loss of nuclear PTEN (in immunohistology). For PTEN the area under the curve is 82.0%, for the loss of nuclear PTEN 75.6%.

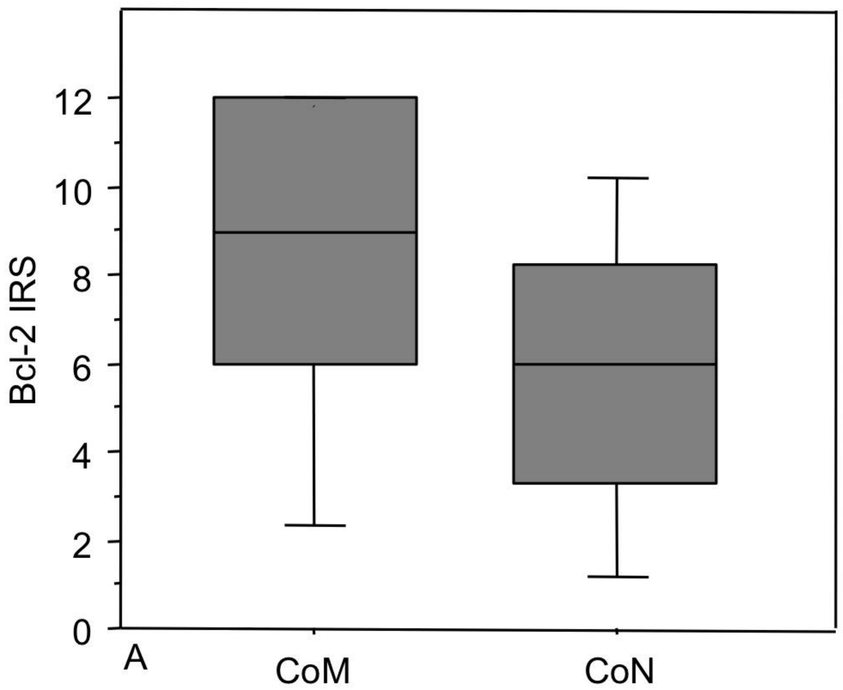
Attribute	**Diversity**	**n=70 (%)**
Time of presentation	First diagnosis	40 (57.1)
	Local recurrence	22 (31.4)
	Unknown	8 (11.5)
Gender	Female	40 (57.1)
	Male	30 (42.9)
Tumour origin	De novo	10 (14.3)
	PAM w.a.	23 (32.9)
	Nevi	16 (22.9)
	Recurrence	10 (14.3)
	Unknown	11 (15.6)
Pigmentation	Pigmented	39 (55.7)
	Amelanotic	10 (14.3)
	Mildly pigmented	16 (22.9)
	Unknown	5 (7.1)
Recurrence during follow-up	Yes	36 (51.4)
	No	17 (24.3)
	Unknown	17 (24.3)
Predominant cell type (n=54)	Epitheloid	35 (50.0)
	Spindlecell	12 (17.1)
	Pleomorph	7 (10.0)
	Unknown	16 (22.9)

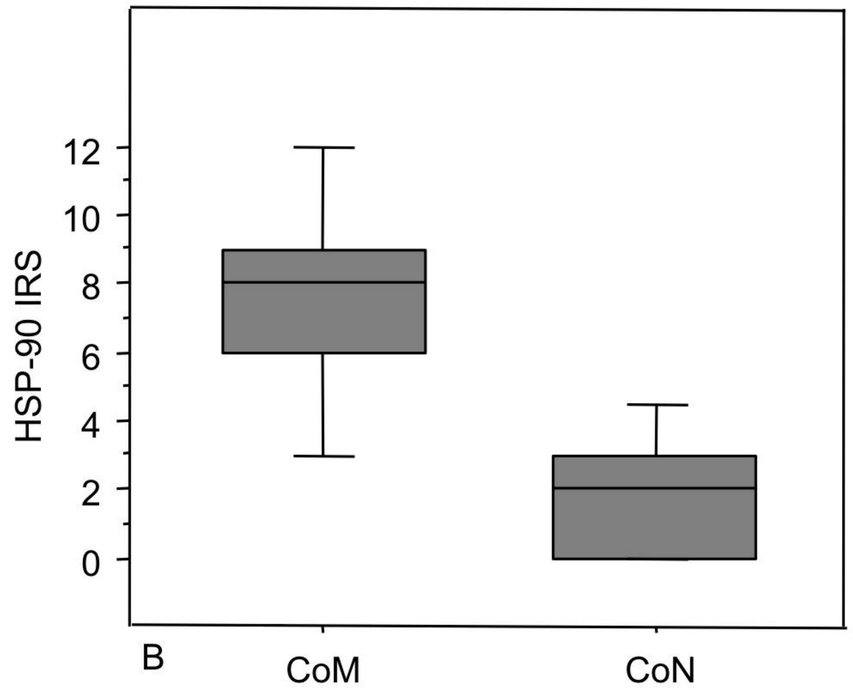
p-TNM	pTX	18 (25.7)
	pT0	0
	pT1	3 (4.3)
	pT2	30 (42.9)
	pT3	15 (21.4)
	pT4	4 (5.7)
c-TNM	TX	13 (18.6)
	T0	0
	T1	18 (25.7)
	T2	18 (25.7)
	T3	17 (24.3)
	T4	4 (5.7)
Metastases	Yes	10 (14.3)
	No	41 (58.6)
	Unknown	19 (27.1)

Table 1: Location of primary tumour: favourable: epibulbar-, limbal involvement, nodular tumour. Unfavourable: diffuse, multifocal tumour, fornical-, caruncle-, muscle-, scleral-, lid-, orbital- involvement. PAM : primary acquired melanosis with atypia.

Points	Intensity of staining	% positive cells
0	No staining	0 %
1	Weak	<10 %
2	Moderate	11 %-50 %
3	Strong	51 % - 80 %
4	-	81 % - 100%

Table 2: The Remmele-Score (also: immunoreactive score (IRS)) is used to estimate the grade of immunoreaction. The product of staining-intensity and percentage of stained cells results in a score from 0-12.


Bcl-2 IRS
12
10
8
6
4
2
0
A
CoM
CoN


HSP-90 IRS
12
10
8
6
4
2
0
B
CoM
CoN

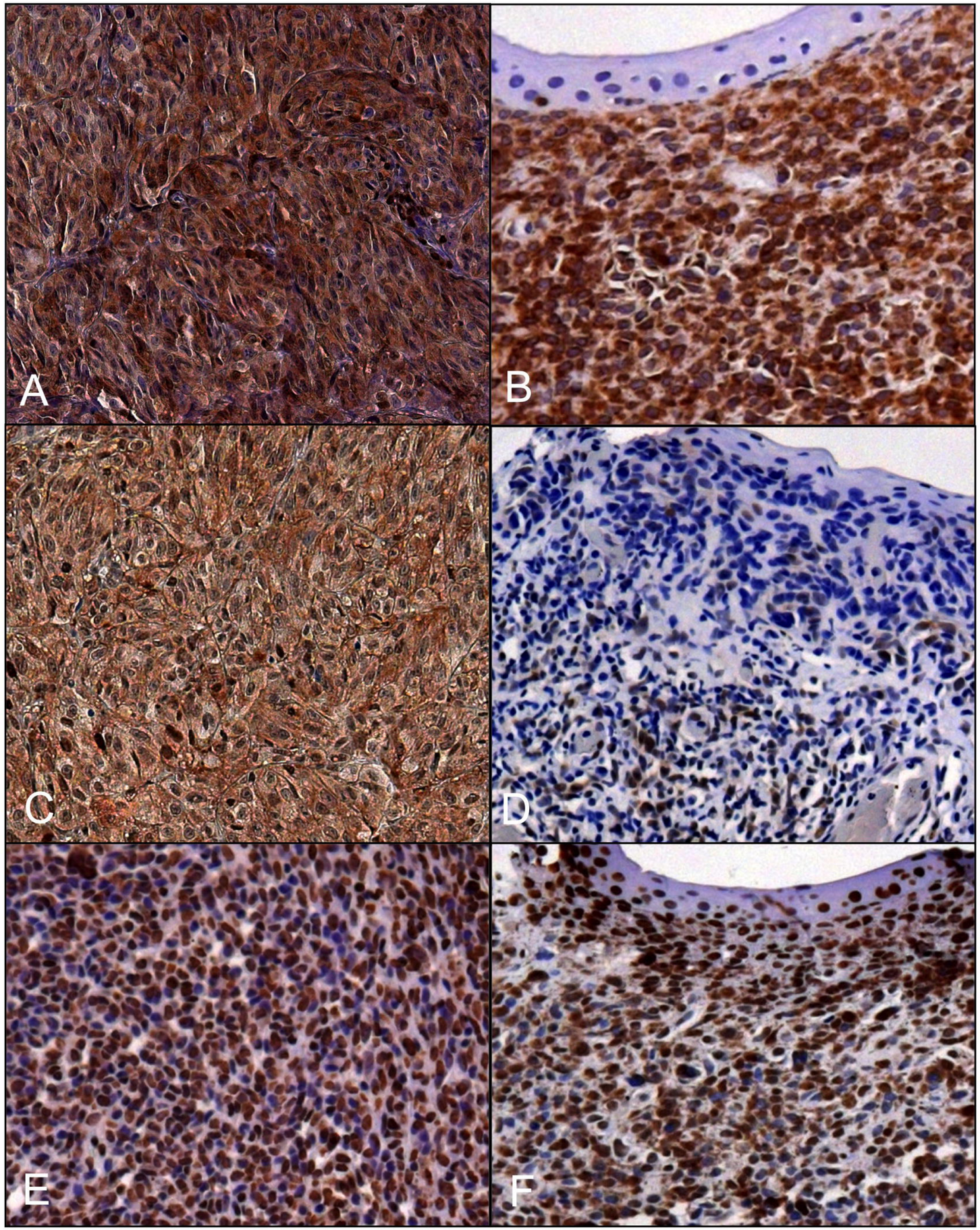
CoM

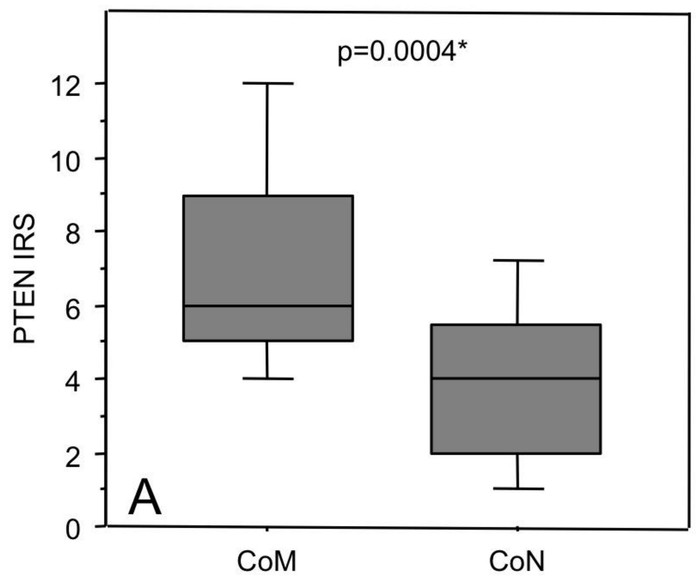
CoN

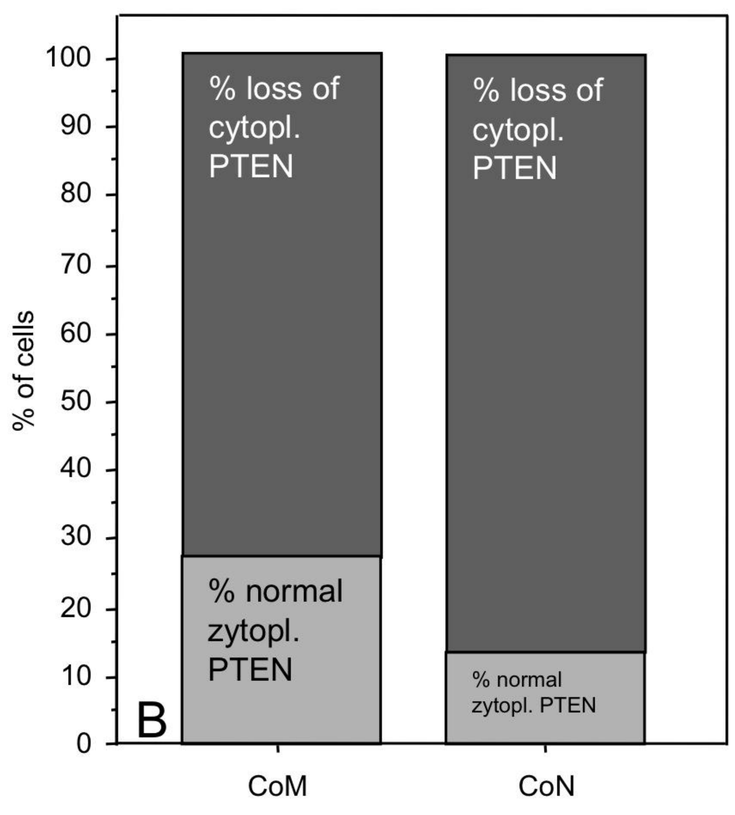
Bcl-2

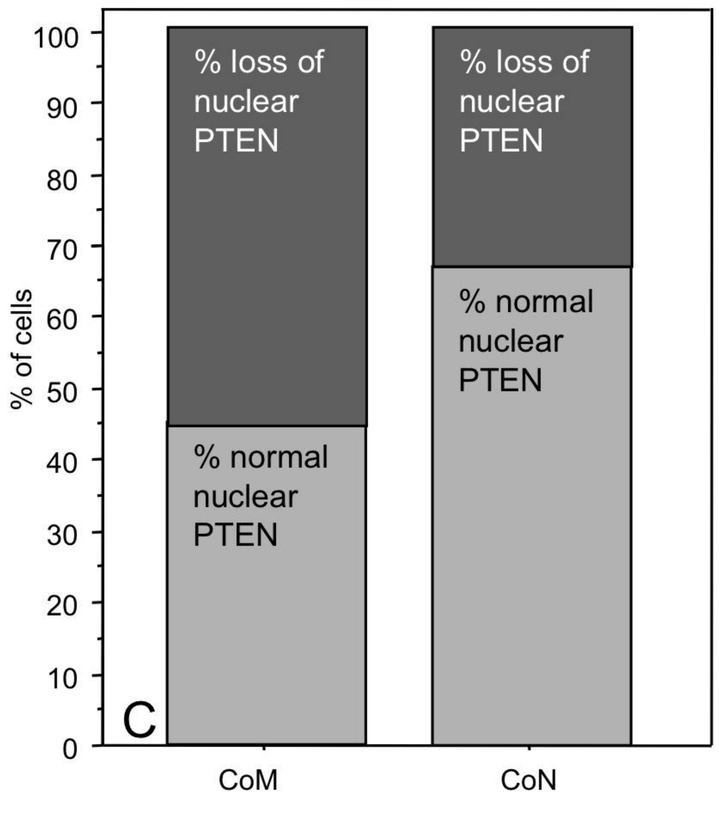
HSP-90

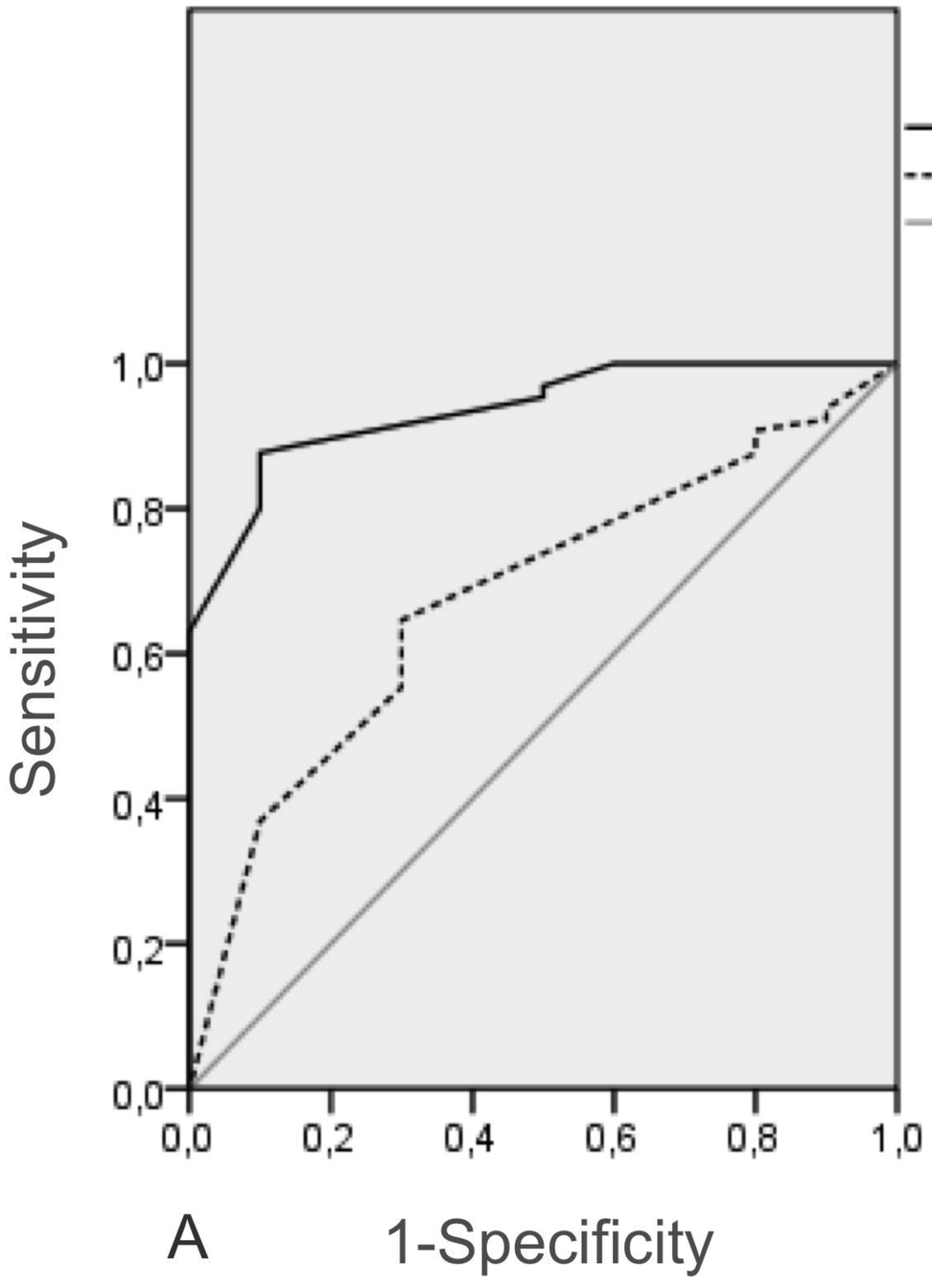
PTEN



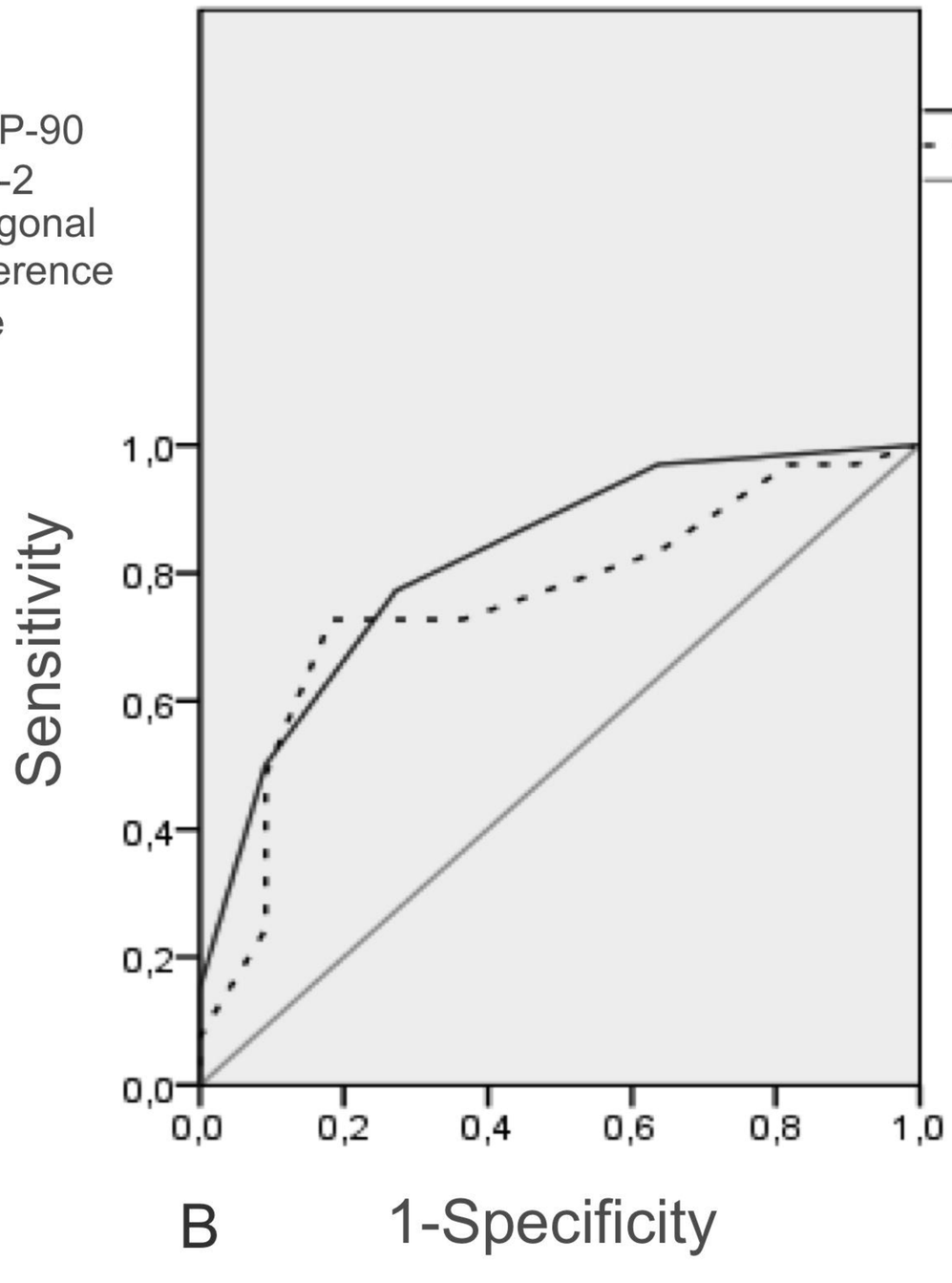

p=0.0004*
PTEN IRS
0
2
4
6
8
10
12
A
CoM
CoN


% of cells
0
10
20
30
40
50
60
70
80
90
100
% loss of cytopl. PTEN
% normal zytopl. PTEN
% loss of cytopl. PTEN
% normal zytopl. PTEN
B
CoM
CoN


% of cells
0
10
20
30
40
50
60
70
80
90
100
% loss of nuclear PTEN
% normal nuclear PTEN
% loss of nuclear PTEN
% normal nuclear PTEN
C
CoM
CoN


HSP-90
Bcl-2
diagonal reference line
Sensitivity
1,0
0,8
0,6
0,4
0,2
0,0
0,0
0,2
0,4
0,6
0,8
1,0
1-Specificity

A


PTEN
loss of nucl. PTEN
diagonal reference line
Sensitivity
1,0
0,8
0,6
0,4
0,2
0,0
0,0
0,2
0,4
0,6
0,8
1,0
1-Specificity

B