

## Experimental Research

# The Utility of Claudin-3 in Diagnosis of Prostatic Adenocarcinoma: A Comparative Immunohistochemical Study with AMACR

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### ABSTRACT

**Objective:** Claudin family of proteins has been shown to be overexpressed in various cancers. We aimed to investigate one member of this family, the Claudin-3 expressions in prostate tissue and evaluate its utility as a diagnostic marker for prostatic adenocarcinoma.

**Material and Method:** A total number of 50 cases of prostatic adenocarcinoma were reviewed from the files of M.H. Ankara Dışkapı Research and Training Hospital. One paraffin block was selected for each case. For separate immunohistochemical staining with Alpha-methyl-acyl-CoA racemase (AMACR) and Claudin-3, two slides were sectioned from each block. Prostatic carcinoma (PC), Benign gland, and High grade prostatic intraepithelial neoplasia (HGPIN) components in each slide were evaluated. Afterwards the intensity of stainings were scored on a scale of 0-2 separately for each component.

**Results:** After immunohistochemical evaluation, 94.1% of PC showed positivity with Claudin-3 whereas this value was 77.4% for AMACR. Furthermore in the areas of HGPIN, the positivity rates with Claudin-3 was also higher than those of AMACR ( $p < 0.005$ ).

**Conclusion:** In statistical analysis, Claudin-3 appeared to be a reliable immunohistochemical marker for PC at least as much as AMACR. We think that our findings strongly suggest the use of Claudin-3 as an alternative for AMACR in the routine tissue diagnosis of PC.

**Keywords:** Claudin-3, AMACR, Prostate, Cancer, Diagnosis.

### ÖZET

**Prostatik Adenokarsinomlarda Claudin-3' ün Tanısal Değeri: AMACR ile Karşılaştırılabilir İmmünohistokimyasal Çalışma**

**Amaç:** Claudin protein ailesinin çeşitli kanserlerde aşırı ekspresyonu göstermiştir. Prostatik dokularda Claudin-3 ekspresyonunu araştırmayı ve prostatik kansinomlarda tanısal önemini değerlendirmeyi hedefledik.

**Gereç ve Yöntem:** Sağlık Bakanlığı Ankara Dışkapı Yıldırım Beyazıt Eğitim ve Araştırma Hastanesi arşivine ait 50 prostatik adenokarsinom olgusu değerlendirilmiştir. Herbir vakaya ait bir parafin blok seçilmiştir. Alpha-methyl-acyl-CoA racemase (AMACR) ve Claudin-3 immünohistokimyasal belirteçler için her blokta iki kesit hazırlanmıştır. Adenokarsinoma, benign gland ve yüksek dereceli prostatik intraepitelyal neoplazi alanları her kesitte ayrı ayrı değerlendirilmiş olup, boyanma yoğunlukları 0-2 aralığında skorlanmıştır.

**Bulgular:** İmmünohistokimyasal değerlendirme sonrası, Prostatik kansinomların %94.1'i Claudin-3 ile pozitiflik gösterirken, bu oran AMACR ile %77.4'dür. Ayrıca Prostatik intraepitelyal neoplazi alanlarında, Claudin-3 ile pozitif boyanma oranları, AMACR'a göre daha yüksektir ( $p < 0.005$ ).

**Sonuç:** İstatiksel olarak prostatik kansinomlarda Claudin-3'ün en az AMACR düzeyinde güvenilir bir immünohistokimyasal belirteç olduğu ortaya konmuştur. Bulgularımız rutin patolojik tanı da Claudin-3'ün AMACR belirteğine iyi bir alternatif olduğunu kuvvetle göstermektedir.

**Anahtar Sözcükler:** Claudin-3, AMACR, Prostatik Kanser, Tanı.

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Prostate cancer is one of the leading causes of death in men and probably because of the use of prostate specific antigen (PSA) screening, its incidence has increased massively in last decades. The current standard method for diagnosis of prostate cancer is transrectal ultrasound-guided core biopsy. In addition, it can also be incidentally detected in resection materials such as transurethral resections (TUR) or prostatectomies. Although it is a standard method in the diagnosis of prostate cancer, transrectal core biopsy has certain limitations particularly because of small specimen size and biopsy induced mechanical

distortion. These tissue samples may contain areas of benign prostatic hyperplasia (BPH), adenosis or atypical adenomatous hyperplasia (AH), atrophic glands (AG), prostatic intraepithelial neoplasia (PIN) and prostatic carcinoma (PC). The morphological similarities among these lesions present another diagnostic challenge for the pathologist. So it can be quite difficult to appreciate an infiltrative architectural pattern of growth in thin core biopsy specimens. For this reason some small foci of atypical glands suspicious but not diagnostic for malignancy in core biopsies are often considered as atypical small acinar proliferation

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(ASAP) (1). Although it is known that ASAP has high predictive value of subsequent adenocarcinoma, it is not a discrete pathologic entity and warrants repeat biopsy. Furthermore repeating the diagnosis of ASAP in the subsequent biopsy is not an exceptional situation in daily practise. In order to avoid repeat biopsies and to make differential diagnosis between adenocarcinoma and its benign histological mimics, IHC evaluation has great importance. For this purpose antibodies directed against basal cells (34Be12 and p63) are used to demonstrate the absence of basal cell layer in carcinoma. However the use of negative staining by basal cell markers has many restrictions because benign mimics of PC often have a discontinuous basal layer and prolonged formalin fixation can cause false negative staining. Alpha-Methylacyl-coA racemase (AMACR) also known as P504S, is another widely used immunohistochemical marker and several groups have assessed strong AMACR immunoreactivity in PC. Currently many laboratories combine basal markers with AMACR to form an immunohistochemical cocktail. On the other hand it is also known that; AMACR immunostaining distinguishes most but not all cases of AH from PC and its sensitivity was reported at a range of 62-100%. Furthermore foci of atrophic prostate have been noted to show moderate and focal AMACR positivity as much as 36%. Another concern about AMACR is that certain subtypes of PC, such as foamy gland carcinoma, atrophic carcinoma, pseudohyperplastic and treated carcinoma show limited or no AMACR expression (2-4).

Tight junctions are expressed on the apical end of the lateral membrane surface and form the epithelial barrier against paracellular transport; moreover they maintain epithelial cell polarity via their fence function (5). Changes in the expression of tight junction proteins are characteristic of many human diseases, including cancer. Among tight junction proteins, claudins are the most important structural and functional components of tight junction strands. Alterations in the expression levels of tight junction proteins continue to be reported in several cancers (5-7). At least 27 subtypes of claudins have been identified. These subtypes are expressed in an organ-specific manner and regulate the tissue-specific physiological functions of tight junctions. One of these subtypes is Claudin-3. Although, like other members of this family, its role in carcinogenesis is still controversial, Claudin-3 has been shown to be overexpressed in various cancers, but most thoroughly studied in ovarian cancer. Claudin-3 expressions has been shown to be up-regulated in ovarian cancer cells more than 80-fold in comparison to non-neoplastic cells in ovary (8-10). In addition to the studies defining Claudin-3 as a useful IHC marker in differential diagnosis of tumors, there are also some reports about its prognostic value. For example in serous adenocarcinomas of ovary, Claudin-3 expressions has been shown to be associated with shorter survival (9). In clear cell renal

carcinomas an increase in expression of Claudin-3 was reported with increasing grades (11). Paradoxically, there are also some studies reporting the down-regulation of Claudin-3 in some cancers. For example in early gastric carcinoma, Claudin-3 was reported to be down regulated (12). Similarly in a study with esophageal carcinoma, distant metastases were reported to be associated with a decrease in Claudin-3 and Claudin 4 expressions (13). In PC specifically, there are studies demonstrating overexpression of Claudin-3 in primary and metastatic prostatic adenocarcinomas (14-16).

In this study we aimed to investigate expression of Claudin-3 in prostatic adenocarcinoma and High-grade prostatic intraepithelial neoplasia (HGPIN). In order to evaluate its utility as a diagnostic marker, we compared its expressions with those of a widely used immunohistochemical marker, AMACR.

## MATERIAL AND METHOD

A total number of 120 cases of prostatic adenocarcinoma diagnosed between years of 2009 and 2011, were reviewed from the files of M.H. Ankara Dışkapı Research and Training Hospital and 50 cases were included to the study. The reviewed materials were needle biopsies, TURs and radical prostatectomies. Haematoxylin and Eosin (H&E) stained slides from paraffin embedded tissue blocks were re-evaluated by a pathologist experienced in uropathology. The diagnosis and Gleason scoring of the tumors was based on the WHO classification of urological tumors (17). One paraffin block were selected for each case. Attention was paid to select blocks which contain some amount of benign glandular structures in neighbourhood of neoplastic tissue (PC or HGPIN). The 5 cases out of 50 had both adenocarcinoma and HGPIN. The patient ages ranged between 41 to 69. Gleason scores of adenocarcinomas ranged from 2+3=5 to 4+5=9. Considering the Gleason scores, the PC was grouped as high Gleason score tumors (HGST) and low Gleason score tumors (LGST). The HGST consisted of the ones with a score higher than or equal to 7. The others, which had a score lower than 7, composed the LGST. The distribution of all the neoplastic and non-neoplastic items that have been immunohistochemically examined is shown on table 1.

**Table 1.** The distribution of the tissue components evaluated immunohistochemically.

HGST		LGST	HGPIN	BG (BPH+AH)	AG
5	28	22	50	20	

*HGST: High gleason score tumor.*

*LGST: Low gleason score tumor.*

### Immunohistochemistry

For separate immunohistochemical staining with AMACR (rabbit monoclonal, Neomarker) and Claudin-3 (rabbit monoclonal, ThermoScientific), two slides were sectioned from each block. After deparafinization and rehydration the slides were treated with hydrogen peroxide and heated at 530 watt microwave oven for 20 minutes in sodium citrate buffer (ph 6.0) for antigen retrieval. Then the slides were rinsed in phosphate-buffered saline (PBS) for 5 minutes. Incubation of the slides with primary antibodies were done for 90 minutes at room temperature. Dilutions were 1/100 for both antibodies. Then again the slides were washed with PBS and incubated with "horseradish peroxidase- labelled rabbit anti-mouse immunoglobulin" (Dako) for 1 hour at room temperature. After washing with PBS, the slides were treated with solution of diaminobenzidine (DAB). Finally counterstain with haematoxylin was applied and the slides were let to dry before mounting. The positive controls were prostate adenocarcinoma proved to show strong AMACR positivity for AMACR and basal cell carcinoma of skin for Claudin-3.

Immunohistochemical evaluation was done under light microscope. All tissue components (PC, HGPIN and benign gland) in each slide were evaluated. Then the intensity of stainings were scored on a scale of 0-2 separately for each component. For both Claudin-3 and AMACR, the score 0 was attained in the case of complete absence of staining. The weak staining is designated by score 1 and moderate to strong staining by score 2. AMACR expressions were evaluated according to cytoplasmic positivity. On the other hand for Claudin-3, membrane bound staining was primarily evaluated. Score 1 was assigned for mild membranous positivity whereas the moderate to strong membranous staining was score 2. Moderate to strong cytoplasmic staining without any membranous activity was scored as 1 for Claudin-3.

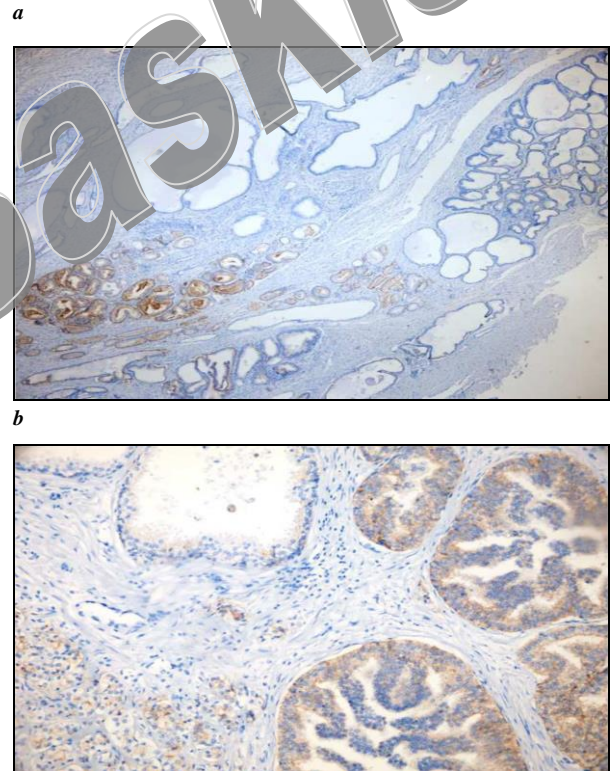
### Statistical analysis

Data analysis was performed by using SPSS for Windows, version 20. Data were shown as number of cases and percentages. The differences in prevalence of staining between antibodies were compared by McNemar test. Sensitivity, specificity, positive and negative predictive values for AMACR and Claudin-3 were also calculated to discriminate benign and malign groups each other. Multiple Logistic Regression analysis was applied for determining the superiority of two antibodies detecting malignancy. Odds ratios and 95% confidence intervals for each antibody were also calculated. A p-value less than 0.05 was considered statistically significant.

### RESULTS

**AMACR:** AMACR was positive in 77.4% of PC. According to the subgroups of HGST and LGST, the positivity rates were 80% and 76.9% respectively.

Concerning the staining intensities, 35.5% of PC had score 2 with AMACR, showing moderate to strong expression. Regarding the HGPIN, 40% of cases showed immunostaining with AMACR. The 24% of these cases had intensity score 2. Among 50 BG foci, all from different slides, only 7% of BG showed weak AMACR positivity (score 1). Atrophic foci did not show any positivity with AMACR at all. The statistical analysis demonstrated a significant difference between AMACR positivities of PC and BG in frequency ( $p < 0.001$ ). AMACR positivities in HGPIN was again higher than that of BG ( $p = 0.016$ ). The analysis did not demonstrate any statistical difference between staining frequencies of PC and HGPIN ( $p = 0.063$ ). The intensity scores among HGST, LGST and HGPIN were not analysed statistically. Examples of AMACR positivities are shown in figure 1.

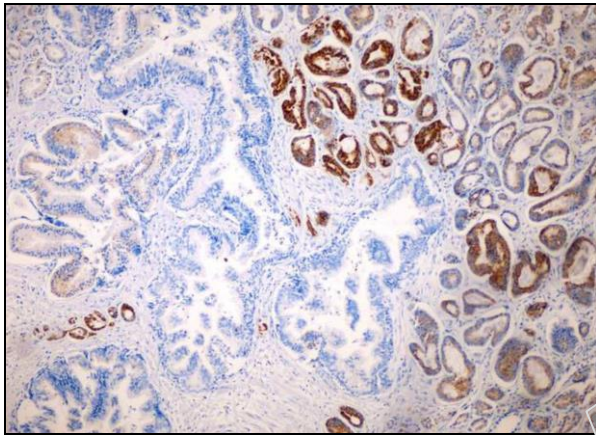


**Figure 1.** Examples of AMACR positivities: Mild positivity of AMACR in PC with Gleason score 3+3 (a) Moderate positivity of AMACR in PC with Gleason score 2+3 (b) AMACR positivity in a focus of PIN (c).

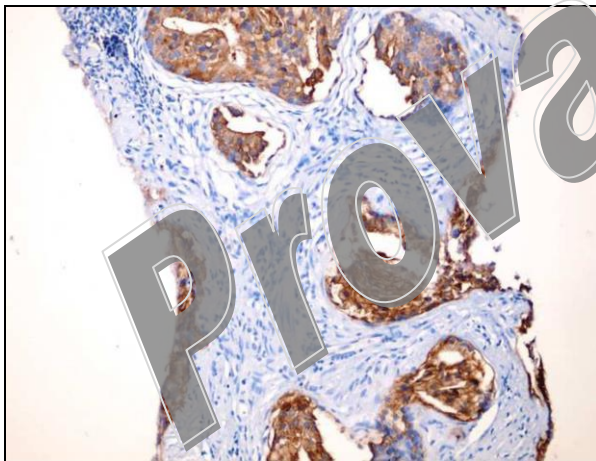
**Claudin-3:** Claudin-3 was positive in all but two cases of PC (94.1%). According to the subgroups of HGST and LGST the positivity rates were 100% and 92.8% respectively. Concerning the staining intensities, 58.8% of PC had score 2, showing moderate to strong Claudin-3 expression. This rate was 57.1% and 60% for HGST and LGST subgroups respectively. Regarding the HGPIN, 87.9% of cases showed immunostaining with Claudin-3. Among 50 BG foci, all from different slides, the rate of Claudin-3 positivity was 14.4% and all showed weak staining as score 1.



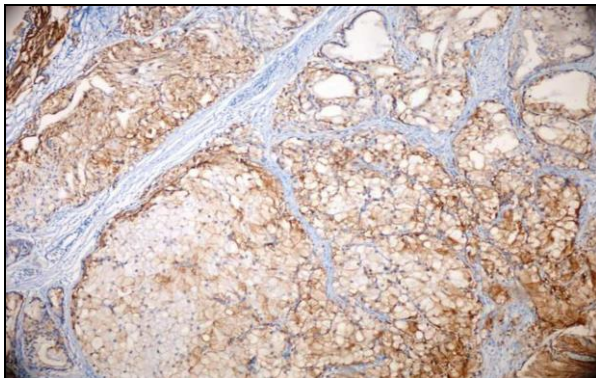
The statistical analysis demonstrated a significant difference between claudin-3 positivities of PC and BG in frequency ( $p < 0.001$ ). Comparing the Claudin-3 positivities of HGPIN and BG, HGPIN demonstrated a significantly higher positivity rate ( $p < 0.001$ ). No statistical difference was observed between the PC and HGPIN with regard of both frequency and intensity of staining ( $p = 1.000$ ,  $p = 1.000$ ). Examples of Claudin-3 positivities are shown in figure 2.



a



b



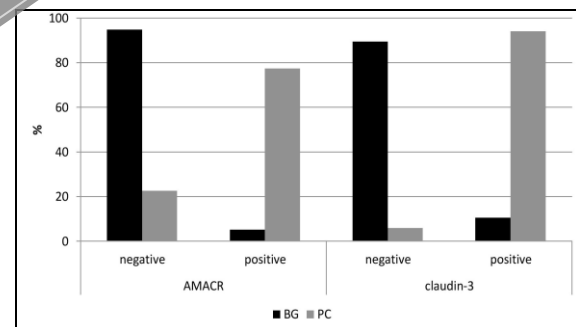
c

**Figure 2.** Examples of Claudin-3 positivities: Moderate to strong positivity of Claudin-3 in PC with Gleason score 3+3 (a) Moderate to strong positivity of Claudin-3 in PIN (b) The Claudin-3 positivity in foamy gland carcinoma of prostate (c).

**Claudin-3 versus AMACR:** Among PC group, 94.1% of cases showed positivity with Claudin-3 whereas this value was 77.4% for AMACR. Using multiple logistic regression analysis Claudin-3 appeared as a more reliable marker than AMACR in differentiating PC from BG ( $p < 0.001$ ). This analysis demonstrated a 38.724 times increase in probability of malignancy in case of Claudin-3 positivity. On the other hand, this probability was calculated as 9.006 times with AMACR ( $p = 0.004$ ). Furthermore the frequency of Claudin-3 positivity was also found to be higher than that of AMACR, considering PC and HGPIN groups as a whole ( $p = 0.006$ ). In HGPIN group, Claudin-3 and AMACR positivities were 87.9% and 40% respectively and while AMACR positivity did not produce a meaningful result between BG and HGPIN, Claudin-3 demonstrated a significant difference ( $p < 0.001$ ). Table 2 demonstrates the statistical data of 3 and AMACR. The graphics in figure 3 represents the distribution of BG and PC according to AMACR and Claudin-3 expressions.

**Table 2.** Multiple logistic regression analysis of the effects of Claudin-3 and AMACR in discriminating PC from BG.

	Odds Ratio	95% Reliability range	Wald Statistics	p-value
AMACR	9,006	1,997-40,609	8,181	0,004
Claudin-3	38,724	6,953-215,669	17,415	<0,001



**Figure 3.** Distribution of BG and PC according to AMACR and Claudin-3 expressions.

## DISCUSSION

Transrectal core biopsy, the main diagnostic tool for PC, has certain limitations due to small specimen size and frequent mechanical distortion of tissue. Furthermore histological similarities between adenocarcinoma and benign glandular components may sometimes be quite challenging even for experienced uropathologists. For these reasons IHC markers have great value in the diagnosis of PC. AMACR is a widely used IHC marker which is known to be positive in PC. On the other hand, while sensitivity of AMACR was reported at a range of 62-100%, AMACR is also known to be expressed by some other entities (eg. AG, AH). Furthermore the knowledge of AMACR negative adenocarcinomas including some rare types makes its interpretation further complicated (18-22).

In this study we aimed to investigate Claudin-3 expressions in prostate tissue and evaluate its utility as a diagnostic marker for PC. For this purpose we compared Claudin-3 expressions with those of AMACR in each group (PC, HGPIN and BG). In our study the sensitivity of AMACR for PC was calculated as 77.4% with 94.8% positive predictive value (ppv) and 82.85 negative predictive value (npv). This rate was within the range indicated by other studies. The statistical analysis demonstrated a significant difference between PC and BG in terms of AMACR positivities. The difference in AMACR profiles of HGST and LGST was not statistically analysed. For BG and HGPIN on the other hand, positivity rates were 7% and 40% respectively and for HGPIN this rate was slightly lower than the previous reports (16-19). One case of foamy gland carcinoma -a rare subtype of PC- in our study did not stain with AMACR and this finding was also consistent with the previous data.

Molecular studies demonstrated an elevation in Claudin-3 genes in PC (8). Correspondingly, immunohistochemical studies were also demonstrated an overexpression of Claudin-3 in PC. Bartholow et al. (14) reported Claudin-3 overexpression both in primary and metastatic PC. Vare et al. (16) studied 5 members of Claudin family (1-5) in PC and reported strong overexpression with Claudin-3 in 97% of cases. Furthermore although they found an association between high Gleason score and low Claudin expression (combined expressions of 1, 2, 3, 4, 5), no statistical difference was obtained between the Claudin-3 expressions of high and low Gleason score tumors. In our study the sensitivity of Claudin-3 for PC was 94.1% (ppv: 89.4, npv:74.4). In terms of staining intensity, 38.8% of positively stained PC had score 2. The difference among Gleason groups was not statistically analysed because of the small size of HGST group. On the other hand Claudin-3 positivity among BG was 14.4% and statistical analysis showed a significant difference between Claudin-3 expressions of PC and BG in terms of both frequency and intensity. None of BG showed moderate to strong positivity. Considering the expressions in HGPIN, the positivity rate was 87.9% and comparing with BG, this was also significantly higher. In the study of Bartholow et al. (14) PIN (not specified as HGPIN or LGPIN) has also been shown to have higher Claudin-3 expressions with respect to BG.

Claudin-3 versus AMACR. 94.1% of PC showed positivity with Claudin-3 whereas this value was

77.4% for AMACR. Using multiple logistic regression analysis, Claudin-3 appeared as a more reliable marker than AMACR in differentiating PC from BG ( $p < 0.001$ ). This analysis demonstrated a 38.724 times increase in probability of malignancy in case of Claudin-3 positivity. On the other hand, this probability was calculated as 9.006 times in AMACR. Furthermore considering the expressions of both antibody in HGPIN, the positivity rate with Claudin-3 was higher than AMACR. Likewise, although statistical comparison of AMACR expressions in BG and HGPIN did not result in a meaningful difference, Claudin-3 positivity in HGPIN were found to be significantly higher than BG. The positivity of Claudin-3 in a case of foamy gland carcinoma was another notable point in our study since this subtype is known to be AMACR negative.

In summary, although the exact roles of Claudin family proteins in carcinogenesis are still being uncovered, it is clear that they represent promising targets for diagnosis and therapy of cancer. In prostate cancer, overexpression of Claudins, particularly Claudin-3, was previously reported. But to the best of our knowledge, as being a comparative immunohistochemical study with AMACR, this is the first report revealing the utility of Claudin-3 as an immunohistochemical marker in biopsy diagnosis of prostatic adenocarcinoma. According to our results, Claudin-3 appeared as a more reliable marker than AMACR in differentiating malignant glands from benign ones. We think that our findings strongly suggest the use of Claudin-3 in needle biopsies of prostate, at least as an alternative for AMACR. Considering the pure cytoplasmic positivity in benign prostate tissue in our study, a strict search for membrane bound staining could aid to reduce false positivities. Future studies with large groups including different subtypes of prostate cancer would be important for the use of Claudin-3 in daily pathology practise.

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#### Ethical Standards

This study have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

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