Immunohistochemical Analyses in the Diagnostics of Breast Cancer

Ph. D. Thesis

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LIST OF PAPERS THAT SERVED AS THE BASIS OF THIS PH.D. THESIS

I. **Szintia Almási**, Levente Kuthi, Anita Sejben, András Vörös, Ákos Nagy, Tamás Zombori, Gábor Cserni. TRPS1 expression in cytokeratin 5 expressing triple negative breast cancers, its value as a marker of breast origin. Virchows Arch. 2023;482:861-868. doi:10.1007/s00428-023-03535-4

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II. **Szintia Almási**, Gábor Cserni. The value of oestrogen receptor, progesterone receptor and keratins 5 and 14 immunohistochemistry in the evaluation of epithelial proliferations at cauterised margins in breast-conserving surgery specimens. Pathol Res Pract. 2024;257:155280. doi:10.1016/j.prp.2024.155280

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III. **Szintia Almási**, Tibor Krenács, László Krenács, Gábor Cserni. Galectin-1 expression in breast cancer stroma – prognostic value in triple-negative breast cancer. Pathobiology. 2025; doi:10.1159/000546206

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1. INTRODUCTION

Breast cancer (BC) is the most common malignant tumour in females with an explicit cancer-related mortality worldwide. By definition, triple-negative BC (TNBC) lacks oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression and is associated with high-grade histological features, an aggressive clinical course, and a high metastatic potential. Immunohistochemistry (IHC) panels might be useful in the case of metastasis, to identify the origin of the tumour. Trichorhinophalangeal syndrome type 1 (TRPS1) is a transcription factor of the GATA family and has been proposed as a new and relatively specific marker of BCs.

The well-known prognosticators for BC are tumour stage (derived from tumour size, lymph node metastasis and distant metastasis), histologic grade, lymphovascular invasion (LVI), ER, PR, and HER2 status. Galectin-1 emerges as a negative prognosticator in a wide-range of solid tumours, due to its immunosuppressive role in the tumour microenvironment. The role of galectin-1 expression is unknown in TNBCs and HER2-positive carcinomas, where tumour

infiltrating lymphocytes (TILs) related data suggest the involvement of the immune system in prognosis.

Nowadays, BC treatment includes local radiotherapy, surgical excision and a wide range of systemic treatments. The aim of oncological surgery is to remove tumours with a rim of tumour free margin, but ablative surgery can lead to tissue alterations, especially cautery-related changes. In cautery artefacts, due to the morphologic changes, the lesions at the resection margins may remain uncertain. In these cases, the morphology alone is not sufficient to judge, and complementary methods may be required for the qualification of altered tissues at the margin.

1.1. TRPS1 EXPRESSION IN CYTOKERATIN 5 EXPRESSING TRIPLE-NEGATIVE BREAST CANCERS, AND ITS VALUE AS A MARKER OF BREAST ORIGIN

Common breast markers include mammaglobin (MGB), GATA binding protein 3 (GATA3), SRY-box transcription factor 10 (SOX10), and gross cystic disease fluid protein 15 (GCDFP-15), but neither of these are uniquely specific to BC. GCDFP-15 is also a marker of apocrine differentiation if diffusely expressed, and therefore obviously labels a subset of TNBC, that show apocrine differentiation. On the other hand, non-apocrine breast cancers may also be stained with this antibody. As breast marker, MGB has proven to be more sensitive than GCDFP-15, but it is also expressed in other tumours, mainly in endometrial carcinomas. GATA3 is probably one of the most sensitive markers of BC, but it is expressed in several other types of tumours, including the majority of urothelial carcinomas. In contrast to the previous markers, SOX10 turned out to be a reliable marker of TNBCs, which also labels myoepithelial cells and shares high specificity for melanocytic tumours. TRPS1 has been found to be a relatively specific marker of breast cancers through TCGA (The Cancer Genome Atlas) data mining. It is not only expressed in a high proportion of ER-positive or HER2-positive BCs, but also in TNBCs of different types, including metaplastic carcinomas, whereas its expression in other cancer types was absent or negligible.

1.2. THE VALUE OF OESTROGEN RECEPTOR, PROGESTERONE RECEPTOR, KERATINS 5 AND 14 IMMUNOHISTOCHEMISTRY IN THE EVALUATION OF EPITHELIAL PROLIFERATIONS AT CAUTERISED MARGINS IN BREAST-CONSERVING SURGERY SPECIMENS

The safety of breast conservation has been proven even in the case of oncoplastic techniques. A factor linked to local recurrences is a tumour-transecting (positive) surgical excision margin

and the associated residual cancer. The definition of a negative (tumour-free) surgical margin has evolved over time. Currently, “no ink on tumour” is widely accepted as a negative margin for invasive breast cancer (IBC), while a tumour-free rim of at least 2 mm is considered sufficient for pure ductal carcinoma *in situ* (DCIS) by some. Others regard a 1 mm margin as adequate for both IBC and DCIS. While surgery with traditional scalpels and blades can give the best-preserved material for histological assessment, they have been widely replaced by electric cutting and sealing devices. Although these make surgery easier with less blood loss, they traumatize the tissues at a higher rate and grade, making margin assessment more troublesome. At times, it is difficult to decide whether the cauterised tissue at the inked margin represents normal, hyperplastic, or neoplastic tissue.

Normal breast tissue and usual-type hyperplasia is characterized by a mosaic-like staining pattern for ER, PR, and some high-molecular-weight keratins, like the pair of CK5 and CK14, whereas low-grade neoplastic lesions, like atypical ductal hyperplasia and low-grade DCIS are typically ER and PR diffusely positive and CK5 and CK14 negative. Invasive carcinomas may have various patterns of ER and PR staining, but CK5 and CK14 are generally negative, though sometimes they can be diffusely positive, like in many TNBCs.

1.3. GALECTIN-1 EXPRESSION IN BREAST CANCER STROMA – PROGNOSTIC VALUE IN TRIPLE-NEGATIVE BREAST CANCER

Galectin-1 is a β-galactoside-binding mammalian lectin with carbohydrate-recognizing domain that interacts with β-galactoside-containing glycans. Galectin-1 plays a role in cell adhesion, regulation of cell growth, cell motility, and invasion. It is expressed in a wide range of normal human tissues and in several malignant tumours, including BC. Overall, galectin-1 participates in tumoral immune escape mechanisms, which might be tumour-type dependent. Galectin-1 expression is associated with a worse prognosis, and patients may potentially benefit from galectin-1-targeted therapies. According to Dalotto-Moreno et al., galectin-1 helps the development of immunosuppressive niches in breast cancer, through the accumulation of CD4-, CD25-, and forkhead box P3- (FoxP3) positive regulatory T cells in the tumour stroma. Galectin-1 also inhibits the re-organization of the actin filament cytoskeleton in T cells, leading to insufficiency of T cell adhesion. These data suggest that galectin-1 has an immunosuppressive role that could deleteriously influence the prognosis of tumours with high expression of this molecule.

2. AIMS

The aims of the thesis are:

I. To evaluate TRPS1 expression in CK5-positive TNBC and compare its diagnostic performance with other established breast markers, including SOX10, GATA3, MGB, and GCDFP-15.

II. To retrospectively assess the value of ER, PR, CK5, and CK14 IHC in clarifying the nature of cauterised tissues at the resection margins.

III. To investigate galectin-1 expression in TNBC and to determine its prognostic value.

3. MATERIALS AND METHODS

3.1. TRPS1 EXPRESSION IN CYTOKERATIN 5 EXPRESSING TRIPLE-NEGATIVE BREAST CANCERS, ITS VALUE AS A MARKER OF BREAST ORIGIN

This study was approved by the Human Investigation Review Board, University of Szeged (Approval No. 133/2019-SZTE RKEB).

Immunostaining was done on tissue microarrays (TMAs) constructed from TNBCs with CK5 expression as described earlier. Antibody details for breast markers other than TRPS1 (polyclonal rabbit clone, Invitrogen, Waltham MA, 1:250) and staining results (GATA3, MGB, GCDFP-15, and SOX10) were reported previously, but the cut-offs for positivity were also tested as ≥ 10% rather than > 5% as this higher percentage allows easier detection and interpretation. For TRPS1, nuclear staining of any intensity in at least 10% of the tumour cells was interpreted as positive. Six pathologists evaluated the IHC with TRPS1. All participants have classified the cases as positive or negative using this cut-off value, to estimate the reproducibility of interpretation. Majority opinions were selected for labelling a case negative or positive. Reproducibility was assessed by ONEST (observers needed to evaluate subjective tests) using an open-source software and kappa statistics. Ninety-five percent confidence intervals (CIs) of proportions were calculated with the VassarStats software (vassarstats.net).

3.2. THE VALUE OF OESTROGEN RECEPTOR, PROGESTERONE RECEPTOR, AND KERATINS 5 AND 14 IMMUNOHISTOCHEMISTRY IN THE EVALUATION OF EPITHELIAL PROLIFERATIONS AT CAUTERISED MARGINS IN BREAST-CONSERVING SURGERY SPECIMENS

We examined specimens from BC patients treated by primary surgery at the Department of Surgery, Bács-Kiskun County Teaching Hospital between 2020 and 2023.

All specimens were received fresh at the Department of Pathology. Large resection specimens were inked and sliced parallelly before fixation, whereas small samples were submersed in the fixative *in toto*. Each sample was fixed in 10% neutral-buffered formalin for at least one day. Margins were generally assessed through one to several blocks taken perpendicularly to the painted surface, unless considered to be significantly distant from it.

During microscopic evaluation of routine diagnostic slides, IHC for ER, PR, CK5, and CK14 were ordered to clarify the neoplastic or non-neoplastic nature of artefactually distorted epithelium at the inked margin or very close to it. The results were evaluated in the context of the lesions and were interpreted as positive margins (ink on tumour), negative margins (ink on non-tumorous epithelium) or uncertain margins (when no firm statement could be reached even with the ancillary studies). Cases were retrospectively collected and analysed as a group to see the value of these stains individually and in combination. Photomicrographs were taken with a Nikon digital camera mounted on a Nikon Eclipse Ci-L microscope to allow archiving of the lesions analysed.

All cases were evaluated in retrospect by the two authors, and consensus was reached on each lesion. The cases were classified into the five categories of neoplastic, favour neoplastic, uncertain, favour non-neoplastic, and non-neoplastic on the basis of the immunostains and the histological context.

No ethical approval and informed consent were deemed necessary, as no intervention was done, no patient data were assessed and only slides from cases with relevant immunohistochemical stains -originally ordered to assess cautery artefacts- were reviewed retrospectively.

3.3. GALECTIN-1 EXPRESSION IN BREAST CANCER STROMA – PROGNOSTIC VALUE IN TRIPLE-NEGATIVE BREAST CANCER

This study was approved by the Human Investigation Review Board, University of Szeged (Approval No. 91/2021 SZTE RKEB)

We examined TNBC cases treated at the Bács-Kiskun County Teaching Hospital between 2005 and 2016. Patients with sufficient BC tissue to build TMAs, and at least 6 months of follow-up time were included. Patients who had disseminated disease at the time or within 6 months of BC diagnosis were excluded. From the selected formalin-fixed, paraffin-embedded tumour blocks, two to four tissue cores were taken. Galectin-1 (Leica/Novocastra, clone 25C1, 1:200) IHC was performed on 2-μm-thick TMA sections at room temperature for 60 minutes.

Since we wanted to evaluate the immunosuppressive shield formed by galectin-1, we specifically looked for staining at the stromal interface around tumour cell nests. Stromal galectin-1 staining intensity was qualified as nil, weak, moderate, strong, and very strong. As the number of cases was rather low in each category of this five-teared system, we have lumped no staining, weak and moderate staining into one category (non-intense), and strong and very strong (intense) staining into another. Additionally, we have also estimated the percentage of the peritumoral stroma that showed any intensity of staining and have used less than 50% and 50% or above, to separate low versus high percentage of staining.

Stromal tumour-infiltrating lymphocytes (sTILs) were determined according to the recommendations of the International Immuno-Oncology Working Group and a cut-off of ≥30% was adopted for segregating tumours with high sTILs and those with low sTILs. The sTILs evaluation was performed on whole histological slides for every case.

We compared the overall survival (OS) and the progression-free survival (PFS) of patients with galectin-1 expression and those without. The follow-up data were taken from the medical charts. We used the SPSS (IBM, SPSS 23.0, Armonk, NY, USA) software package for the statistical analyses and Kaplan-Meier curve generation, and we used the log-rank test for the survival-curve comparisons. The level of significance was set at $p<0.05$. We set the time of the last visit as the end-point of the follow-up time when the patient was lost to follow-up or alive. Univariable and multivariable Cox regressions were analysed for identifying independent prognostic factors. Prognosticators, which had a statistical impact on survival in the univariable analysis, were entered into a multivariable Cox proportional hazards model. When several galectin-1 evaluations turned out to be significant in univariable analysis, all were entered in the multivariable model first, but as these variables reflect the same thing, a second calculation was performed with only the galectin-1 related evaluation yielding the lowest *p*-value in the univariable test. Any association of significant prognostic factors with galectin-1 staining was tested with the Fisher's exact test.

4. RESULTS

4.1. TRPS1 EXPRESSION IN CYTOKERATIN 5 EXPRESSING TRIPLE-NEGATIVE BREAST CANCERS, ITS VALUE AS A MARKER OF BREAST ORIGIN

Of the 120 cases, only 117 had evaluable samples. The majority (n=112) of the tumours were IBCs of no special type (NST), inclusive of 6 cases with medullary pattern and 2 with mixed invasive micropapillary component, but a few metaplastic carcinomas were also part of the tumours investigated, including 4 with squamous and 1 with heterologous mesenchymal differentiation. For the ONEST plot analysis, all permutations (6!=720) of the observers were used, rather than only 100 randomly chosen as suggested by the first descriptions and uses. The main descriptors of ONEST included 72.6% overall percent agreement, and a minimum of 4 observers needed to assess reproducibility. The Cohen's kappa coefficient was 0.67, reflecting substantial agreement. Majority opinions were used for categorization as positive or negative, and for the two cases with 50–50% split of opinions, revision of the slides was done to categorize the cases as positive.

Of the 117 evaluable tumour samples, 92 samples (79%; 95% CI 70–85%) showed nuclear staining with TRPS1 IHC in 10 to 100% of tumour cells. Generally, a diffuse staining was seen; 78/92 cases (85%; 95% CI 75–91%) showed ≥ 50% nuclear labelling, and this was the case in 3/5 metaplastic carcinomas, of which the remaining two (with squamous metaplasia) turned out to be negative. The remainder, i.e., 25 samples, were completely negative, or showed at times strong cytoplasmic staining without nuclear labelling with the exception of 1 case which showed very weak labelling in about 5% of nuclei. Discrepant interpretations were generally seen in cases of non-diffuse labelling. As concerns the other markers, their number (rate) of positivity (with the same 10% cut-off) were as follows: SOX10, 82 (70%; 95% CI 61–78%); GATA3, 11 (9%; 95% CI 5–17%), MGB 10 (9%; 95% CI, 4–16%), and GCDFP-15, 7 (6%; 95% CI 3–12%). This order was taken into account when organizing the IHC markers in hierarchy. Of the 25 TRPS1-negative cases, 11 samples were positive, and 13 were negative with SOX10. To continue this line, out of 13 TRPS1 and SOX10 dual-negative cases, none showed GATA3 positivity, and one was not assessable. Of the 12 TRPS1, SOX10, and GATA3 triple-negative cases, only 1 was positive with MGB. The remaining 11 cases were divided into 3 GCDFP-15-positive and 8 GCDFP-15-negative cases. Of the 92 TRPS1-positive cases, 20 were positive with only this marker (22%, 95% CI 14–32%); the rest showed dual or triple positivities, and a single case was positive for all 5 markers.

The 25 TRPS1-negative cases displayed various stainings with other breast cancer markers. One case had no available results for the rest of the markers, and one had only positive SOX10 results available. With a general 10% cut-off for positivity, 7 cases were positive with SOX10 only. One case showed dual positivity with SOX10 and GATA3. Two cases were positive with both SOX10 and MGB. One case was positive with MGB only. Neither of the cases was positive with just GATA3. Four cases were positive with GCDFP-15. Eight cases were negative with all of the examined breast cancer markers. As the proportion of positive TNBC cases is much influenced by the cut-off values of GATA3, MGB, and GCDFP-15, we have also assessed a 5% cut-off (only for these 3 markers) used by several other studies to see the labelling of our TRPS1 negative cases.

4.2. THE VALUE OF OESTROGEN RECEPTOR, PROGESTERONE RECEPTOR AND KERATINS 5 AND 14 IMMUNOHISTOCHEMISTRY IN THE EVALUATION OF EPITHELIAL PROLIFERATIONS AT CAUTERISED MARGINS IN BREAST-CONSERVING SURGERY SPECIMENS

In total, we analysed 34 lesions from 23 patients. The neoplasms for which surgery was performed included 14 IBC NST, 6 of which showed an extensive intraductal component (EIC); 4 pure DCIS; 1 tubular carcinoma with EIC; 1 case of microinvasive Paget's disease of the nipple with EIC; and a tumour bed following neoadjuvant systemic therapy for an IBC NST. Additionally, a case of atypical ductal hyperplasia and an intraductal papilloma were also included, as the identification of (further) neoplastic lesions in the cauterised area would have upgraded their diagnosis. All of the invasive carcinomas and 3 of 4 DCIS cases were ER- and PR-positive. The remaining case of DCIS was ER- and PR-negative and HER2-positive with apocrine differentiation confirmed by the expression of androgen receptors and GCDFP-15. HER2 positivity was also a feature of the Paget's disease (ER- and PR-positive) and the invasive carcinoma treated previously with systemic therapy; this latter had also been ER-positive with unknown PR status.

Seven lesions served as controls, 3 obviously representing cauterised neoplastic tissues and 4 representing non-neoplastic tissues with cautery artefacts. The remaining 27 cases belonged to lesions that could not be adequately classified on the basis of the haematoxylin and eosin (HE) stains. Following the quadruple immunostaining, all but one control case showed the expected pattern of staining with the keratin antibodies; i.e. no staining in neoplastic and mosaic-like staining in non-neoplastic epithelium with myoepithelial labelling. The deviating case was one with no ER and CK14 staining but mosaic pattern of CK5 and PR staining. In contrast, steroid

hormone receptor stainings were somewhat less often supportive, either due to negativity or partial positivity in neoplastic lesions or complete lack of staining in the cauterised tissue.

Of the cases that were uncertainly classifiable on HE-stained slides, two thirds could be classified as either non-neoplastic or neoplastic, and two thirds of the remaining could be favoured as neoplastic or non-neoplastic, with 3 out of 27 cases remaining uncertain.

All 4 IHC reactions proved helpful in classifying the lesions as neoplastic or non-neoplastic in nearly half of the cases, though this proportion was smaller when uncertain, i.e. challenging cases were considered. However, 3 or 4 immunostains were supportive of the classification in 19 out of 27 (0.70; 95% CI: 0.50–0.86). The most useful stains were the keratins, generally demonstrating a matching pattern of cell labelling with CK5 and CK14; however, in a few cases (n=4), the two antibodies yielded divergent results. ER and PR, especially the first were less useful in classifying uncertain lesions.

Considering all 27 questionable lesions, IHC with ER, PR, CK5, and CK14 clarified the lesions at the cauterised margins in 23 cases (0.85; 95% CI: 0.66–0.96), and in a further case (ER+, PR+, and HER2+ DCIS with Paget's disease and microinvasion) none of the 4 antibodies showed staining, but a strong HER2 staining allowed classification as neoplastic on this contextual basis. Another lesion is worth mentioning, namely an apocrine DCIS, in which CK5 and CK14 negativity was associated with ER and PR negativity, and this was interpreted as fully supportive of a neoplastic nature.

4.3. GALECTIN-1 EXPRESSION IN BREAST CANCER STROMA – PROGNOSTIC VALUE IN TRIPLE-NEGATIVE BREAST CANCER

We evaluated 95 cases, and all of them were from female patients without metastasis at the time of the diagnosis. The median age of the patients was 69 years (range: 29-91 years). Almost the same number of cases belonged to the pT1c (40) and pT2 (43) categories. All but 3 cases were of histological grade 3. The majority of the studied cases had pN0 disease, and among the node-positive tumours, most belonged to the pN1 category. The median follow-up time was 62 months (range: 11 to 220 months). During the follow-up, 37 deaths were recorded, including 22 deaths due to breast cancer. Locoregional or distant recurrence was noted in 57 patients. Accordingly, 38 patients were disease-free until the endpoint of the follow-up.

Surgery included mastectomy (n=26) or BCS (n=69) with sentinel lymph node biopsy (n=50) or BCS with axillary lymph node dissection (n=43); no axillary procedure was done in two patients. Seven patients received neoadjuvant chemotherapy but showed no regression or even

progressed. Most of the patients got adjuvant chemotherapy (either a taxane-containing therapy or an anthracycline-based therapy). A smaller part of the patients did not get adjuvant chemotherapy, due to their known comorbidities, or their refusal to the recommended therapy.

According to the log-rank test, there was a significant difference in OS (but not PFS) in the nil/weak/moderate/strong/very strong intensity staining 5-tiered model (degree of freedom: 4, p=0.006). When only two intensity groups were separated and compared, we also observed a significant difference in OS (p=0.008). For the low sTILs subset, we found a significant difference in OS between intensively staining cases versus the rest of the cases (p=0.005). With the application of the 50% cut off value for peritumoral stromal staining, the log-rank analysis resulted in a significant difference in both OS (p=0.02), and PFS (p=0.007). For the low sTILs subset, we found a significant difference in PFS (p=0.025), but not in OS.

With prognostic factors (pT and pN, grade, LVI, sTILs, and galectin-1 staining) entered in univariable Cox regression, pT (p=0.036), and pN categories (p=0.003), intense galectin-1 stromal staining (p=0.01) and ≥50% stromal positivity (p=0.024) proved to be significantly associated with OS. In multivariable analysis, only pN emerged as independent prognosticator when all significant variables were entered in the model. When of the two galectin-1 staining parameters only the one with the lower p-value was entered in the model (i.e. the 2-tiered intensity scoring), both pN and galectin-1 staining intensity emerged as independent prognosticators. Galectin-1 staining intensity and percentage were not associated with the pT and pN categories (p=0.08 /intensity, pT/; p=0.70 /intensity, pN; p=0.53 /percentage, pT/; p=0.61 percentage, pN/).

Regarding the low sTILs group, with a smaller number of cases (n=43) in the univariable Cox regression analysis, stromal staining intensity (p=0.008), ≥50% galectin-1 peritumoral positivity (p=0.011) and the pN category (p=0.046) proved to be significantly associated with OS. Similarly to the above-described approach, two multivariable analyses were performed with the variables emerging as significant influencers of prognosis in the univariable tests first, and with only the most relevant galectin-1-related classification entered in the second, and with this approach, only galectin-1 intense staining had a statistical impact on survival (p=0.009).

In the univariable Cox regression analysis, the pT categories (p=0.004), pN categories (p=0.001), and ≥50% stromal galectin-1 expression proved to be significantly associated with PFS. In the multivariable analysis the pN category (p=0.004) and stromal galectin-1 staining percentage (p=0.008) proved to be independent prognosticators. For the low sTILs subset, only

pN and galectin-1 staining percentage were significant prognosticators in univariable analysis, and both proved to be independent on multivariable analysis.

5. DISCUSSION

5.1. TRPS1 EXPRESSION IN CYTOKERATIN 5 EXPRESSING TRIPLE NEGATIVE BREAST CANCERS, ITS VALUE AS A MARKER OF BREAST ORIGIN

GCDFP-15, MGB, and GATA3, as the first reported breast markers, all show decreased sensitivity in TNBCs. Low percentage of weakly staining cells always cast some doubt about the interpretation of the given IHC reactions, despite the fact that even low positivity rates may point to BC origin in relation to TNBCs. Diminishing the cut-off to 5% did not greatly impact on SOX10 and TRPS1 positivity rates, 82 vs 86/117 and 92 vs 93/117, respectively.

Even with the potential variation with different antibody clones in mind, literature data and the presented results indicate that SOX10 and TRPS1 are more sensitive breast markers than GATA3, MGB, and GCDFP-15 for TNBCs, including those expressing CK5 and overlapping in phenotype with squamous carcinomas. In this set, TRPS1 was the most expressed breast marker, followed by SOX10, GATA3, MGB, and GCDFP-15. The sensitivity of TRPS1 was 0.86 whereas that of SOX10 was 0.69 (with a 10% cut-off, and these values would have been 0.87, and 0.72, respectively with a 5% cut-off).

There was substantial agreement between the observers in rating the cases as positive or negative with TRPS1, and the ONEST analysis suggested over 70% overall agreement. The number of observers needed to reliably reflect reproducibility was 4, and this is more than for ER or PR, but less than for Ki67 with a similar 10% cut-off, all being nuclear staining proportions evaluated.

5.2. THE VALUE OF OESTROGEN RECEPTOR, PROGESTERONE RECEPTOR AND KERATINS 5 AND 14 IMMUNOHISTOCHEMISTRY IN THE EVALUATION OF EPITHELIAL PROLIFERATIONS AT CAUTERISED MARGINS IN BREAST-CONSERVING SURGERY SPECIMENS

At present a tumour-free margin at BCS is considered to be no ink on tumour, or 1- or 2-mm tumour-free band of non-tumorous tissue. Cautery artefacts interfere with the pathology reporting of margins, as it is not easy to decide whether the “burnt”, traumatised tissue represents part of a neoplasm or is an innocuous bystander.

As one could expect, the use of the quadruple IHC stain did not make all the decisions black and white, although in the majority (two thirds) of the cases, it helped to make a firm categorization, and in two thirds of the remaining cases it helped to reach a conclusion that could be taken into account in treatment planning. In a limited number of cases (about one tenth), the IHC did not help at all. The antibodies were not equivalent, and all four were supportive of the classification in only about 40% of the cases. In general, keratins were found more helpful. In fact, there was no case, where neither of the two keratins were of help and the steroid receptors assisted in the clarification, but there were a few cases, where one of the keratins failed to give (strong) evidence in favour of either a neoplastic or a non-neoplastic nature, and here the ER and PR stains had some additional value. The keratins may also be helpful in distinguishing between cauterised invasive and carcinoma *in situ* at the margin owing to the presence or absence of the peripheral (myoepithelial) staining.

5.3. GALECTIN-1 EXPRESSION IN BREAST CANCER STROMA – PROGNOSTIC VALUE IN TRIPLE-NEATIVE BREAST CANCER

From the point of our research, it is important to highlight that sTIL numbers are generally higher in TNBCs and HER2-positive tumours, compared to the luminal types. TNBCs with higher sTIL numbers have better prognosis, than those with low sTIL counts, suggesting that this inflammatory infiltrate may bear greater antitumoral effect than tumour promoting effect. Thus, an immunosuppressive molecule may have prognostic relevance in these subgroups of BC.

Galectin-1 has an immunosuppressive role in the tumour microenvironment, which could be the reason behind the association of galectin-1 expression intensity and/or percentage and shorter survivals. In our study, galectin-1 expression intensity showed a significant association with worse OS in the whole series, but there was no significant association with PFS. In contrast, galectin-1 stromal staining percentage was associated with both worse OS and PFS. Our results therefore support the negative prognostic nature of galectin-1 in TNBC.

We examined separately the high and the low sTILs groups with a cut-off of 30%. The statistical analysis showed a significant difference in OS for the low sTILs subset according to galectin-1 staining intensity, and in PFS according to galectin-1 staining percentage. In these cases, the lower sTILs number and the higher galectin-1 expression could both explain the worse survivals. Our study seems to be the first to look at galectin-1 expression and its impact on

survival in TNBCs, where immune mechanisms and immune components of the malignant phenotype are important and affect the outcome of the disease.

The observations that high sTILs correlate with better prognosis in TNBCs suggest that the immune system plays an anti-tumour activity in these tumours. Therefore, the immunosuppressive effects of galectin-1 may form an immune-escape mechanism as part of the principal hallmarks of malignancy and can explain the worse OS (and in some approaches also the worse PFS) we demonstrated in TNBC. The multivariable analysis supported the survival curve analysis and indicated that intense staining and ≥50% galectin-1 stromal positivity in TNBC are independent prognosticators. We found that not only the percentage of galectin-1 expression in the peritumoral stroma has an impact on PFS and OS, but also that the more intensive the staining, the worse the OS. In the low sTILs subgroup, galectin-1 expression (by percentage of staining) turned out to be a significant prognostic factor in the multivariable analysis for PFS.

6. CONCLUSIONS

Based on our results, of the five markers compared, TRPS1 seems to be the most sensitive marker for the mammary origin of CK5-expressing TNBCs (most likely to coincide with basal-like breast carcinomas) and might be best exploited in the metastatic setting. Cases that are negative are most often labelled with SOX10, whereas the dual-negative subset may still be positive for one of the additional breast markers. The reproducibility of the evaluation indicates that TRPS1 is a reproducible and readily interpretable marker.

The clarification of whether a triple-negative carcinoma in the breast is a primary breast carcinoma or not is dependent on several contextual features, like the presence of corresponding carcinoma *in situ*, the morphological alignment with typical histological types of breast cancer, and presence of unconventional morphologies. Consequently, these may reduce the need for breast marker testing. Core biopsies may be less representative and may require more extensive testing. Of course, both in the primary and the metastatic settings, relevant clinical history is of prime importance. But when breast marker testing becomes a need, a panel of markers is best to be used, as even the least sensitive marker can be of value. On the basis of our results and the cited literature, TRPS1 is a valuable marker to be included in these panels.

Considering cauterized surgical margins, CK5, CK14, PR, and ER IHC may help in distinguishing between cautery-damaged neoplastic and non-neoplastic tissues. All four IHC

may yield the best support for decision making, but sometimes, CK5 and/or CK14 alone may be sufficient. To avoid misinterpretations, the patient's disease must always be put into context.

Intense and/or diffuse galectin-1 stromal positivity around tumour cell nests in TNBC was associated with worse OS and/or PFS and proved to be an independent prognosticator on multivariable analysis. We found that not only the percentage of galectin-1 expression in the peritumoral stroma has an impact on PFS and OS, but also that the more intensive the staining, the worse the OS. In the low sTILs subgroup, galectin-1 expression (<50% versus more) turned out to be a significant prognostic factor in the multivariable analysis for PFS.

The results of our exploratory study suggest that the immunosuppressive effects of galectin-1 may form an immune-escape mechanism as part of the principal hallmarks of malignancy. As the mechanisms regulating anti-tumour immunity are complex, further confirmatory studies are needed before the results can be exploited as having possible therapeutic implications with galectin-1 targeting treatments.

7. MAJOR NEW FINDINGS

TRPS1 seems the most sensitive marker for the mammary origin of CK5-expressing TNBCs (most likely to coincide with basal-like breast carcinomas) and might be best exploited in the metastatic setting. The reproducibility of the evaluation showed substantial agreement, indicating that it is a reproducible and readily interpretable marker.

CK5, CK14, PR, and ER IHC may help in distinguishing between cautery-damaged neoplastic and non-neoplastic tissues. All four IHC may yield the best support for decision making, but CK5 and/or CK14 alone may be sufficient in many cases.

Worse OS (and PFS) were found in TNBCs demonstrating a more intense and/or diffuse peritumoral stromal immunostaining with galectin-1, and this could be explained by the immunosuppressive effects of galectin-1 forming a shield around tumour nests indicating an immune escape mechanism.

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