Immunohistochemical Analyses in the Diagnostics of Breast Cancer

Ph. D. Thesis

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LIST OF ABBREVATIONS:

AMACR: Alfa-Methylacyl Coenzyme A Racemase

BC: Breast cancer

BCS: Breast conserving surgery

CI: Confidence interval

CK: Cytokeratin

DCIS: Ductal carcinoma in situ

ECM: Extracellular matrix

EIC: Extensive intraductal component

ER: Oestrogen receptor

FFPE: Formalin-fixed, paraffin-embedded

GATA3: GATA binding protein 3

GCDPF-15: Gross cystic disease fluid protein 15

HE: Haematoxylin and eosin

HER2: Human epidermal growth factor receptor 2

HMWCK: High molecular weight cytokeratin

IBC: Invasive breast cancer

IHC: Immunohistochemistry

LCA: Leukocyte common antigen

LVI: Lymphovascular invasion

MGB: Mammaglobin

NST: No special type

NYBR-1: New York Breast-1

ONEST: Observers needed to evaluate subjective tests

OS: Overall survival

PAM50: Prediction Analysis of Microarray 50

PFS: Progression-free survival

PR: Progesterone receptor

SOX10: SRY-box transcription factor 10

sTILs: Stromal tumour-infiltrating lymphocytes

TCGA: The Cancer Genome Atlas

TNBC: Triple-negative breast cancer

TMA: Tissue microarray

TRPS1: Trichorhinophalangeal syndrome type 1

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1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Breast cancer (BC) is the most common malignant tumour with an explicit cancer-related mortality worldwide [1]. Based on gene expression profiles, BCs have been divided into intrinsic subtypes with different prognosis: the oestrogen receptor- (ER) driven luminal A and luminal B tumours, the human epidermal growth factor receptor 2 (HER2)-enriched, and the basal-like carcinomas [2-4]. Owing to the limited availability of gene expression profiling, immunohistochemistry (IHC) based surrogate subtypes have become key parameters in predicting prognosis and planning treatment of the disease. According to hormone receptor and HER2 expression as well as proliferation, BC is divided into luminal A-like (ER and progesterone receptor (PR) positive, HER2 negative and low Ki67), luminal B-like (ER and/or PR positive, HER2 positive or negative with high proliferation rate (Ki67)), ER negative HER2 positive, and triple-negative subtypes. There are huge differences between the biological behaviour and treatment options of these subtypes, and triple-negative BCs (TNBCs) are usually high-grade, aggressive tumours with high metastatic potential [3-4], therefore, due to their worse prognosis, we decided to centre our research on this subtype.

Per definition, TNBC lacks ER, PR, and HER2 expression. These are not specific markers of BCs, but as the majority of BCs express them in some combination, in cases of a distant metastasis, they might be helpful in orienting the attention to the breast as primary site. In such cases, IHC panels may be helpful to distinguish the origin of the tumour. These IHC panels usually contain mammaglobin (MGB), GATA binding protein 3 (GATA3), SRY-box transcription factor 10 (SOX10), and gross cystic disease fluid protein 15 (GCDFP-15). These well-known markers are not specific enough for BC. Trichorhinophalangeal syndrome type 1 (TRPS1) is a transcription factor of the GATA family, and has been proposed as a new, relatively specific marker of BCs [5].

In daily pathology practice, we can usually decide on the diagnosis based on the morphology and IHC markers. We face challenges when these two components are affected. When tumours are high-grade and do not express well-known specific and sensitive markers, we are happy to find new IHC markers that can help us decide on the primary or metastatic nature of a malignancy, and TRPS1 in TNBC seems to be such a marker.

BC is a common malignant tumour with well-known prognosticators, such as the tumour stage, tumour size, lymph node metastasis, 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 [6]. As data on this marker are limited, 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 [7].

Nowadays, BC treatment includes local and systemic treatments. The multidisciplinary team decides about the personalised treatment in each case. A wide-range of systemic therapies are available, such as chemotherapy, endocrine therapy, targeted-drug therapy, and immunotherapy. Local treatments include radiotherapy and surgical treatment.

Regarding the breast itself, the surgical treatment can be divided into breast-conserving techniques, including oncoplastic surgery, and different mastectomies with or without reconstruction. The axillary procedures, if performed, range from sentinel lymph node biopsy to complete axillary lymph node dissection.

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.

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

TNBCs are often high-grade tumours with poor prognosis that overlap with carcinomas classified as basal-like on the basis of gene expression profiling [2-4] especially if they express cytokeratin 5 (CK5) and/or epidermal growth factor receptor [8]. However, the two categories are not identical, as TNBCs can be subdivided according to gene expression into distinct categories [9-11] and also include rare tumours with a relatively indolent behaviour [12]. Common breast markers include GCDPF-15, GATA3, MGB, and SOX10, 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 (and skin appendage tumours), that show apocrine

differentiation [11, 13-14]. On the other hand, non-apocrine breast cancers may also be stained with this antibody, and this phenomenon may serve as evidence of breast origin in metastatic cases. Besides cutaneous tumours, other neoplasms that have been reported to be GCDFP-15 positive include salivary gland adenocarcinomas [15, 16] and prostatic adenocarcinoma [15 ,17].

As breast marker, MGB has proven to be more sensitive than GCDFP-15, but is also expressed in other tumours, mainly in endometrial carcinomas, but rarely also in some sweat and salivary gland tumours, pancreatic, and ovarian carcinomas along with other tumours including some melanomas [14].

GATA3 is probably one of the most sensitive markers of BC [18] but is expressed in numerous other types of tumours, including the majority of urothelial carcinomas [19]. As GATA3 is a key component of the ER-alpha-GATA3-FOXA1 transcriptional network [20], it is logical that TNBCs might have this marker expressed less frequently than luminal or luminal-like BCs. Indeed, our previous analysis of CK5-expressing TNBCs suggested that GATA3 was expressed in 71% of the 115 cases tested, but only 23 cases (20%) expressed this protein in > 5% of the tumour cells [21]. Due to the relatively low proportion of tumour cells and frequently weak immunoreactivity, GATA3 is considered less reliable in CK5-expressing TNBCs, despite demonstrating better performance than other markers such as MGB, GCDFP-15, and NYBR-1. Notably, NYBR-1 labelled only 6% of CK5-positive TNBC cases, with more than 5% staining observed in just 3% of cases [21] consistent with earlier findings showing a correlation between NY-BR-1 expression and ER positivity, and an inverse association with EGFR expression [22].

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 [23-25]. Other SOX10 positive tumours include salivary and skin adnexal gland tumours [26-27]. In keeping with the above, SOX10 outperformed GATA3, MGB, and GCDFP-15 as a mammary origin marker in TNBC [28-29].

TRPS1 (trichorhinophalangeal syndrome type 1) is a transcription factor of the GATA family and has been found to be a relatively specific marker of breast cancers through TCGA (The Cancer Genome Atlas) data mining [5]. 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 [5, 30].

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

In the treatment of BC, breast conserving surgery (BCS) and adjuvant radiotherapy have long proven to be equivalent with mastectomy in terms of local disease control and outcome [31-32], and there is even a suggestion that they are associated with better outcome [33]. The safety of breast conservation has been proven even in the case of oncoplastic techniques [34]. A factor that has been linked to local recurrences, is a tumour transecting (positive) surgical excision margin and the deduced residual cancer [35-38]. The practical approach and perceptions of what constitutes a negative (tumour free) surgical margin have changed over time, and currently "no ink on tumour" is widely considered the negative margin for invasive breast cancer (IBC) and a tumour free rim of at least 2 mm is considered sufficient for pure ductal carcinoma *in situ* (DCIS) [39-41] by some, whereas others are happy with a 1 mm wide margin for both IBC and DCIS [41].

While surgery with traditional scalpels and blades can give the best-preserved material for histological assessment, this has disadvantages because of more bleeding and intraoperative blood loss than electrocautery [43-44]. This is why "cold scalpels" have been widely replaced by electric cutting and sealing devices (electrocautery devices, electric or harmonic scalpels). 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 [45-46] whereas low-grade neoplastic lesions, like atypical ductal hyperplasia and low-grade DCIS are typically ER and PR diffusely positive while CK5 and CK14 negative [45-47]. 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.

1.4. 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 [48]. Within the cells, galectin-1 is positioned in the cytoplasm, the inner surface of the cell membrane, and the nucleus [49-50]. The cells are also capable of secreting galectin-1 into the extracellular matrix (ECM), however, this does not happen through the usual endoplasmic reticulum/Golgi complex pathway [51-52], but by a direct translocation through the plasma membrane [50, 53].

Galectin-1 plays a role in cell adhesion, regulation of cell growth, cell motility, and invasion [50]. It is expressed in a wide range of normal human tissues, such as the thymus, lymph nodes, prostate, spleen, lung, placenta, endothelial cells, keratinocytes, fibroblasts, and Langerhans cells [49, 54-55].

Galectin-1 expression has been described in several malignant tumours including cholangiocarcinoma [56], cervical squamous cell carcinoma [57], colorectal adenocarcinoma [58-59], gastric adenocarcinoma [60], gliomas [61-62], hepatocellular carcinoma [63], laryngeal squamous cell carcinoma [64], non-small cell lung carcinoma [65], oral cavity squamous cell carcinoma [66], ovarian carcinoma [67], pancreatic ductal adenocarcinoma [68-69], prostate adenocarcinoma [70], renal cell carcinoma [71-72], thyroid carcinoma [73], bladder urothelial carcinoma [74], and breast carcinoma [75-80].

Overall, galectin-1 participates in tumoural immune escape mechanisms, which might be tumour-type dependent [6]. Galectin-1 expression is associated with a worse prognosis, and patients may benefit from galectin-1-targeted therapies [6].

In head and neck cancer, galectin-1 secreted to the ECM can influence the endothelial cells blocking T cell migration through the blood vessels, leading to immune suppression in the tumour stroma. The tumour cell-derived galectin-1 can also upregulate programmed death-ligand in the cell surface and galectin-9 expression in the endothelial cells, which also leads to the inhibition of immune cell infiltration [81].

Furthermore, galectin-1 sensitizes the T cells to the FAS/caspase-8 mediated cell death [82] and suppresses the immune response-mediated by helper T cells 1 and 17 (Th1, Th17). As a human lectin, galectin-1 can bind specific carbohydrates and induce apoptosis in the terminally differentiated T cells, expressing carbohydrate ligands [83].

Galectin-1 is also involved in B cell regulation by reducing proliferation and depressing B cell receptor-mediated signal transduction [84].

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 [75]. Galectin-1 also inhibits the re-organization of the actin filament cytoskeleton in T cells, leading to insufficiency of T cell adhesion [85]. 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

Magnifications for the microscopic figures in this thesis are reflected by the microscope objectives which were used to make the IHC figures, these are mentioned in each figure legend, and do not include the camera objectives.

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 constructed from TNBCs with CK5 expression as described earlier [21]. Antibody details for breast markers other than TRPS1 were reported previously and are summarized in *Table 1*. Staining results for GATA3, MGB, GCDP-15, and SOX10 were used from previous work [21, 29], but the cut-offs for

positivity were also tested as ≥ 10% rather than > 5% (as used in several previous works) as this higher percentage allows easier detection and interpretation.

Table 1. **Antibodies used for breast marker assessment**

Antibody	Clone	Source	Dilution
TRPS1	Polyclonal rabbit	Invitrogen, Waltham, MA	1:250
SOX10	A-2	Santa Cruz, Dallas, TX	1:500
GATA3	HG3-31	Santa Cruz, Dallas, TX	1:50
MGB	1A5	Biocare, Concord, CA	RTU
GCDFP-15	23A3	Cell Marque, Rocklin, CA	1:200

RTU: ready to use

For TRPS1, nuclear staining of any intensity in at least 10% of the tumour cells was interpreted as positive. Six pathologists (SzA, LK, AS, AV, TZ, and GCs – see list of authors of the related publication on page 2) 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.

In addition, we studied 6 BC cases demonstrating HER2 positivity and CK5 expression (basal HER2 carcinomas by IHC). Among these, half were classified as basal-like and half as HER2-enriched on the basis of the expression of the Prediction Analysis of Microarray 50 (PAM50) gene expression pattern. This was determined from formalin-fixed paraffin-embedded (FFPE) tissues by the Breast Cancer 360™ Panel (Nanostring, USA) on the NanoString nCounter® FLEX platform (Nanostring, USA) following the manufacturers' instructions.

Reproducibility was assessed by ONEST (observers needed to evaluate subjective tests) [86-89] using an open-source software [89-90] and kappa statistics [91]. 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, larger resection specimens were inked (posterior margin stained blue, while the others stained black) and sliced parallelly before fixation, whereas smaller 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.

The following antibodies were used for the IHC (ER: clone 6F11, Leica Biosystems Newcastle, UK, 1:200; PR: clone PgR312, Leica Biosystems Newcastle, UK, 1:400; CK5: clone XM26 Thermo-Fisher-Epredia-LabVision, Kalamazoo, MI, USA, 1:40 (or earlier Labvision, Fremont, CA, USA, 1:25, developed manually); CK14, clone LL002, Leica Biosystems Newcastle, UK, ready-to-use; (in earlier cases the same, but developed manually). The IHC stains were carried out on a BOND-MAX Fully Automated IHC Staining System (Leica Biosystems, Deer Park, IL), all but the CK5 reaction used ER1 (pH=6), whereas CK5 used ER2 (pH=9) as epitope retrieval solution. Incubation time was 20 minutes for all antibodies. All cases were evaluated in retrospect by the two authors (SzA ang GCs), and consensus was reached on each lesion.

On occasions, one patient could have had several slides assessed and/or one slide could have had several questionable areas investigated. These latter areas formed the lesions referred to throughout the article. Some identified lesions could be classified without the use of the IHC stains, but as they were included on the slides and cauterised at some degree, they were used as controls of known nature (neoplastic *vs* non-neoplastic) whereas others were lesions of uncertain nature requiring clarification. These latter changes 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 perform TMA, 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 FFPE tumour blocks, two to four tissue microarray (TMA) cores were taken. One TMA contained ten columns and seven rows with some non-tumorous and non-mammarian tissue cores for orientation.

Galectin-1 IHC was performed on 2-µm-thick TMA sections. In brief, sections were routinely deparaffinized and heat-treated with appropriate antigen retrieval buffer solutions using a household electronic pressure cooker. After protein blocking (RE7102, Leica/Novocastra), the sections were incubated with the anti-human mouse galectin-1 monoclonal primary antibody (Leica/Novocastra, clone 25C1, 1:200) at room temperature for 60 minutes. Detection was performed using the Novolink polymer kit (Leica/Novocastra). Each IHC staining was performed using a 4-channel TECAN Freedom Evo liquid handling platform.

The IHC stained slides were scanned with a Pannoramic Midi Scanner (3DHistech, Budapest).

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, while galectin-1 staining in tumour cells was ignored and not evaluated in this study.

We adapted the method used by De Oliveira et al for the evaluation of stromal intensity [92]. Stromal galectin-1 staining intensity was qualified as nil, weak, moderate, strong, and very strong (illustrated in *Figure 1*). 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 (*Figure 1*).

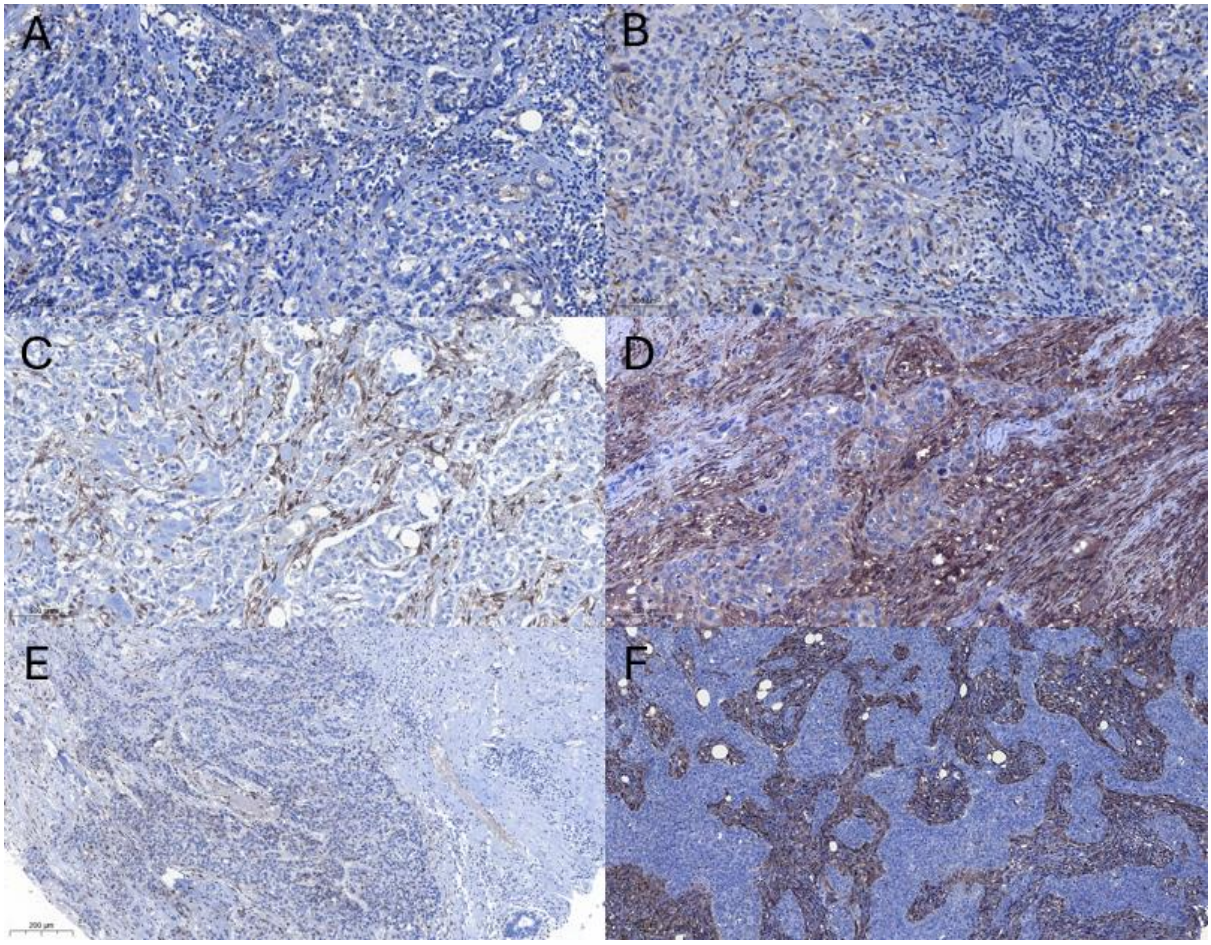

Figure 1. **Examples of galectin-1 immunohistochemistry with different stromal staining intensity: weak (A), moderate (B), strong (C) very strong (D) (x20). Less than 50% stromal positivity (E) and more than 50% stromal positivity (F) is also illustrated (x10).**

Stromal tumour-infiltrating lymphocytes (sTILs) were determined according to the recommendations of the International Immuno-Oncology Working Group [93] and a cut-off of ≥30% was adopted for segregating tumours with high sTILs and those with low sTILs. The cut-off selection was based on the meta-analysis suggestive of a prognostic role of sTILs in the adjuvant setting [94]. 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. Examples of TRPS1 immunostains are shown in *Figure 2*.

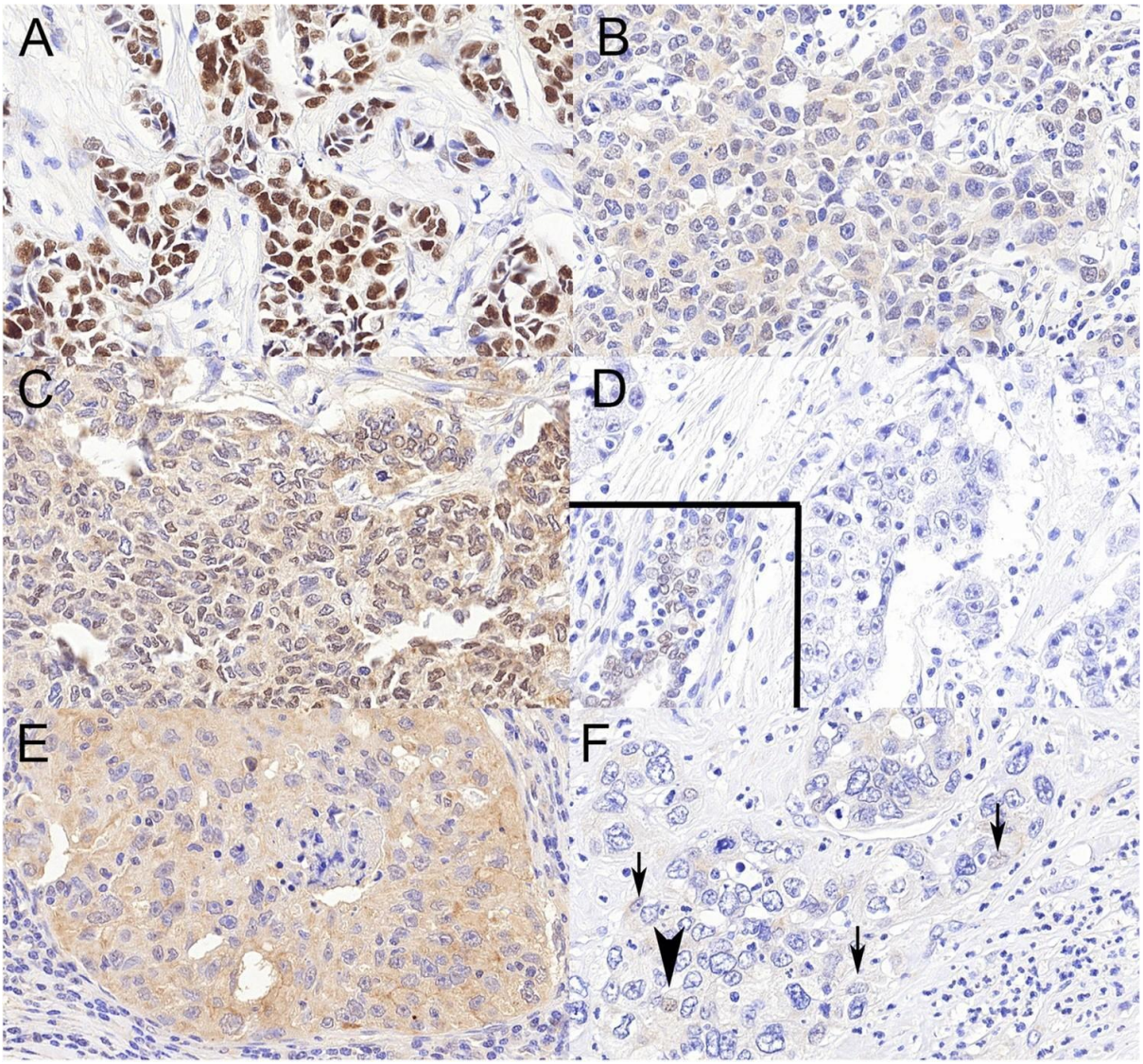

Figure 2. **Examples of TRPS1 positive (ABC) and negative (DEF) immunostainings. A: Obvious positive staining with strong nuclear labelling and no cytoplasmic background. B, C: Weak nuclear staining in > 10% of the cells interpreted as positive or negative by 50% of the observers; note the relatively strong background cytoplasmic staining. D: Completely negative reaction, inset showing positive staining in a normal duct of the same TMA core. E: A case with strong cytoplasmic background staining ignored due to the lack of nuclear staining. F: The case with about 5% of the nuclei staining weakly (bold arrowhead) or in a barely visible fashion (arrows) (x40).**

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 [86-87]. The main descriptors of ONEST included 72.6% overall percent agreement, 19.7% bandwidth (greatest difference in rating by 2 observers) and a minimum of 4 observers needed to assess

reproducibility. The Cohen's kappa coefficient was 0.67, reflecting substantial agreement [91]. 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 case as positive (these are illustrated in *Figure 2 B-C*).

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 (*Figure 2 D–F*). 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 (*Figure 3*).

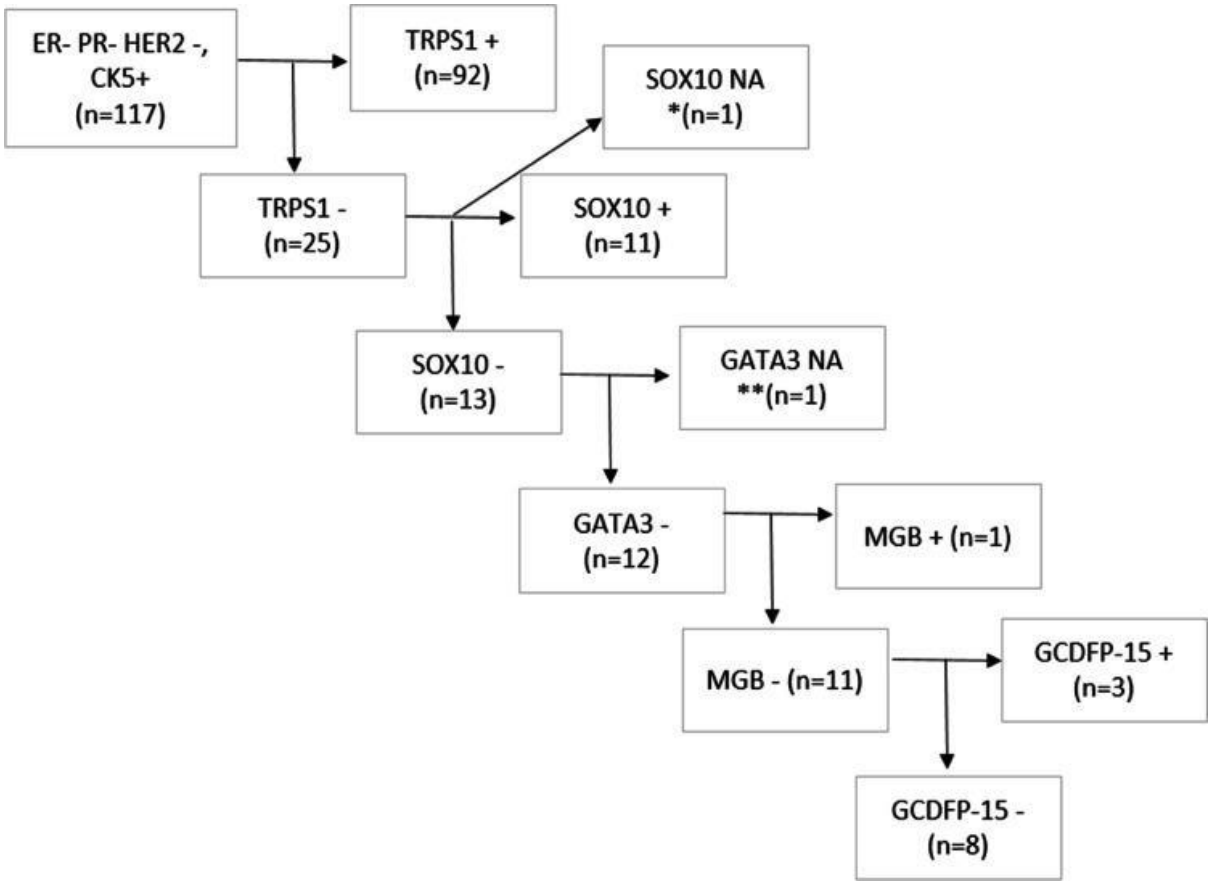

Figure 3. **Hierarchical labelling of CK5+ TNBC cases with TRPS1, SOX10, GATA3, MGB and GCDFP-15 (*none of the other stains were evaluable; ** the case had no GATA3 and MGB slide available, but 80% of tumour cells were GCDFP-15 positive); NA, not available. The order of the markers follows their positivity rate from highest to lowest from left to right**

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 (*Figure 3, Supplementary figure 1*).

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 (*Figure 4*).

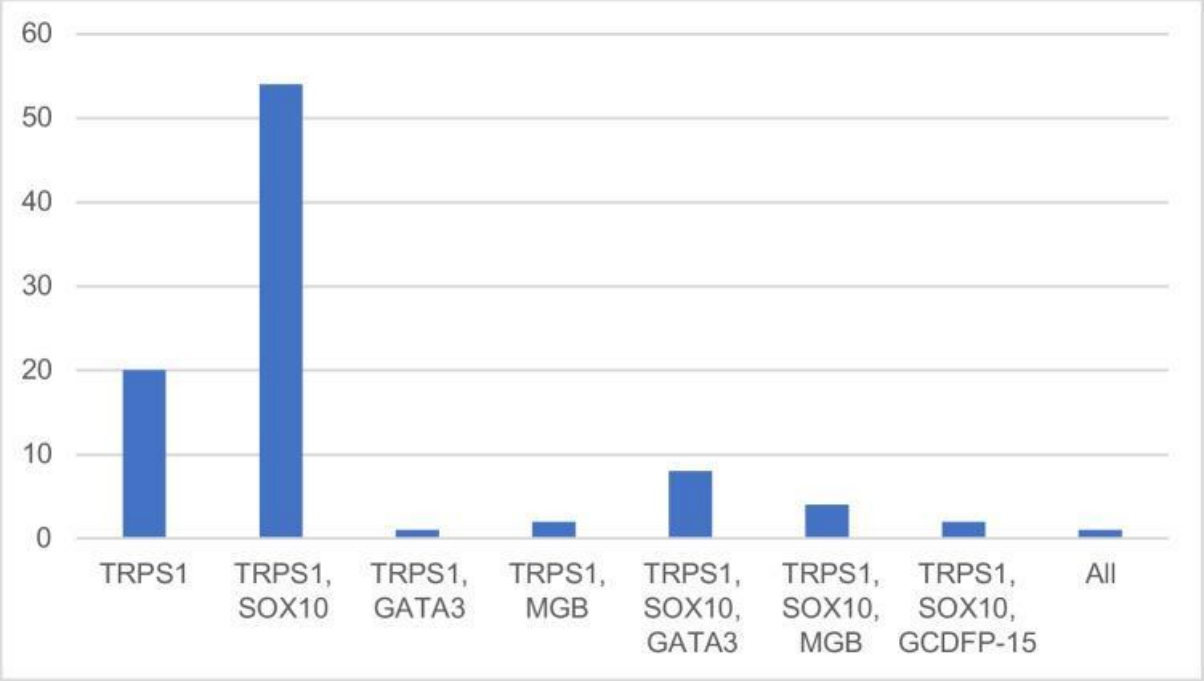

Figure 4. **Distribution of positive stainings with different breast markers in the 92 TRPS1-positive cases (number of cases on axis y)**

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 (*Figure 5 A*). 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. The results are displayed in *Figure 5 B*.

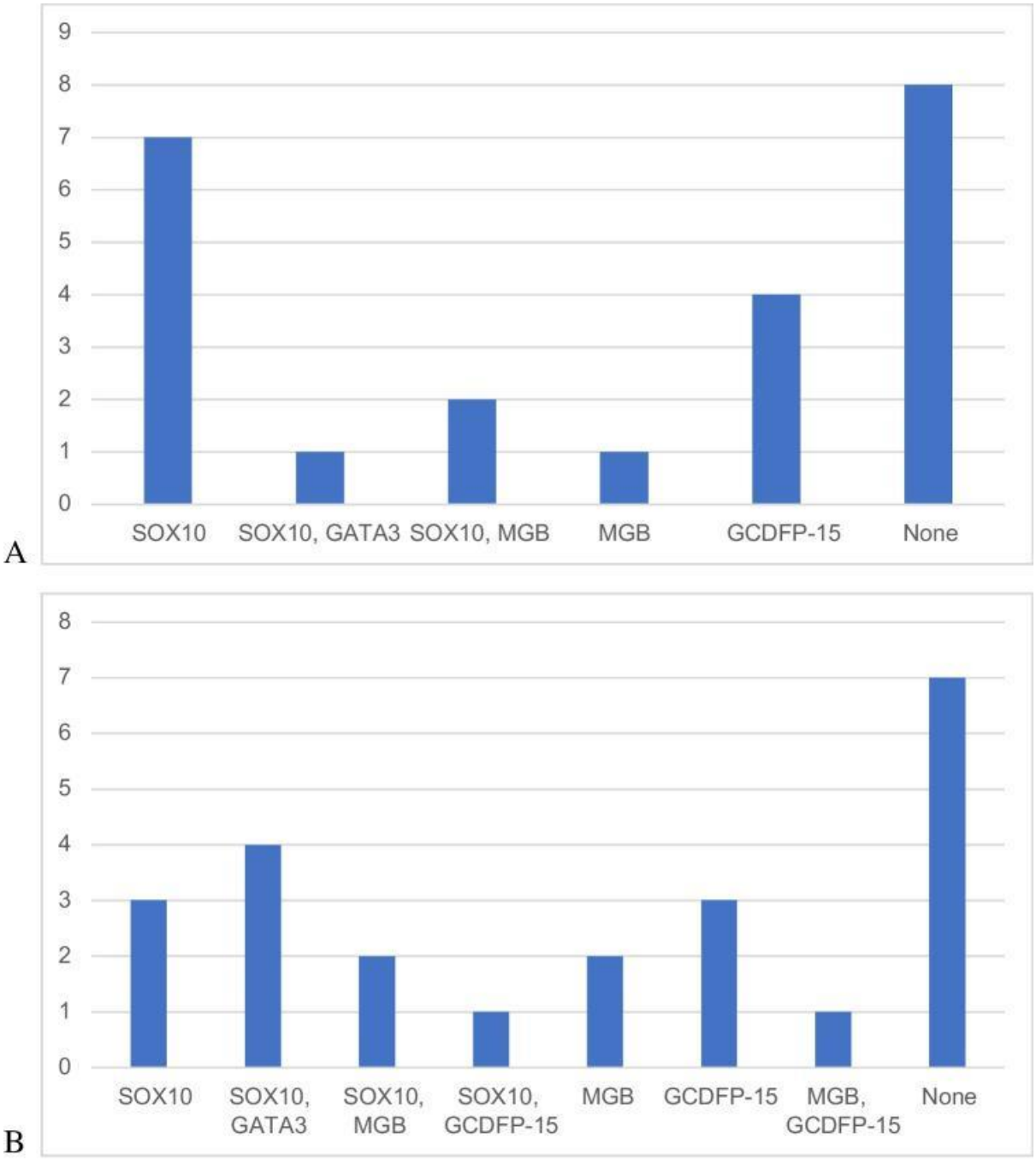

Figure 5. **Distribution of positive stainings with different breast markers in the 23 TRPS1-negative cases with available data for the rest of the markers. A: All markers with 10% cut-off. B: Using 5% positivity cut-off for GATA3, MGB and GCDFP-15. (Number of cases on axis y)**

The 6 cases showing HER2 positivity along with CK5 positivity (independently of being identified as HER2-enriched or basal-like) were all positive for TRPS1 and, in keeping with SOX10 being a marker of TNBCs, were negative for SOX10. The other markers were not tested.

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

Altogether we analysed 34 lesions from 23 patients. The neoplasms for which the operations were performed included 14 IBCs of NST (6 cases with extensive intraductal component - EIC), 4 pure DCIS, 1 tubular carcinoma with EIC, 1 instance of microinvasive Paget's disease of the nipple and 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 3/4 DCIS cases were ER and PR positive, whereas a single case of DCIS was ER and PR negative and HER2 positive with apocrine differentiation substantiated with the expression of androgen receptors and GCDFP-15. HER2 positivity was also a feature of the Paget's disease (ER and PR positive) investigated, 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 (*Table 2*).

Table 2. **Results of the four immunostains in different lesions**

		All	ER helpful	PR helpful	CK5 helpful	CK14 helpful	4 helpful	3 helpful	2 helpful
CTRL Non-neoplastic		4	2/4	4/4	4/4	3/4	2/4	1/4	1/4
CTRL Neoplastic		3	2/3	2/3	3/3	3/3	2/3	0/3	1/3
All certain cases		7	4/7	6/7	7/7	6/7	4/7	1/7	2/7
Uncertain	Non-neoplastic	15	14/15	13/15	14/15	14/15	10/16	6/16	0/16
	Favour non-neoplastic	2	0/2	0/2	2/2	2/2	0/2	0/2	2/2
	Uncertain	3	0/3	0/3	0/3	1/3	0/3	0/3	0/3
	Favour neoplastic	4	0/4	1/4	3/4	3/4	0/4	1/4	2/4
	Neoplastic*	3	1/3	2/3	2/3	2/3	1/3	1/3	0/3
All uncertain cases		27	15/27	16/27	21/27	22/27	11/27	8/27	4/27
All		34	19/34	22/34	28/34	28/34	15/34	9/34	6/34

CK5: keratin 5, CK14: keratin 14, CTRL: control, ER: oestrogen receptor, PR: progesterone receptor; * 1 case was not supported by any of the 4 IHC reactions, but was clarified by the HER2 immunostain being diffusely positive in the cauterised tissue (Case 17, Supplementary material).

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 (*Case 12, Supplementary material*). In contrast, steroid hormone receptor stainings were somewhat less often supportive, either due to negativity (e.g. Case 34, Supplementary material) or partial positivity in neoplastic lesions or complete lack of staining in the cauterised tissue (e.g. *Case 12, Supplementary material*).

Of the cases that were uncertainly classifiable on HE stained slides, two thirds could be classified as either non-neoplastic (*Figure 6*, Case 9) or neoplastic (*Figure 7*, Case 21), and two thirds of the remaining could be favoured as neoplastic or non-neoplastic, with 3 out of 27 cases remaining uncertain (*Figure 8*, Case 4) (*Table 2*).

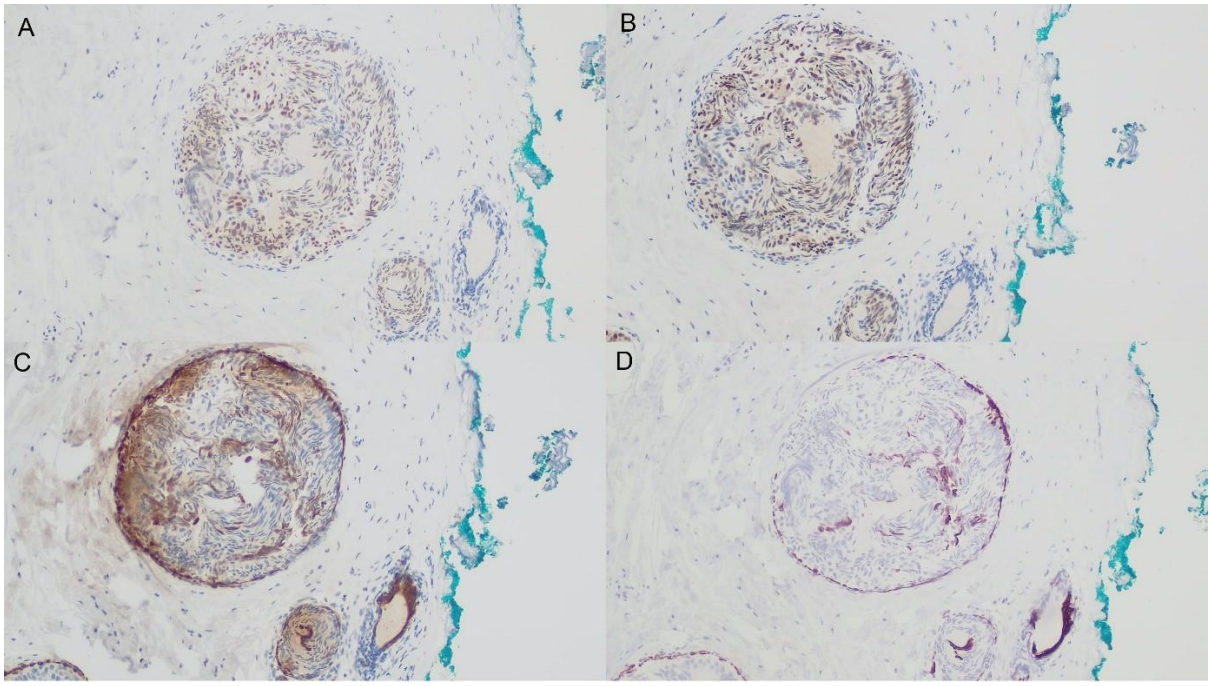

Figure 6. **Cauterised non-neoplastic tissue close to the inked margin demonstrating various degrees of mosaic like staining with all four antibodies; (A) ER, (B) PR, (C) CK5 (D) CK14. (x10) ER: oestrogen receptor, PR: progesterone receptor, CK5: keratin 5, CK14: keratin 14.**

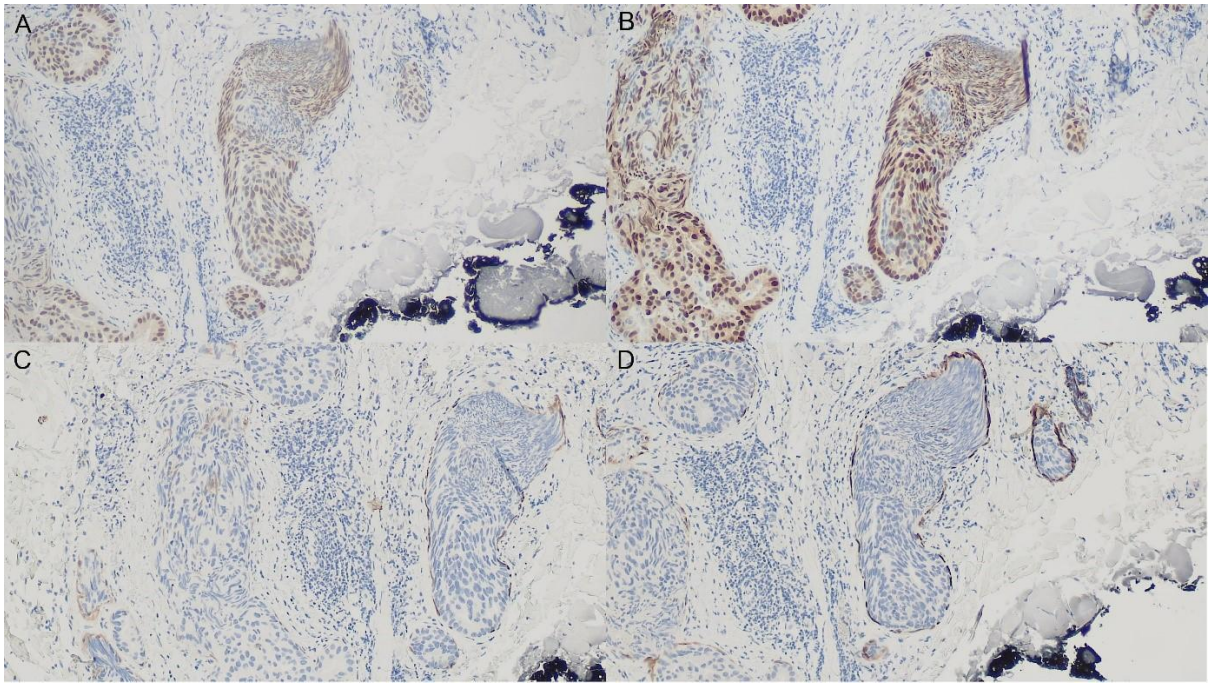

Figure 7. **Cauterised neoplastic tissue close to the inked margin demonstrating various intensities of rather diffuse ER (A) and PR (B) positivity, and various intensity of myoepithelial labelling along with tumour cell negativity with CK5 (C) and CK14 (D)**

(x10). ER: oestrogen receptor, PR: progesterone receptor, CK5: keratin 5, CK14: keratin 14.

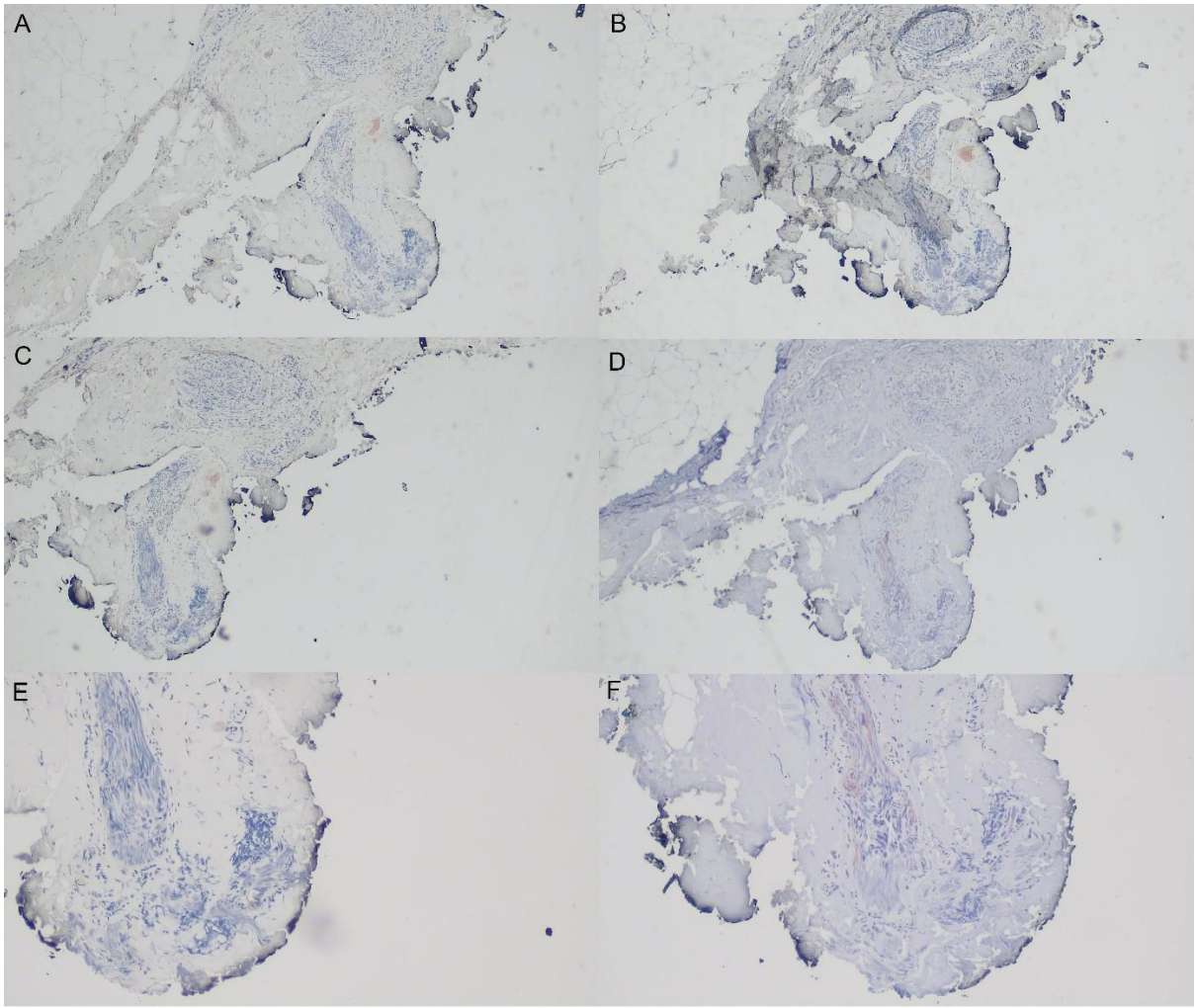

Figure 8. **A case remaining unclassifiable on the basis of the quadruple immunostain with all antibodies being negative; (A): ER (B) PR (C) CK5 (D) CK14 (x4) (E) CK5 (F) CK14 (x10). ER: oestrogen receptor, PR: progesterone receptor, CK5: keratin 5, CK14: keratin 14.**

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, *Case 17, Supplementary material*) 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 (*Case 13, Supplementary material*), 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. All of them were from female patients without metastasis at the time of the diagnosis.

Clinicopathological parameters

Table 3 summarizes the clinicopathological features and the galectin-1 staining results.

Table 3. **Clinicopathological characteristics of the studied cases and their distribution according to galectin-1 staining.**

		Value (range)
Median age (years)		69 (29–91)
Histological type		
	No Special Type	91
	Mixed micropapillary	2
	Metaplastic	2
pT category		
	pT1 (pT1c, pT1b, pT1a)	44 (40-4-0)
	pT2	43
	pT3	4
	pT4	4
pN category		
	pN0	50
	pN1	31
	pN2	10
	pN3	2
	pNx	2
Grade		
	1	0
	2	3
	3	92
Surgery type		
	Breast-conservative	69
	Mastectomy	26
	Sentinel lymph node biopsy	50
	Axillary lymph node dissection	43
	No axillary surgery	2
Lymphovascular invasion present		
	Yes	25
	No	70
Galectin-1 (stromal intensity)		
	Nil to moderate (0-1-2)	53 (6-18-29)
	Strong to very strong (3-4)	42 (24-18)
Galectin-1 (stromal percentage)		
	<50%	36
	≥50%	59

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, indicating limited nodal involvement. In two cases, the lymph node status was not recorded.

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.

In about one-quarter of the cases, mastectomy was performed (n=26), and the majority of the patients underwent BCS (n=69). Sentinel lymph node biopsy was the axillary staging procedure in 50 cases, while axillary lymph node dissection was performed in 43 patients, and no axillary operation 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 anthracyclin-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.

Galectin-1 staining results are also displayed in Table 3.

Survival analyses

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 found a significant difference in OS (p=0.008) (*Figure. 9 A*). For the low sTILs subset, with this categorization, we found a significant difference in OS between intensively staining cases versus cases demonstrating nil to moderate staining (p=0.005) (*Figure 9 B*).

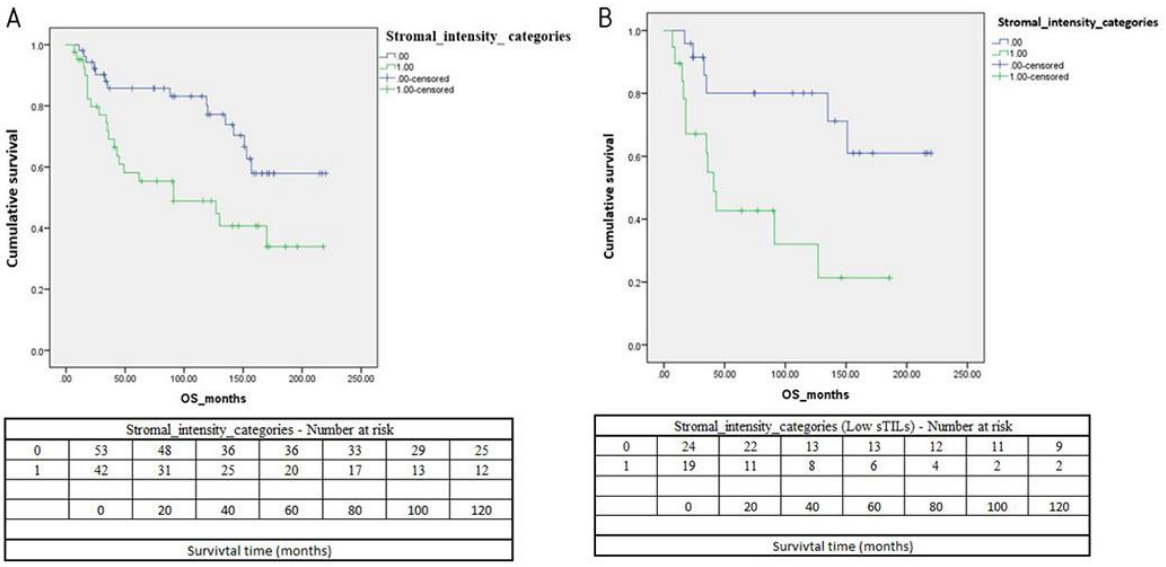

Figure 9. **Overall survival (OS) of cases with galectin-1 (intensity) strong/very strong positivity (1) and nil to moderate staining (0) (A); and overall survival (OS) of cases with low proportions of stromal tumour infiltrating lymphocytes (sTILs) with galectin-1 (intensity) using the same classification, (1) vs (0) (B).**

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=0.020) (*Figure 10*), and PFS (p=0.007) (shown in *Figure 11 A*) For the low sTILs subset, we found a significant difference in PFS (p=0.025) (*Figure 11 B*), but not in OS.

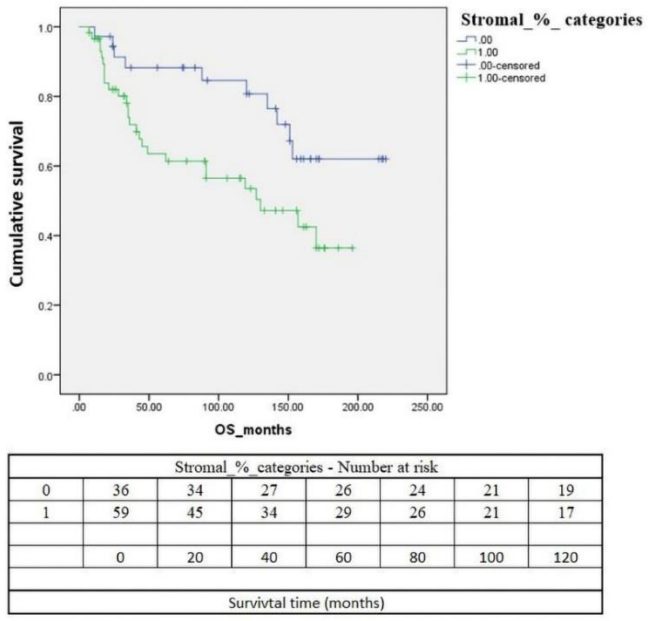

Figure 10. **Overall survival (OS) of cases with the application of the 50% cut off value for peritumoral stromal staining: 50% or more (1) versus less than 50% (0).**

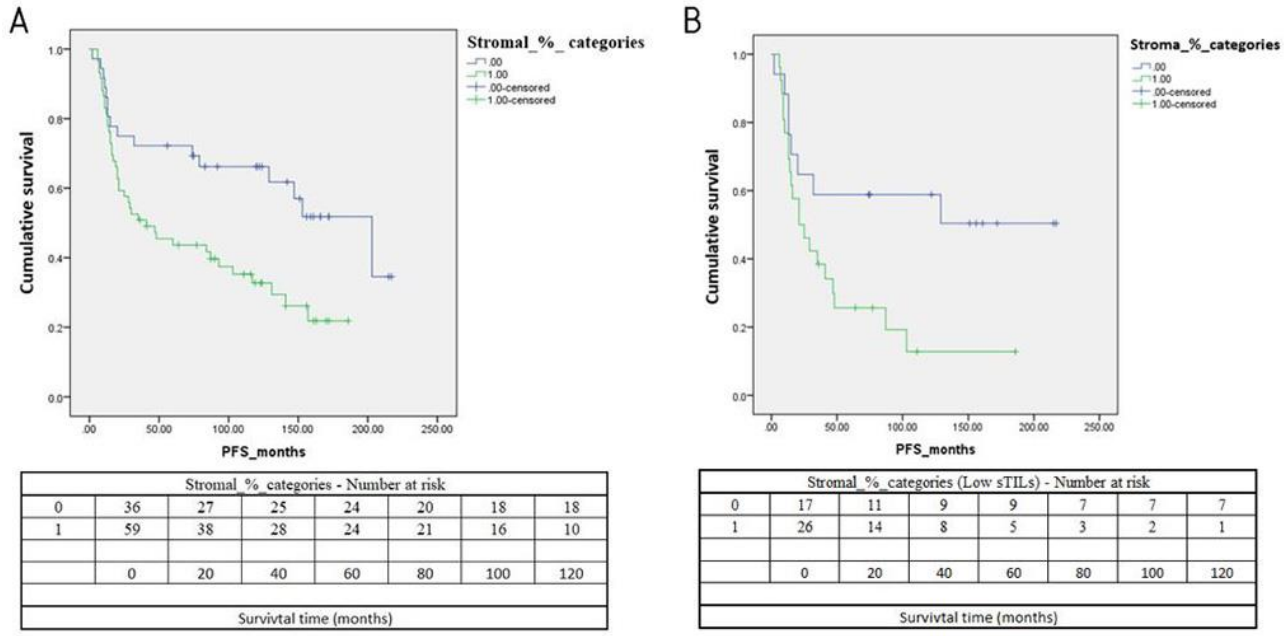

Figure 11. **Progression free survival of cases with the application of the 50% cut off value for peritumoral stromal staining, equal or more than 50% (1) versus less than 50% (0) (A) and PFS of cases with low proportions of stromal tumour infiltrating lymphocytes (sTILs) with galectin-1 equal or more than 50% (1) versus less than 50% (0) (B).**

With prognostic factors (pT and pN categories, grade, lymphovascular invasion, 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=0.010) and ≥50% stromal positivity (p=0.024) proved to be significantly associated with OS (*Table 4*). In multivariable analysis, only pN emerged as independent prognosticator when all significant variables were entered in the model (*Table 4*). 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 (*Table 4, multivariable analysis 2*). 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/).

Table 4. **The results of the univariable and multivariable Cox regression analyses for OS.**

	Univariable analyses	**Multivariable analysis 1**		**Multivariable analysis 2**	
	p-value	HR	*p-value*	HR	*p-value*
pT	**0.036**	1.302	0.252	1.335	0.204
pN	**0.003**	1.831	**0.007**	1.785	**0.009**
Grade	0.694				
LVI	0.611				
sTILs	0.177				
Galectin-1 intensity	**0.010**	1.921	0.152	2.438	**0.010**
Galectin-1 percentage	**0.024**	1.465	0.448		

HR: Hazard ratio, LVI: lymphovascular invasion, sTILs: Stromal tumour infiltrating lymphocytes. Intensity refers to nil to moderate versus strong to very strong staining; percentage to peritumoral stromal staining <50% versus ≥50%.

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) (*Table 5*).

Table 5. **The results of the univariable and multivariable Cox regression analyses for OS in the low sTILs group.**

	Univariable analyses	**Multivariable analysis 1**		**Multivariable analysis 2**	
	p-value	HR	*p-value*	HR	*p-value*
pT	0.331				
pN	**0.046**	1.614	0.185	1.681	0.137
Grade	0.501				
LVI	0.532				
Galectin-1 intensity	**0.008**	2.426	0.261	3.864	**0.009**
Galectin-1 percentage	**0.011**	1.929	0.475		

HR: Hazard ratio, LVI: lymphovascular invasion, sTILs: Stromal tumour infiltrating lymphocytes. Intensity refers to nil to moderate versus intense staining; percentage to peritumoral stromal staining <50% versus ≥50%.

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 (*Table 6 A*). 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 (*Table 6 B*).

Table 6. **The results of the univariable and multivariable Cox regression analyses for PFS (A). The results of the univariable and multivariable Cox regression analyses for PFS in the low sTILs group (B).**

	Univariable analyses (A)	**Multivariable analysis (A)**		**Univariable analyses (B)**	**Multivariable analysis (B)**	
	p-value	HR	*p-value*	*p-value*	HR	*p-value*
pT	**0.004**	1.313	0.125	0.391		
pN	**0.001**	1.711	**0.004**	**0.026**	1.792	**0.048**
Grade	0.895			0.612		
LVI	0.926			0.728		
sTILs	0.066			-		
Galectin-1 intensity	0.094			0.181		
Galectin-1 percentage	**0.008**	2.328	**0.008**	**0.031**	2.664	**0.033**

HR: Hazard ratio, LVI: lymphovascular invasion, sTILs: Stromal tumor infiltrating lymphocytes

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 [95]. Sensitivities vary according to the proportion of staining cells and intensities used as cut-off values for distinguishing negative and positive cases. With low cut-offs, such as any staining, more tumours turn out to be positive (e.g. primary breast carcinomas of mixed phenotype being positive in 94%, 83%, and 89% with GATA3, GCDFP-15, and MGB, respectively [18], but these become less frequent with higher cut-offs. Indeed, our

previous analysis of CK5-expressing (IHC defined basal-like) carcinomas demonstrated 82%, 30% and 23% positivity with any staining for GATA3, MGB, and GCDFP-15, respectively, but this decreased to 23%, 12% and 9% by applying a cut-off as low as > 5% staining [21]. When applying a more readily perceived cut-off of at least 10%, the proportions went down to 9% (GATA3), 9% (MGB) and 6% (GCDFP-15). 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.

The results may also be different by antibody clones. For example, GATA3 clone HG3-31 was shown to be less sensitive to label TNBCs than L50-823 [96]; therefore, the application of other antibody clones may alter the results.

Among markers of breast origin, SOX10 has been shown to have the highest sensitivity and specificity (compared to GATA3, MGB, and GCDFP-15) to discriminate between TNBC and lung adenocarcinomas, reflecting its diagnostic value in differentiating TNBC metastases to the lung [28].

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). We have not investigated other tumour types; therefore, the specificity of TRPS1 could not be determined directly, but on the basis of the limited data available, this is also a rather specific marker.

In 2020, Ai et al. examined 31 different solid tumour types through TCGA data mining and found TRPS1 as a protein specific for breast carcinoma. The 479 cases of various types of breast cancers they analysed with IHC showed a high proportion of staining in ER/PR positive (95% of 176), HER2 positive (79% of 67), and TNBCs (81% of both 52 metaplastic and 184 non-metaplastic cases), being more sensitive than GATA3 for this latter subset. An evaluation of altogether 1234 different solid tumours from different organs revealed TRPS1 to be specific, too. Among carcinomas of the bladder, lung, ovary, salivary duct, pancreas, colon, stomach,

kidney, and thyroid as well as melanomas, strong expression was only seen in 2/77 pulmonary squamous cell carcinomas, 2/165 serous and 1/86 non-serous ovarian adenocarcinomas and 7/143 salivary duct carcinomas. Lesser intensity staining was also identified in a significant minority of the same tumour types (up to 22% in squamous cell carcinoma of the lung and 19% in salivary duct carcinomas), and very rare cases of pulmonary adenocarcinomas (3/122), 1/144 pancreatic adenocarcinomas, and 1/40 melanomas also showed low to intermediate labelling. They concluded as TRPS1 is a highly sensitive and specific marker for breast carcinomas, including TNBC [5].

Similarly, Parkinson et al. have also found TRPS1 to diffusely (≥ 50% of the cells) stain the majority of HER2-positive cancers (91% of 64 cases) and TNBCs (87% of 76 cases), with a minority of tumours being labelled to a lesser degree, and only 3 and 6 cases showing < 10% labelling, i.e. being below the cut-off used in the present study. In addition, in other types of cancer investigated including colorectal (n = 208), hepatocellular (n = 208), endometrial (n = 93) carcinomas, cholangiocarcinomas (n = 106) and pulmonary adenocarcinomas (n = 49), only 3 and 1 of the latter two showed > 1% staining, which again suggests that TRPS1 is not only sensitive, but also specific for breast carcinomas in general, and TNBC is no exception to high sensitivity [30].

Yoon et al, have investigated primary or metastatic TNBCs of no special type or lobular type, and only 1/151 turned out to be negative with TRPS1, the rest demonstrated > 10% staining, with the majority showing > 50% [97]. They also analysed the staining in 141 metaplastic carcinomas, of which only 7 were negative, and 11 showed < 10% staining, i.e., the majority of metaplastic TNBCs were also TRPS1 positive [97], which points to a minor overlap with squamous cell carcinoma labelling described by Ai et al. [5]. The proportion of staining cells of their cases is in keeping with our results suggesting that most cases stain in at least 10% of the cells.

Du et al. have also analysed several subsets of breast carcinomas with TRPS1, and found that altogether, only 8% of 1201 breast carcinomas were completely negative for this marker, and further 13% were weakly positive (≤ 10% staining, i.e., negative according to our criteria) [98]. In contrast to the previously cited studies, metaplastic carcinomas were those that showed the highest positivity rate (129/140, 92% demonstrating over 10% staining), and non-metaplastic TNBCs being the less frequently positive (in 69% of 144 cases).

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 [87]. Although cases such as the one shown in *Figure 2 F*, with low-percentage of weakly staining cells, may escape detection and were considered negative with our 10% cut-off value, it must be remembered that weak and low proportion of cells staining does not exclude mammary origin and is less common with TRPS1 than with other markers. Less than perfect agreement was also related to cases with weaker nuclear and/or cytoplasmic staining as shown in *Figure 2B-C*.

It is well known that IHC is only a surrogate approach to reflect gene expression-based intrinsic subtypes of breast cancer [99]. Therefore, CK5 expression only increases the likelihood of a TNBC to be of the basal-like subtype [8] and cannot substitute just approximate the results of gene expression profiling. This is why we were also interested in the TNBC breast marker expression of 6 cases with CK5 expression that did not satisfy the category of TNBC, but expressed HER2 on IHC, as 3 of these were classified as basal-like on the basis of mRNA expression. The numbers are obviously low, but all were positive for TRPS1, in keeping with this marker being a useful pan-breast cancer marker, including TNBCs and basal-like cancers.

Based on our results, of the five markers compared, 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. Cases that are negative are most often labelled with SOX10, whereas the dual-negative subset may still be positive for one of the additional markers. In our series, interestingly, GATA3 had no additive value (neither with 10% nor with 5% cut-offs for positivity), and GCDFP-15 was the most expressed marker in this minority of TRPS1 and SOX10 negative cases. The reproducibility of the evaluation showed substantial agreement in our current study, which means it is an easily assessable 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 *in situ* carcinoma, the morphology matching histological types of breast cancers (including rare salivary gland-like tumours) versus unconventional morphologies, and these may obviate the need for breast marker testing. Core biopsies may be less representative, and

may require more frequent testing. Of course, both in the primary and the metastatic setting, 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 may be the only positive one. On the basis of our results and the cited literature data, we suggest that TRPS1 has a good place in these panels.

A word of caution needs to be formulated at the end of this discussion. A case of postirradiation angiosarcoma of the breast turned to be positive for TRPS1 in our routine diagnostic work and initiated the exploration of TRPS1 expression in breast angiosarcomas; 60% of 35 angiosarcomas proved to be positive [100], emphasizing the need for context in the use of immunohistochemical markers.

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

Mankind would probably be at ease in making decisions if most things could be categorised along a clear-cut dichotomic (“black or white”) scale. However, this is rarely the case. Biology is complex, and our methods of assessing its features are less than perfect. Therefore, easy decisions are not always possible. Decision making requires awareness of many circumstances and specific judgment for instances that are neither clearly yes nor clearly no (represent a shade of grey in the black and white world).

The perceptions and definitions of what constitutes a safe and tumour-free margin at BCS have changed over time. Early recommendations suggested a free margin of at least 5 mm for invasive carcinoma and 10 mm for DCIS [101-102], but with accumulating evidence, this has changed to a more conservative approach, and at present no ink on tumour [39-40] and 1 or 2 mm tumour-free band of non-tumorous tissue [40-41] are considered sufficient for IBC and DCIS, respectively, in order to reduce local recurrences. 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.

In our daily practice, when challenged by this issue, we used the common knowledge that normal to hyperplastic (non-neoplastic) breast tissue shares a mosaic-like staining pattern with ER, PR, and the CK5/CK14 pair of keratins, whereas low-grade neoplasia has a typical strong

and diffuse ER and PR staining and lacks CK5 and CK14 staining in neoplastic cells [45-47]. In this retrospective analysis, we tried to evaluate the value of this knowledge in practice. Some other contexts have also been part of the series: e.g. an ER- and PR-negative apocrine tumour, where lack of staining for these steroid hormone receptors could also favour the neoplastic nature of the cauterised tissue.

Cauterised and/or necrotic tissues maintain some epitopes useful in their identification with IHC [103-108]. Based on results by Judkins et al., the cytokeratins are well preserved in necrotic tissues, more precisely damaged tissues seem to retain immunoreactivity with CK AE1 and AE1/AE3 antibodies in epithelial tumours; they stained 10/14 necrotic carcinomas [103]. In contrast, leukocyte common antigen (LCA) and S100 showed false-positive labelling in some necrotic carcinomas along with specific or partial specific staining in the examined lymphomas and melanomas, respectively [103]. Although the tissue is undergoing necrosis, CK expression remains specific; no false positivity was detected in non-epithelial tumours [103]. In a separate study, the same authors specifically looked at 20 thyroid neoplasms, including 12 cases with post fine-needle aspiration necrosis and 11 tumours originating from different organs with necrosis serving as controls [104]. CK AE1/AE3 was at least focally positive in nearly half of the examined necrotic areas (9/20), but PanCK (an antibody against keratins 5, 6, 8 and 18 by Novocastra Laboratories, Burlingame, CA) was less useful in necrotic tissues; hence it was positive in viable epithelial tumour cells, but it proved to be negative in the necrotic areas [104]. Thyroglobulin retained positivity in 13/20 necrotic thyroid tumour areas, including foci with AE1/AE3 negativity and was negative in non-thyroid neoplasms [104]. Of note, some necrotic cases demonstrated complete loss of immunoreactivity with all antibodies examined [103-104]. Similar conclusions can be reached in non-epithelial neoplasms, e.g. the IHC expression of melanocytic markers can be maintained in necrotic neoplasms, but non-specific staining may interfere with specific staining; and the more severe the tissue damage, the more unlikely is an IHC marker to be retained [105]. It is believed that coagulative tissue damage caused by electric surgical devices, i.e. cautery artefacts closely resemble tissue necrosis, and IHC can be of help in identifying tissue origin and neoplastic versus non-neoplastic nature.

Antibody panels can be helpful in damaged tissues surrounding the resection margins in different organs. In prostate samples, 34βE12 (HMWCK), p63, and Alfa-Methylacyl Coenzyme A Racemase (AMACR) is used in daily practice to identify carcinomas and distinguish them from non-neoplastic glands. These antibodies can also be used in the

evaluation of areas with artificial damage. Pierconti F et al. examined 30 crushed radical prostatectomy samples and 25 crushed transurethral prostatic resection samples with equal number of control cases applying this antibody panel [106]. While the controls showed the expected results with the complete panel (though with lack of AMACR in 7/25 cases of adenocarcinoma), only HMWCK and AMACR gave the same expected results in the thermally damaged areas, and p63 expression was missing not only in the 5 neoplastic areas, but also in 37/50 instances of benign prostatic glands demonstrating the presence of basal cells by HMWCK and being negative for AMACR [106]. Groisman et al. could distinguish between thermally coagulated adenomatous and non-adenomatous colorectal polyps on the basis of the maintained Ki67 (MIB1) staining pattern [107]. Smoothelin expression (highlighting the muscularis propria of urinary bladders but being only weakly positive in the muscularis mucosae) was maintained in all 46 cauterised transurethral tumour resection specimens and helped in identifying muscle invasive tumours [108].

These results point to the fact that IHC markers differ in retaining their expression in damaged tissues. While p63 is more susceptible to cautery artefacts, keratin, like HMWCK (which also contains the CKs 5 and 14 studied in our series) expression better reflects vital tissue reactivity in both cauterised [107] and necrotic tissues [103].

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 a previous study, Nayak et al., found that CK5/6 and HMWCK were useful in classifying 11 and 11 mildly to moderately cauterised breast tissues at the margin into hyperplastic versus neoplastic lesions, and the IHC also helped them to segregate 11 severely cauterised margins into those involved by neoplasia *vs* hyperplasia [109]. 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 (*Case 10, Supplementary material-CK14, Case 22, Supplementary material-CK5*), and here the ER and PR stains had some additional value. There were also a few cases where all four IHCs failed (e.g. *Figure 8*). The keratins may also be helpful in distinguishing between cauterised invasive and *in situ*

carcinoma at the margin owing to the presence or absence of the peripheral (myoepithelial) staining (See control to *Case 30, Supplementary material*).

It seems that ER staining, at least with the 6F11 clone antibody used in this context may be more compromised by the thermocoagulative alterations caused by cautery, since the reactions were often weaker or completely vanishing. It might happen that alternative antibodies may yield different results. The trouble with this phenomenon may arise from the fact that some of our cauterised tissues became pseudo-negative, and in some contexts, an ER-negative tumour and an ER-pseudo-negative cauterised tissue may lead to a wrong interpretation. Therefore, these immunostains always need to be interpreted in the proper context.

Recognizing the value of the quadruple IHC being at least partially helpful in many cases, we have also identified some practical and potential limitations. Although mosaic like CK5 and CK14 staining supports a non-neoplastic nature of the cauterised tissue, residual luminal epithelial cells (e.g. in case of pagetoid spread or partial involvement of the duct) as well as myoepithelial cells in papillomas involved by a neoplastic process may yield a similar pattern (e.g. *Case 26 and its control, Supplementary material*). A rarity, the tall cell carcinoma with reverse polarity may also display a focal, mosaic-like staining with CK5 (or CK14) [110]. A previous study referred above [26], investigated HMWCK in the same context, but CK5 and/or 14 seem better, as HMWCK also stains the majority of cells in lobular neoplasia, and little positivity has also been found in ductal neoplasia [111]. Some tumours -invasive or their in situ precursors- may also exhibit a staining with these basal keratins, either as a morphological evidence of basal-like differentiation in TNBCs [110, 8] or along with ER positivity and HER2 negativity as reported in up to 8% of this group of tumours in a large cumulative series [112]. The mosaic-like ER and PR staining pattern of usual type hyperplasia and normal breast tissue is a helpful feature in classifying cauterised breast tissues, especially when it can be contrasted with the full-blown diffuse and strong positivity of low-grade DCIS, atypical hyperplasia, some invasive carcinomas and higher grade DCIS or the absolute negativity of apocrine carcinomas or triple-negative cancers. On the other hand, apocrine lesions of any type are ER and PR negative [113] whereas columnar cell lesions share the immunophenotype of low-grade DCIS, and recently a papillary variant has also been described [114], raising the possibility of potential misinterpretation. Many cancers have a heterogeneous labelling for ER and PR, and this should also be considered when interpreting the staining pattern of cauterised areas. At times, other supplementary IHCs may also be of help, like HER2 or apocrine markers (*Cases 13 and 17, Supplementary material*). Ross et al. have used a triple stain (CK7 to identify the epithelial

nature, p63 for myoepithelial cells, and E-cadherin to distinguish between lobular and ductal (in situ) carcinomas) to identify and classify minimal foci of breast cancer in core needle biopsies and excision specimens, and found this to be useful in at least one case where cautery artefact resulted in the distortion of the architecture at the inked margin [115], however, their study was not specifically devised to assess IHC results in cauterised breast tissues, and literature data cited above suggest that p63 may not be ideal in this context [106].

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

Malignant tumours are characterized by various combinations and degrees of features forming the malignant phenotype, i.e. 1./ activating signal independent growth, 2./ insensitivity to growth-inhibitory signaling, 3./ the ability to avoid apoptosis, 4./ limitless replication potential, 5./ angiogenesis to cover oxygen and nutrient supply and drain metabolites, and 6./ the ability to invade and thereby give rise to metastasis [116]. With further understanding of the model and new insights into carcinogenesis, further hallmarks of malignancy have been added to the original sextet, and these include 7./ refining of the cellular metabolism to better support neoplastic proliferation, 8./ genomic instability that allows mutations interfering with other hallmarks, 9./ tumour progression favouring inflammatory pathways and 10./ evasion from antitumour immune mechanisms [117]. The latter two hallmarks are associated with tumour promoting and inhibitory inflammatory events [93, 117], which are strongly associated with the microenvironment and form a very complex system that our study tried to approach with a pragmatic but superficial way.

Among BC subtypes, NST TNBCs have the worst prognosis [118]. Besides tumour size, grade, and nodal status [118], sTILs are also important prognosticators in this BC subset [119].

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 [120-121]. 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 [6]. 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.

Regarding the importance of sTILs, we examined separately the high and the low sTILs groups with a cut-off of 30% [94]. The statistical analysis showed a significant difference in OS for the low sTILs subset according to the 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.

Jung et al. examined the proteome of BC tissues to identify tumour-specific proteins with different methods. They compared the proteomic analysis of BC patients with normal breast tissue from 6 patients. They found that galectin-1 expression is significantly (3.7 times) higher in BC tissues than in normal breast parenchyma. They also examined 105 BC cases using galectin-1 IHC. The majority (n=71) were positive for both oestrogen and progesterone receptor), 14 were negative for both hormone receptors, and the remaining 20 cases were positive with only one hormone receptor (i.e. either oestrogen or progesterone receptor positive). No data on HER2 status was presented. They found that stromal galectin-1 expression was associated with poor prognostic features, including higher TNM stage and tumour invasiveness, i.e. invasive tumours demonstrating higher proportion of stromal staining (58/89; 65%) than in situ carcinomas (4/16; 25%) [122].

According to our literature review, thus far, only one research examined the connection between galectin-1 expression and the clinicopathological parameters in BC [122]. It proved the association between galectin-1 expression and markers of worse prognosis. The above-mentioned study included mainly luminal-type BCs and did not compare galectin-1 expression with survival. 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.

There are data suggesting that galectin-1 expression correlates with tumour aggressiveness [122], and it is considered a poor prognosticator; therefore, patients might gain benefit from galectin-1-targeted therapies [75]. 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 univariable and multivariable Cox regression analysis results supported the survival curve analysis results 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.

Our study is only an exploratory one, but it supports the prognostic value of galectin-1 in TNBC. As the mechanisms that regulate anti-tumour immunity are complex, alternative studies would be required to explore the role of galectin-1-associated immunosuppression and disease outcome along with possible therapeutical implications.

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. In our series, interestingly, GATA3 had no additive value (neither with 10% nor with 5% cut-offs for positivity), and GCDFP-15 was the most expressed marker in this minority of TRPS1 and SOX10 negative samples. The reproducibility of the evaluation showed substantial agreement in our current study, indicating that it 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 *in situ* carcinoma, the morphological alignment with typical histological types of breast cancer (including rare salivary gland-like tumours) 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 frequent 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.

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 CK5 and/or CK14 alone may be sufficient in some cases. The essential approach is that the results must be interpreted with caution, always considering the patient's disease, to avoid misinterpretations.

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 in our current study, 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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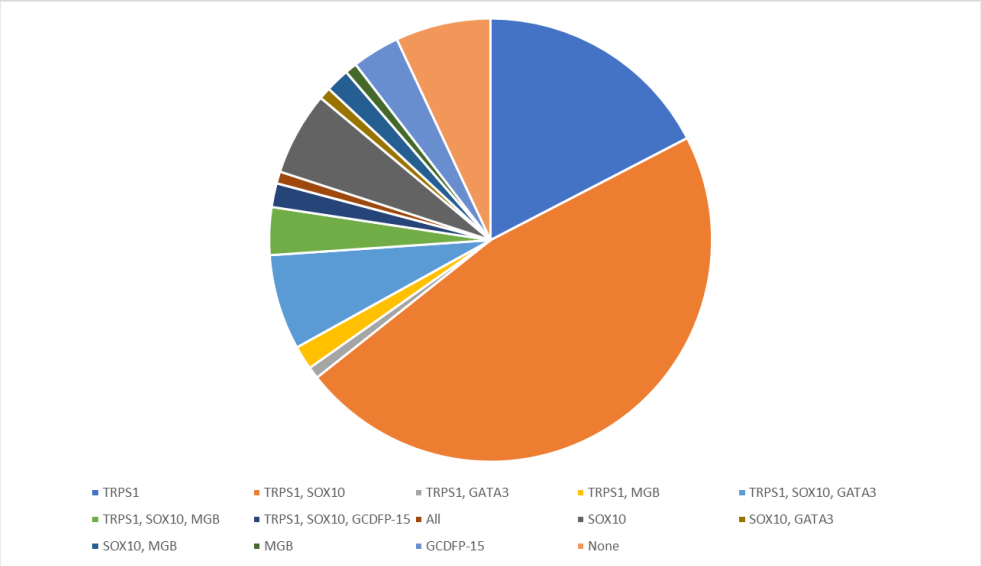
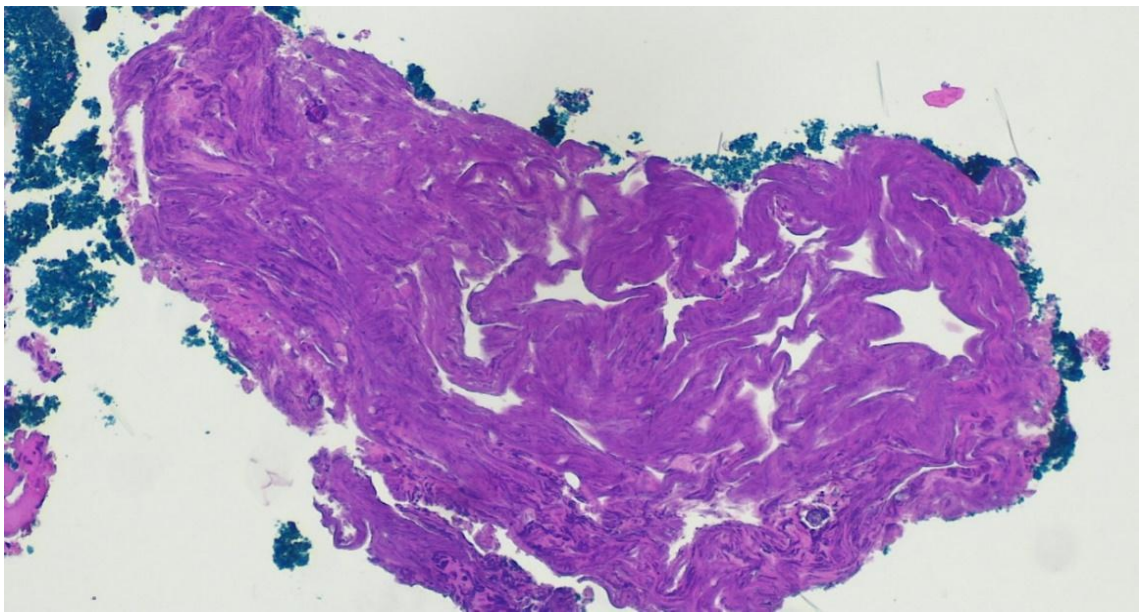
10. SUPPLEMENTARY FIGURES

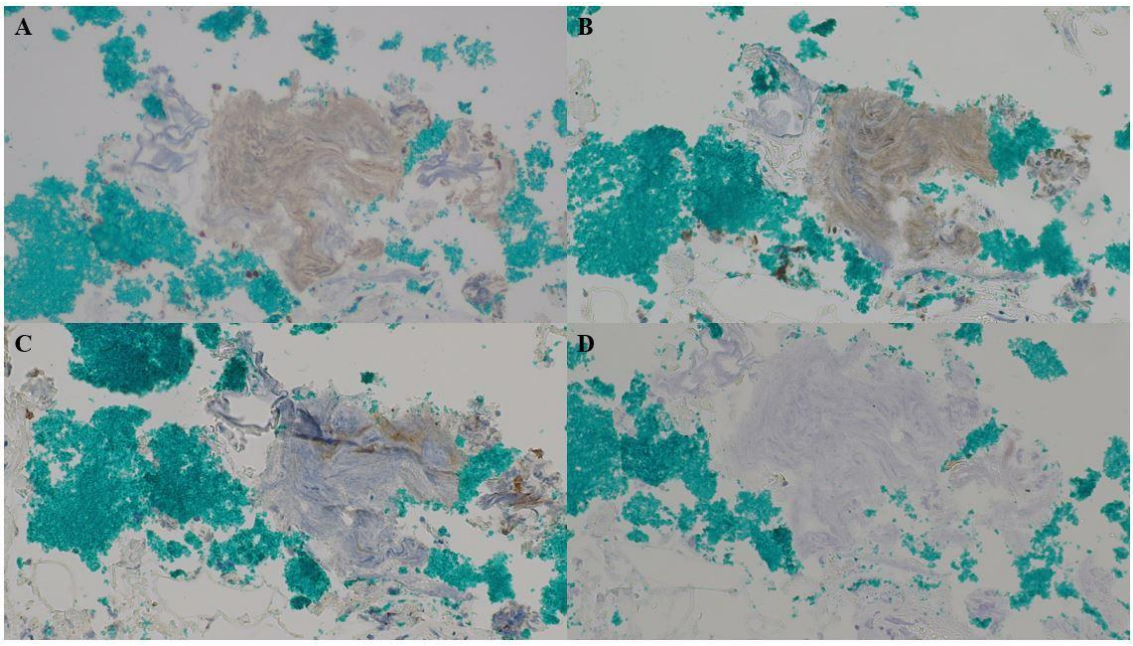


Supplementary figure 1. **Staining patterns of the 117 triple-negative breast cancers investigated (Starting from 12 o'clock position, clockwise from TRPS1 only to none)**

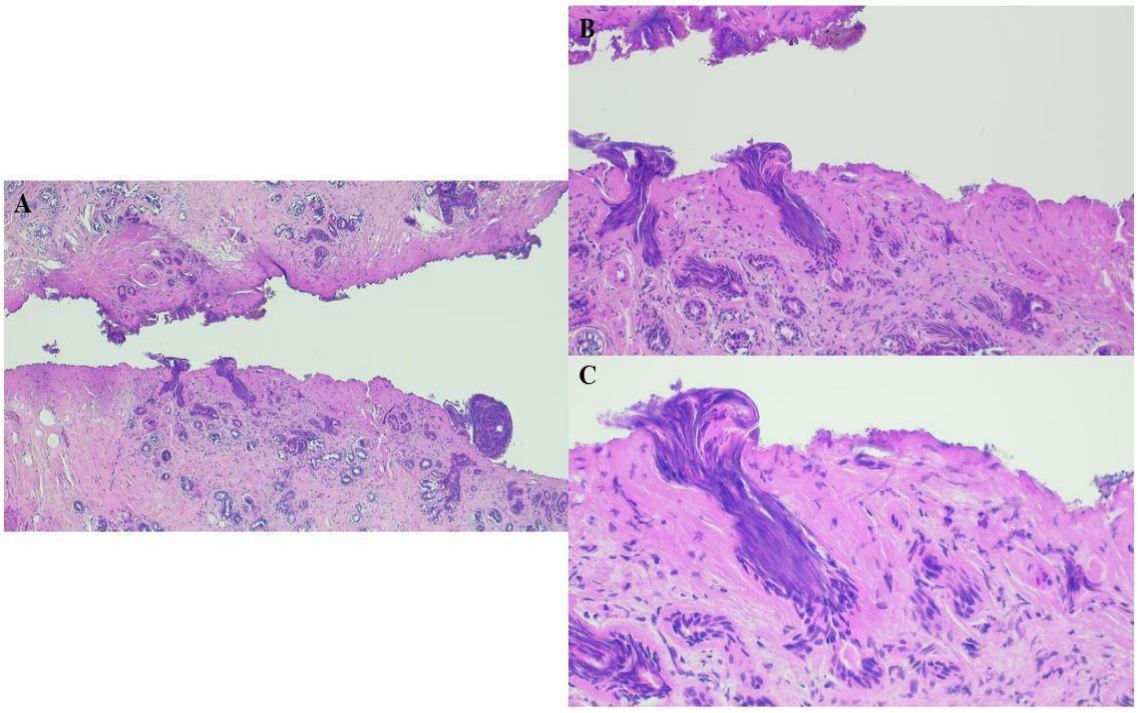
Supplementary figures 2-40: Cases No 10, 12, 13, 17, 22, 26, 30, 34. Magnifications refer to the objectives, x4 (low power), x10 (medium power), x40 (high power). HE: haematoxylin and eosin, IHC: immunohistochemistry, ER: oestrogen receptor, PR: progesterone receptor, CK5: cytokeratin 5, CK14: cytokeratin 14, DCIS: ductal carcinoma in situ, HER2: human epidermal growth factor receptor 2. GCDPF-15: gross cystic disease fluid protein 15



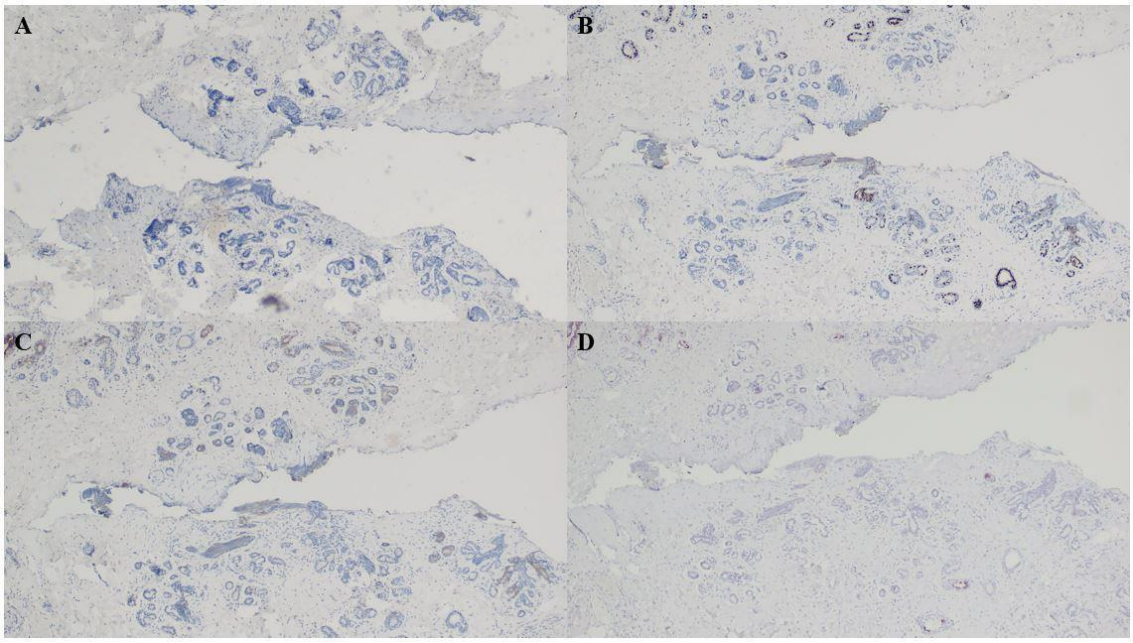
Supplementary figure 2. **Case 10 uncertain, HE (x10)**



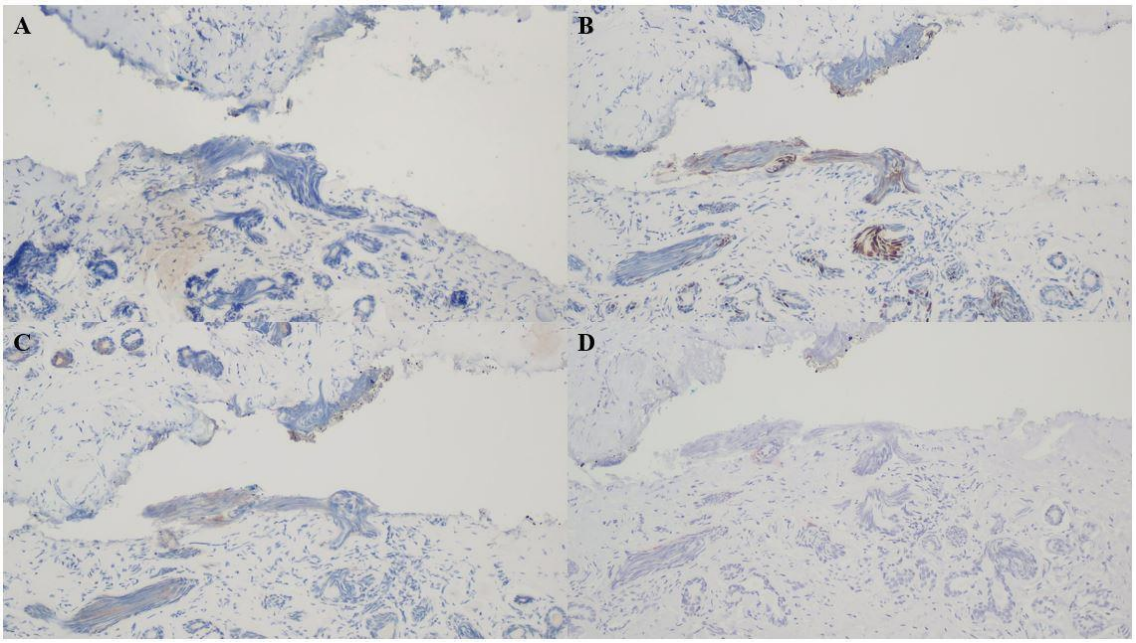
Supplementary figure 3. **Case 10 IHC: A: ER (weak mosaic: support for non-neoplastic), B: PR (weak mosaic: support for non-neoplastic), C: CK5 (mosaic: strongest support for non-neoplastic), D: CK14 (a few cells: deemed insufficient to support anything) (x20)**



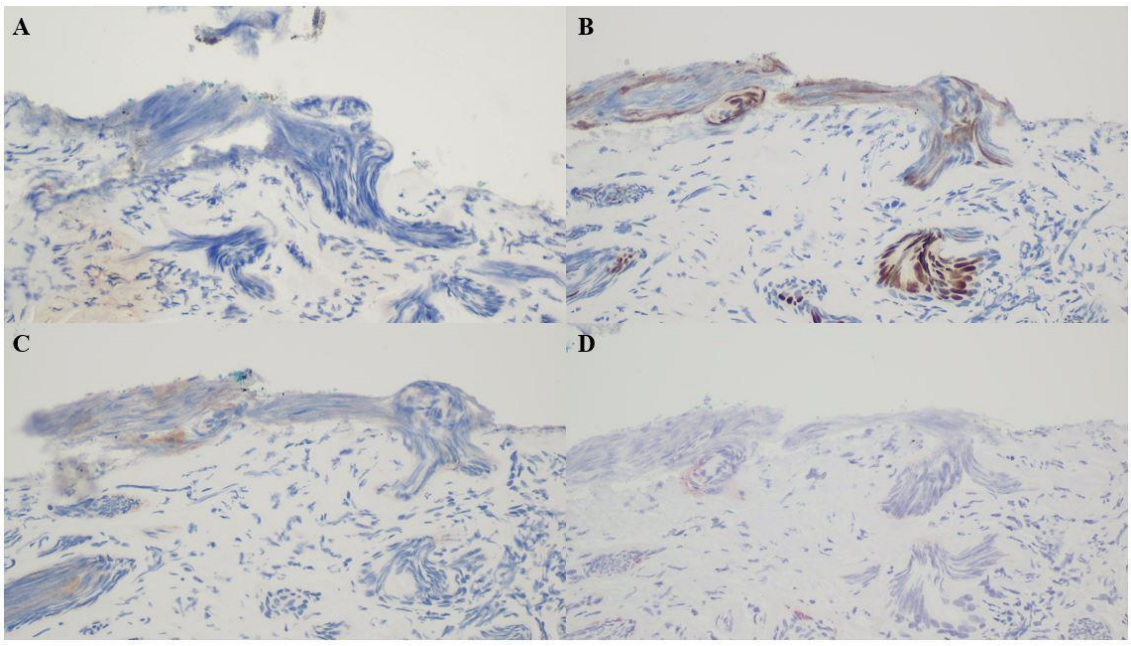
Supplementary figure 4. **Case 12 Non-neoplastic: A: HE (x4), B: HE (x10), C: HE (x20)**



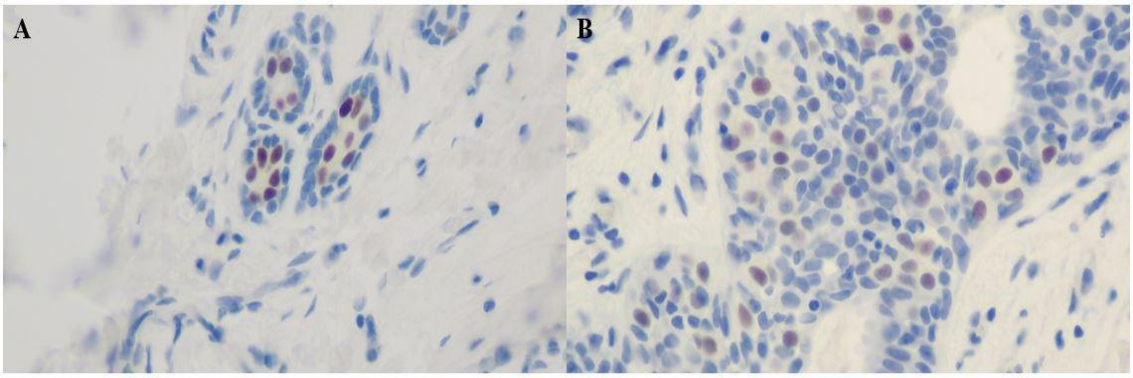
Supplementary figure 5. **Case 12 IHC: A: ER (no staining at all; improper), B: PR (mosaic-like, supportive of non-neoplastic), C: CK5 (mosaic-like, supportive of non-neoplastic), D: CK14 (no staining at all) (x4)**



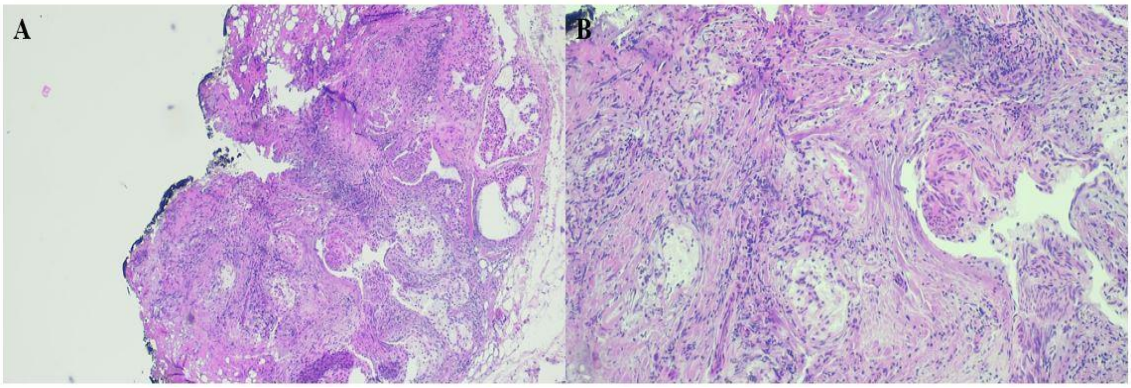
Supplementary figure 6. **Case 12 IHC: A: ER (no staining at all; improper), B: PR (mosaic-like, supportive of non-neoplastic), C: CK5 (mosaic-like, supportive of non-neoplastic), D: CK14 (no staining at all) (x10)**



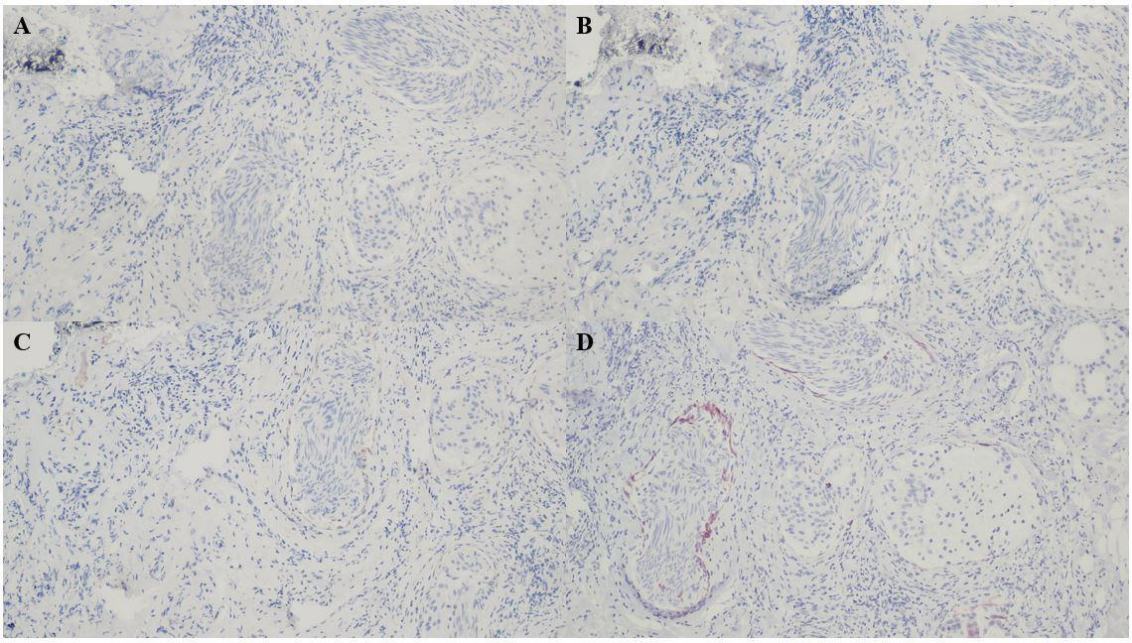
Supplementary figure 7. **Case 12 IHC: A: ER (no staining at all; improper), B: PR (mosaic-like, supportive of non-neoplastic), C: CK5 (mosaic-like, supportive of non-neoplastic), D: CK14 (no staining at all, except myoepithelial cells) (x40)**



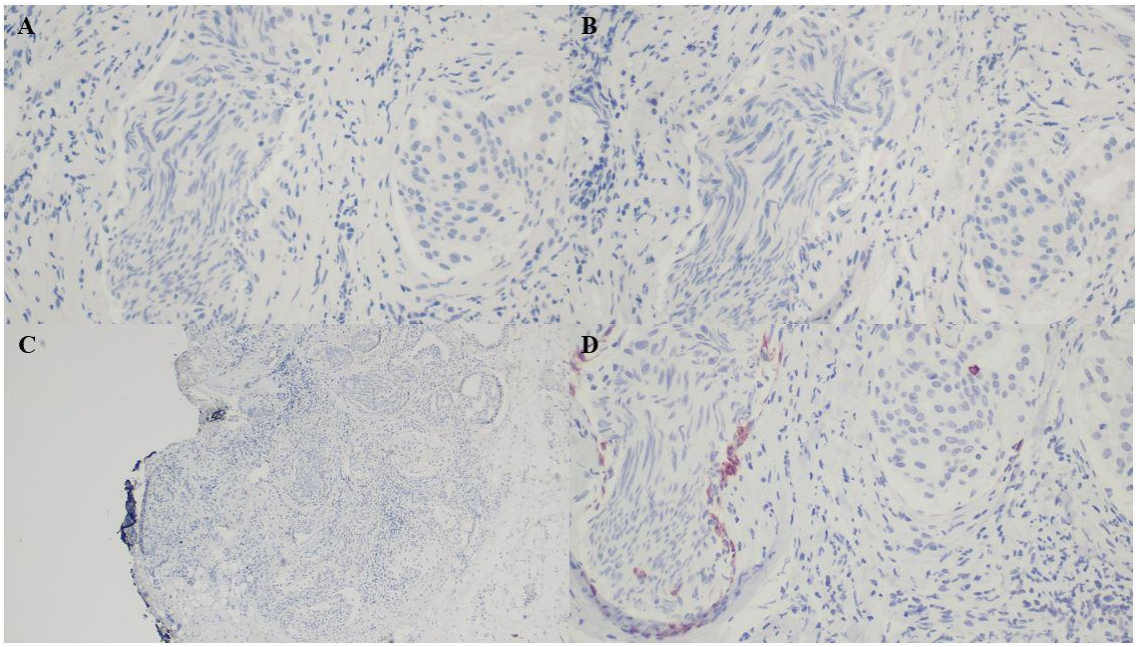
Supplementary figure 8. **Case 12 IHC: A-B: ER control elsewhere in the same section (x20)**



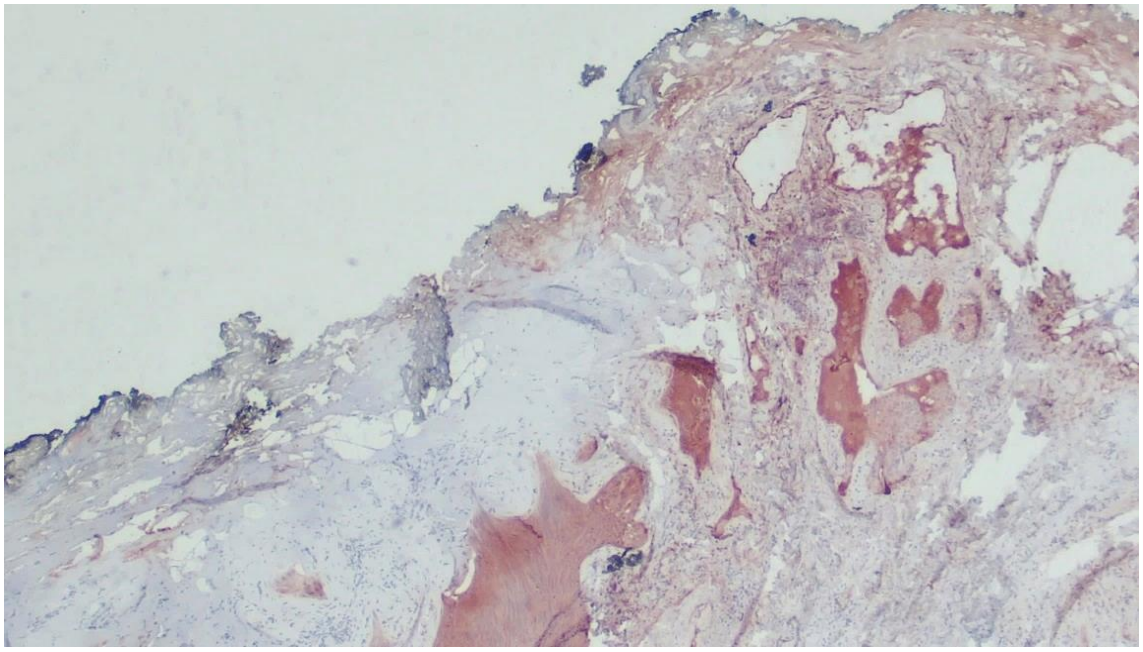
Supplementary figure 9. **Case 13 IHC: Apocrine DCIS, A: HE (x4), B: HE (x10) (All 4 IHCs negative)**



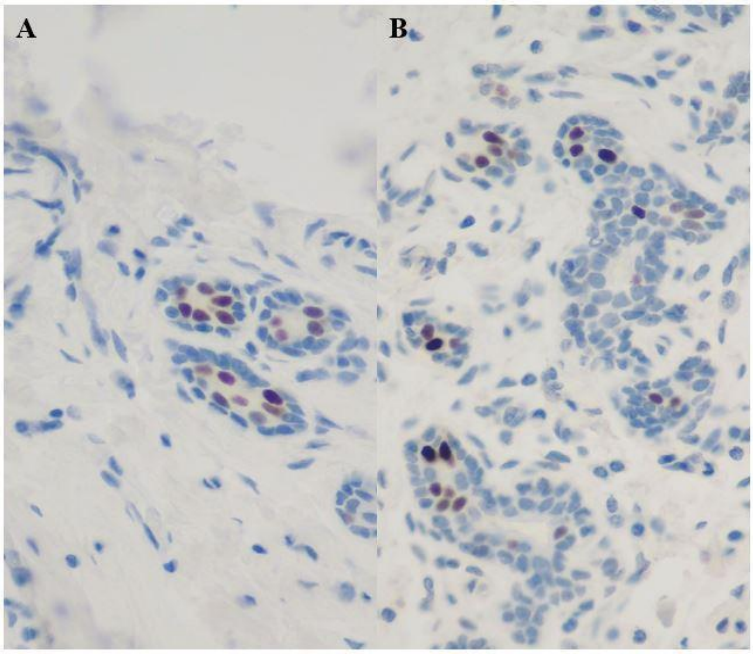
Supplementary figure 10. **Case 13 IHC: A: ER (negative), B: PR (negative), C: CK5 (negative with weak myoepithelial staining), D: CK14 (negative with myoepithelial staining) (x10)**



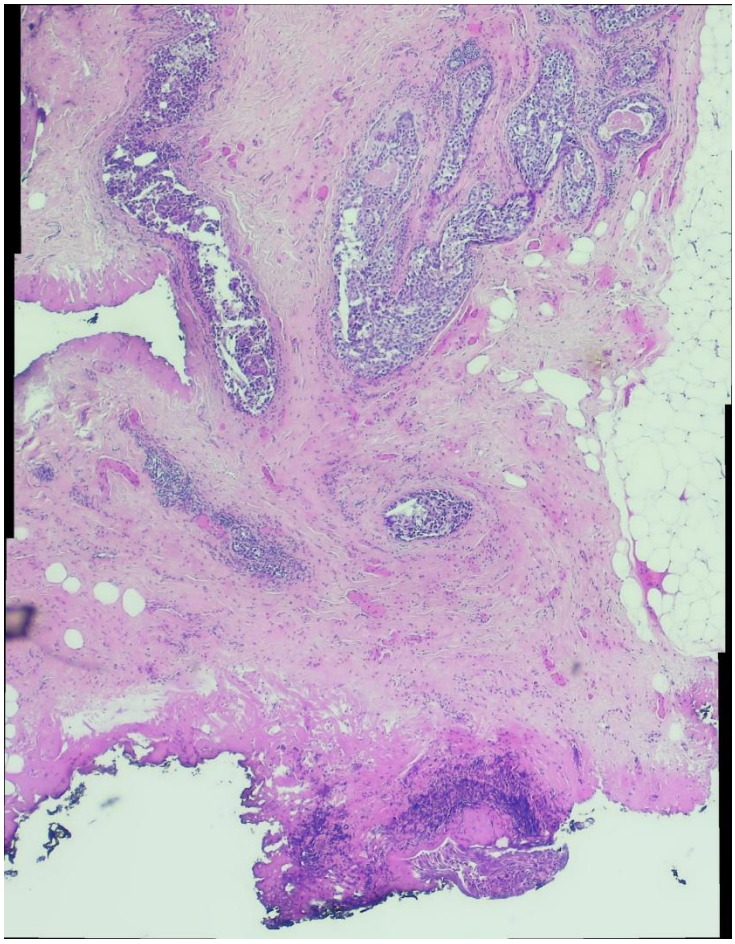
Supplementary figure 11. **Case 13 IHC: A: ER (x20) (negative), B: PR (x20) (negative), C: CK5 (x4) (negative with weak myoepithelial staining), D: CK14 (negative with myoepithelial staining) (x20)**



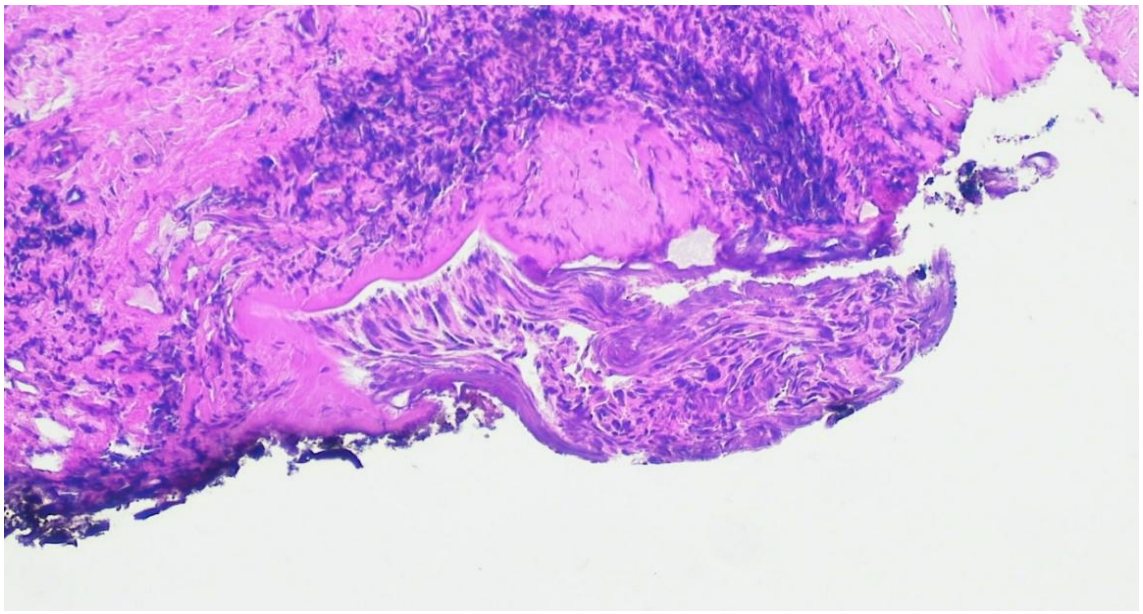
Supplementary figure 12. **Case 13: GCDPF-15 (Clone 23A3, 1:200, CellMarque, Rocklin CA) (x10)**



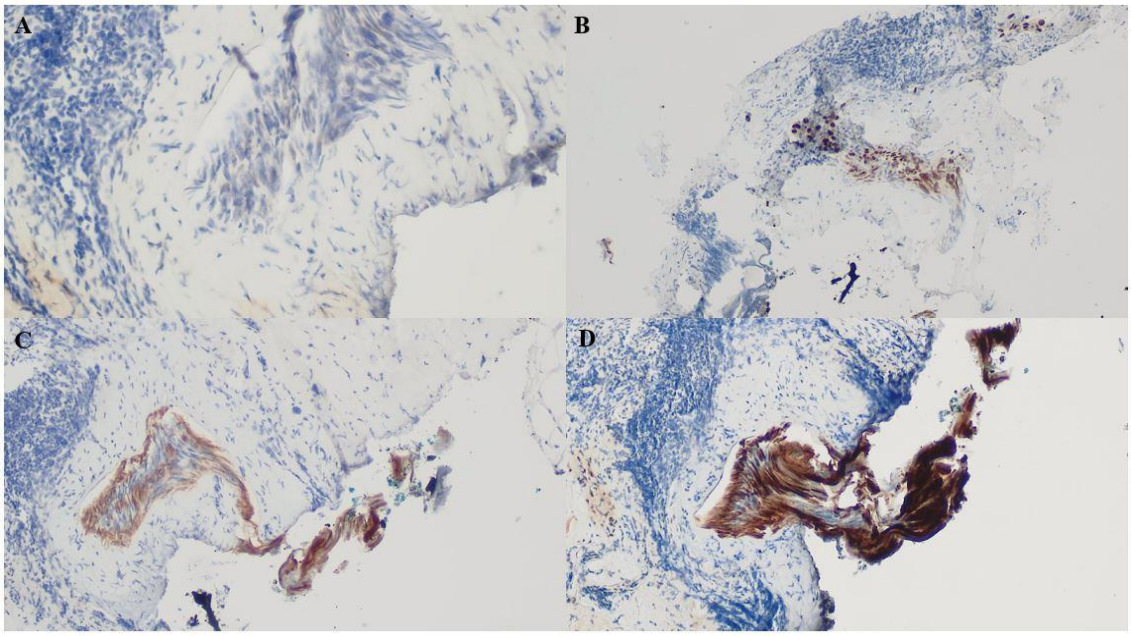
Supplementary figure 13. **Case 13 IHC: ER (A) & PR (B) internal control (elsewhere on the same slide) (x4)**



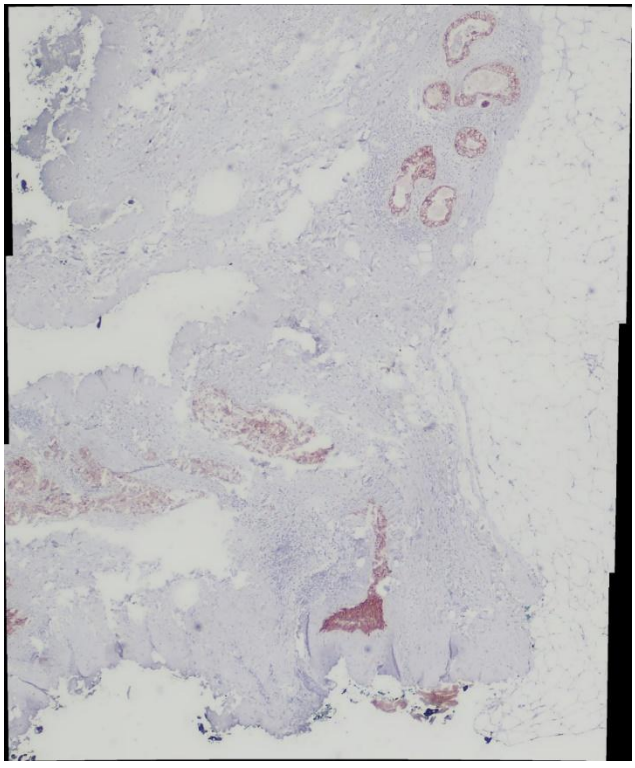
Supplementary figure 14. **Case 17: ER+PR+HER2+ DCIS control, HE (x4 stich)**



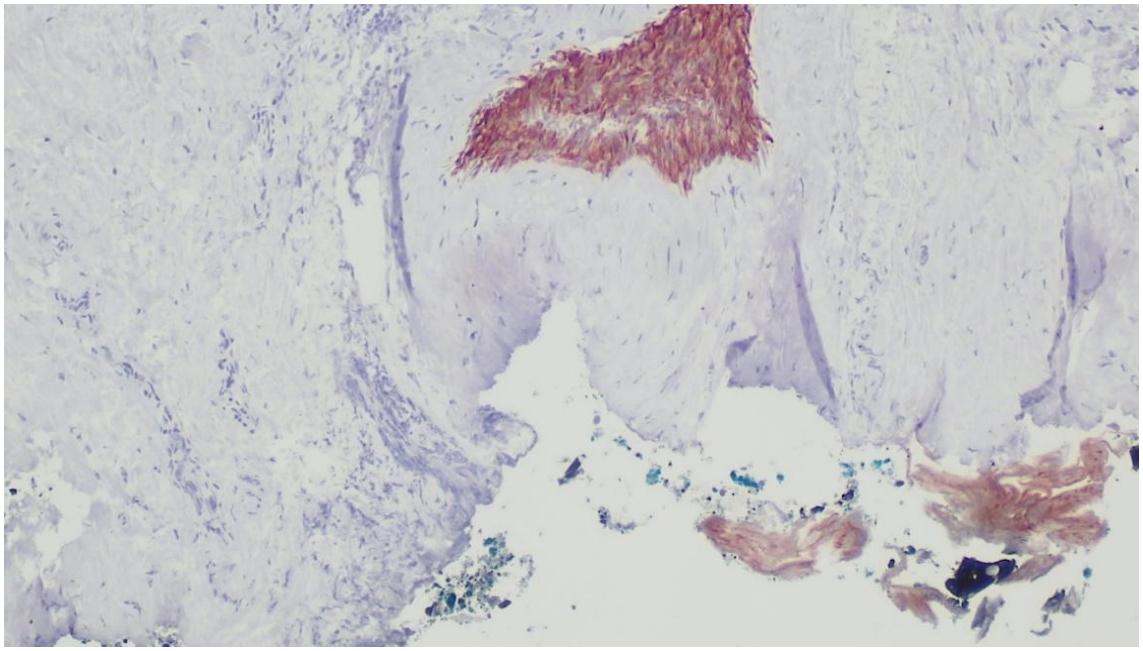
Supplementary figure 15. **Case 17: uncertain lesion; HE (x10)**



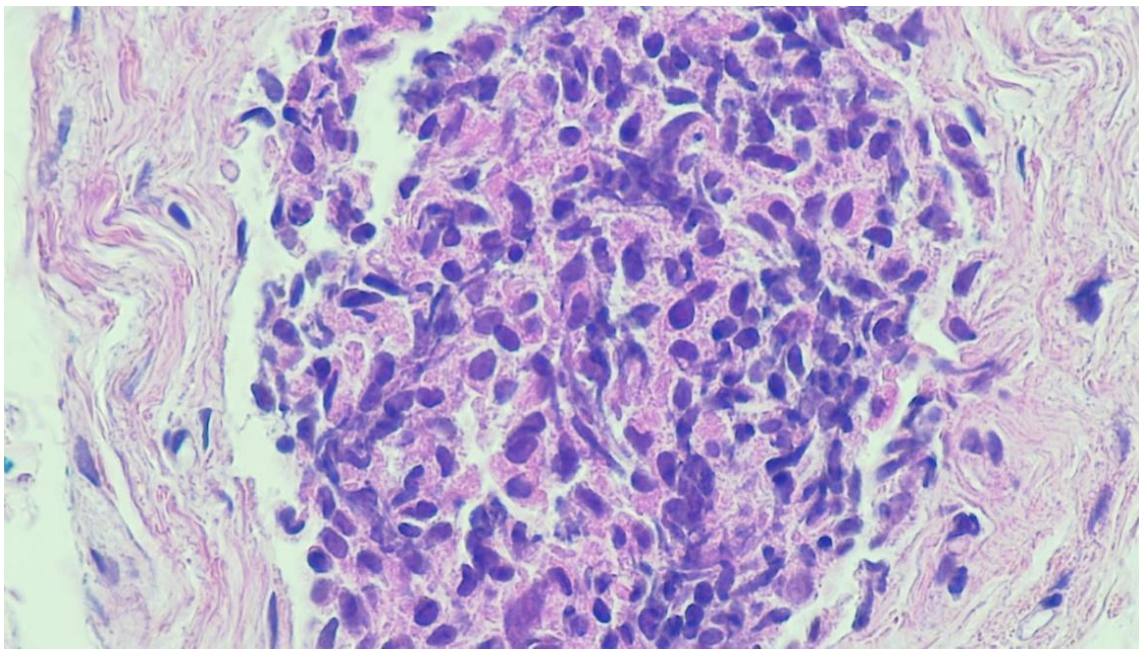
Supplementary figure 16: **Case 13 IHC: A: ER (x20) (no staining; pseudo-negative, improper), B: PR (x4) (mosaic, with majority of cells staining – in keeping with the PR positivity of the tumour, but not conclusive), C: CK5 (x10) (luminal positivity besides myoepithelial staining), D: CK14 (x10) (luminal positivity besides myoepithelial staining)**



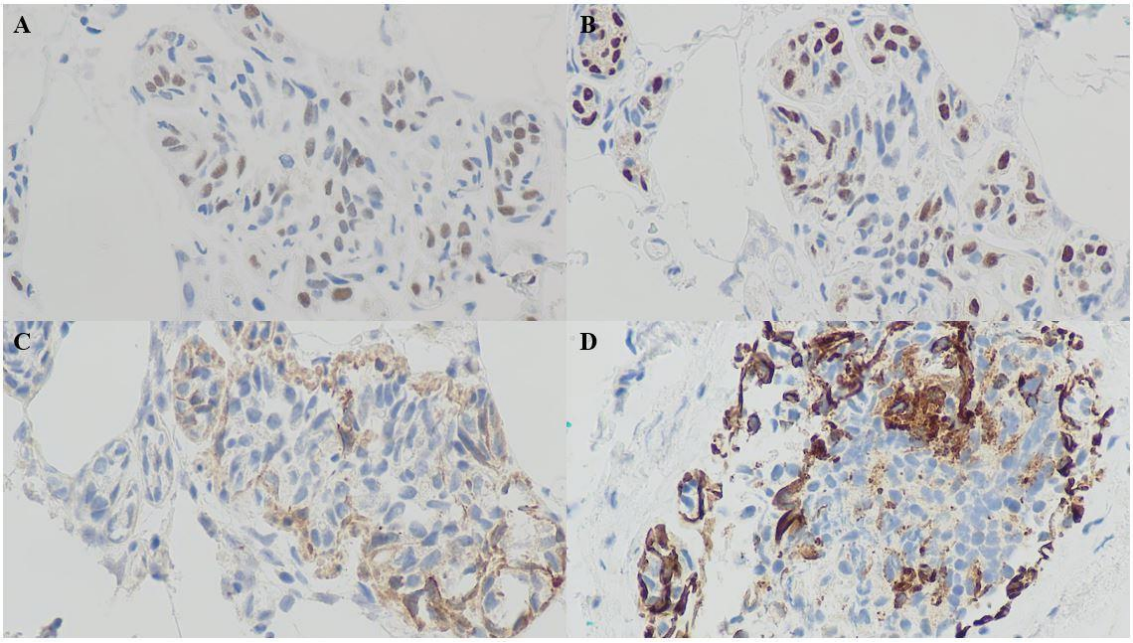
Supplementary figure 17: **Case 13 IHC: HER2 (clone: 4B5, Roche-Ventana, Tucson, pH6), (x4 stich)**



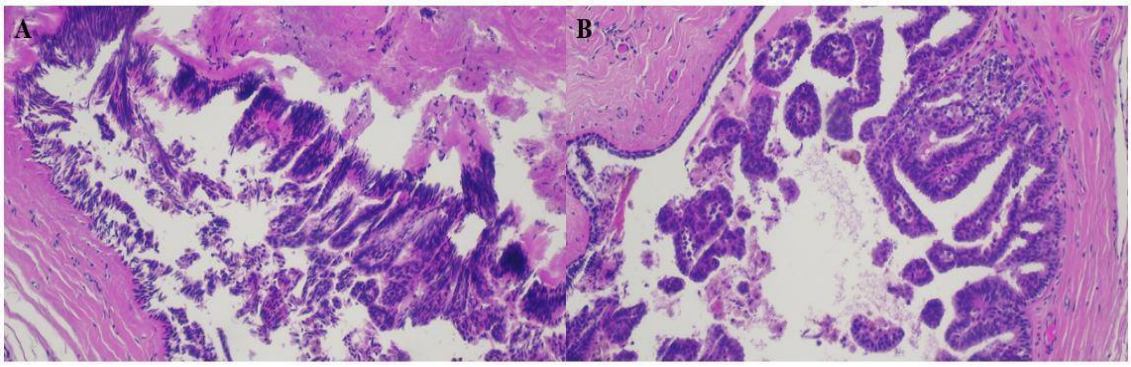
Supplementary figure 18: **Case 13*:* Neither of the 4 IHCs were conclusive, and only PR was in keeping with the diagnosis; HER2 IHC obviously identified the cauterized tissue as neoplastic. HER2, (x10)**



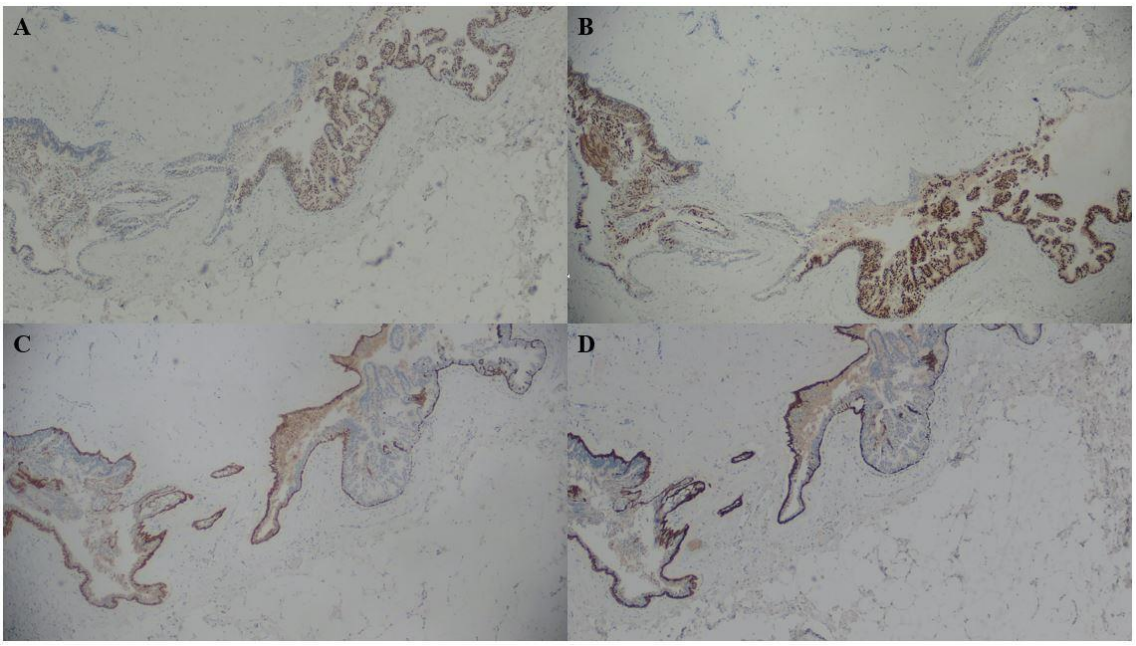
Supplementary figure 19: **Case 22: uncertain (in the context of DCIS ER+PR+ 80-90%++ & LCIS ER+PR+ 80%++;), HE, (x40)**



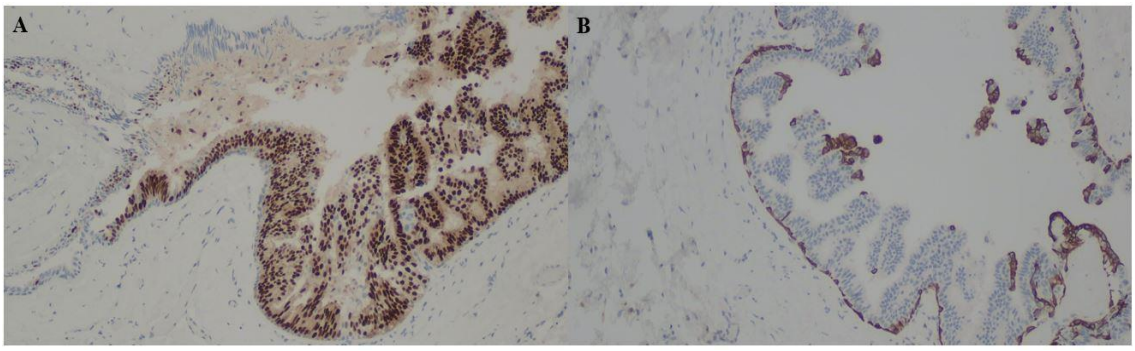
Supplementary figure 20: **Case 22 IHC: A: ER (<80% mosaic, supportive of non-neoplastic), B: PR (<80% mosaic, supportive of non-neoplastic), C: CK5 (besides myoepithelial, 2-3 luminal cells, in keeping with, but not deemed sufficient to support non-neoplastic), D: CK14 (mosaic, supportive of non-neoplastic) (x40)**



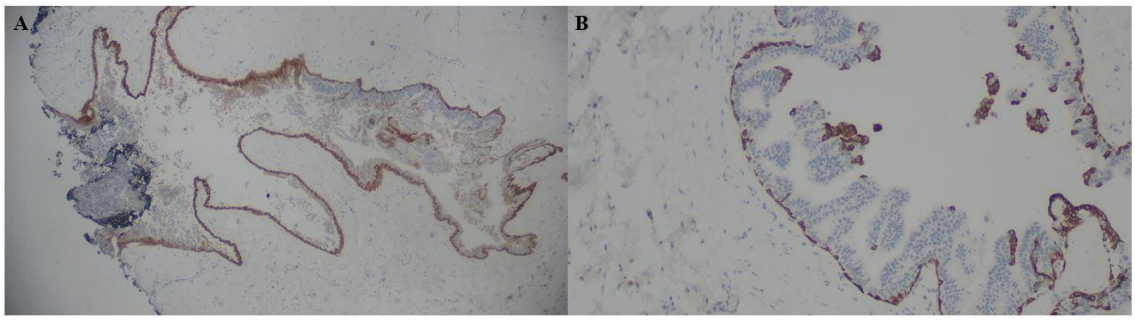
Supplementary figure 21: **Case 26: A: uncertain HE, B: Papillary DCIS HE (x10)**



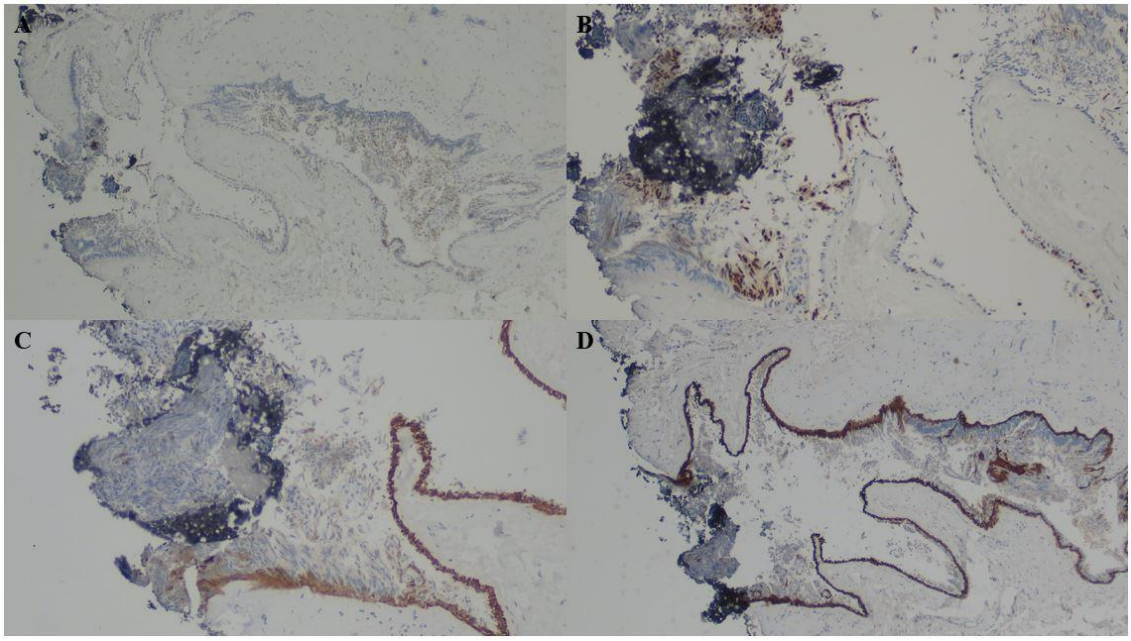
Supplementary figure 22: **Case 26 IHC: Partial involvement of the duct by ER+PR+CK5/14- DCIS (right bottom areas and probably left top areas; note weakening ER towards the left and CK5+ & CK14+ cells among the neoplastic cells), A: ER, B: PR, C: CK5, D: CK14 (x4)**



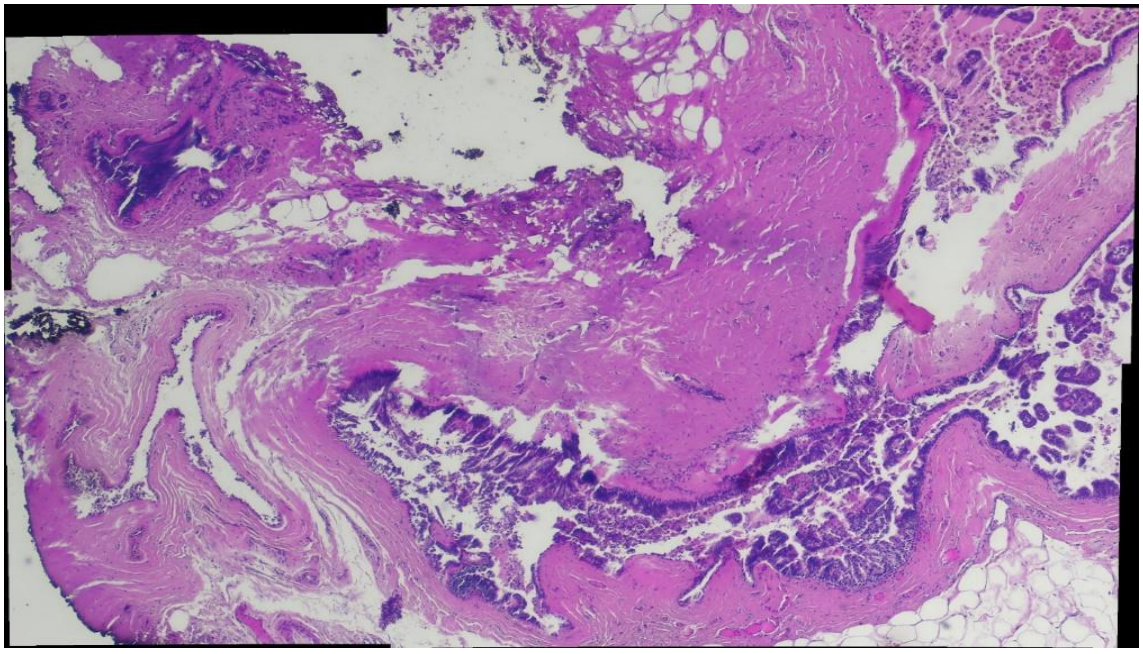
Supplementary figure 23: **Case 26 IHC: A: PR (partial involvement by neoplasia), B: CK5 (residual non-neoplastic cells) (x10)**



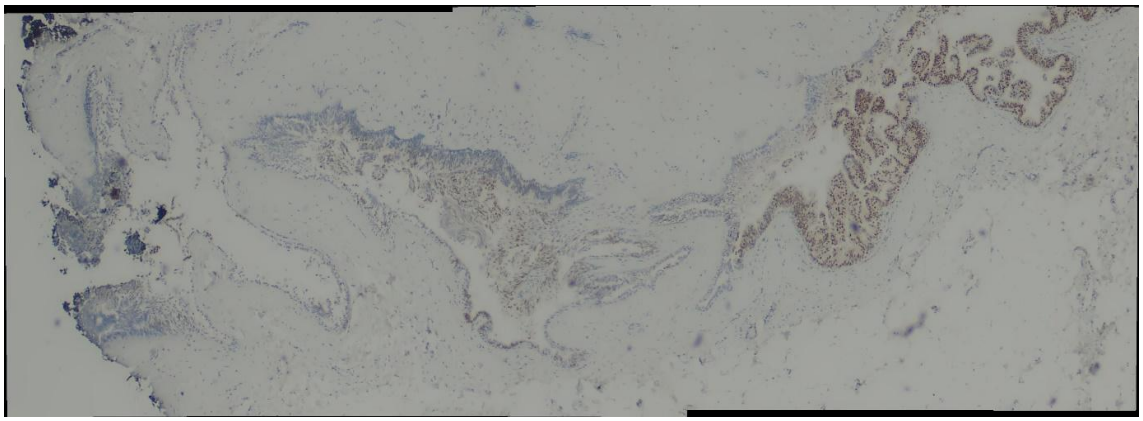
Supplementary figure 24: **Case 26 IHC: CK5, dominantly negative, but still mosaic (hyperplastic-like) staining in the cauterized tissue on the right (B), and its possible explanation on the left (CK5) (A) – interpreted as favouring neoplastic in this context (x10)**



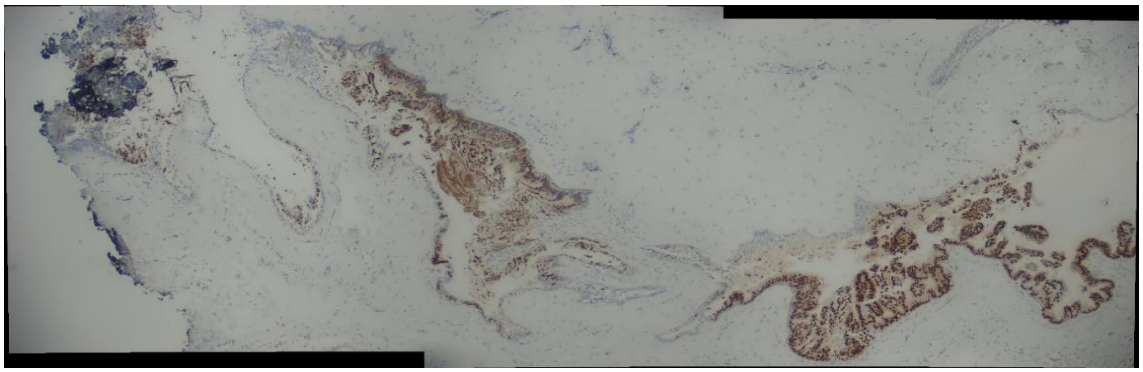
Supplementary figure 25: **Case 26 IHC: A: ER, B: PR, C: CK5, D: CK14 (x4)**



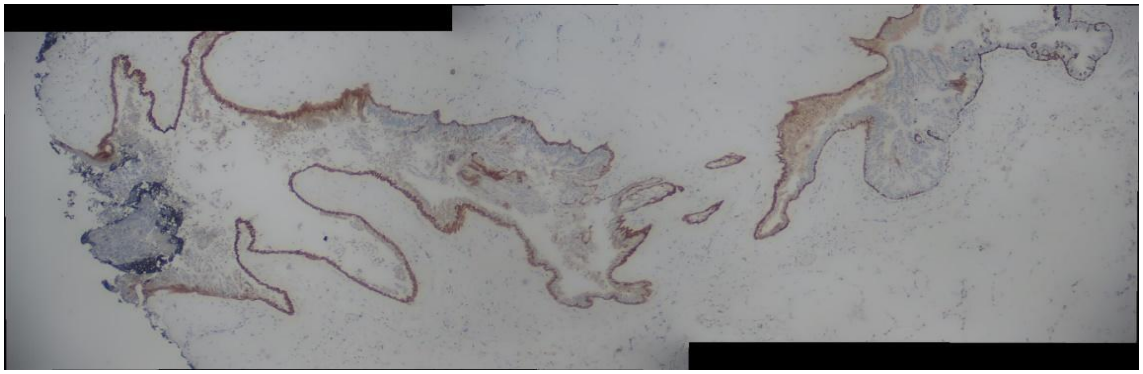
Supplementary figure 26: **Case 26 HE in context, (x4 stich)**



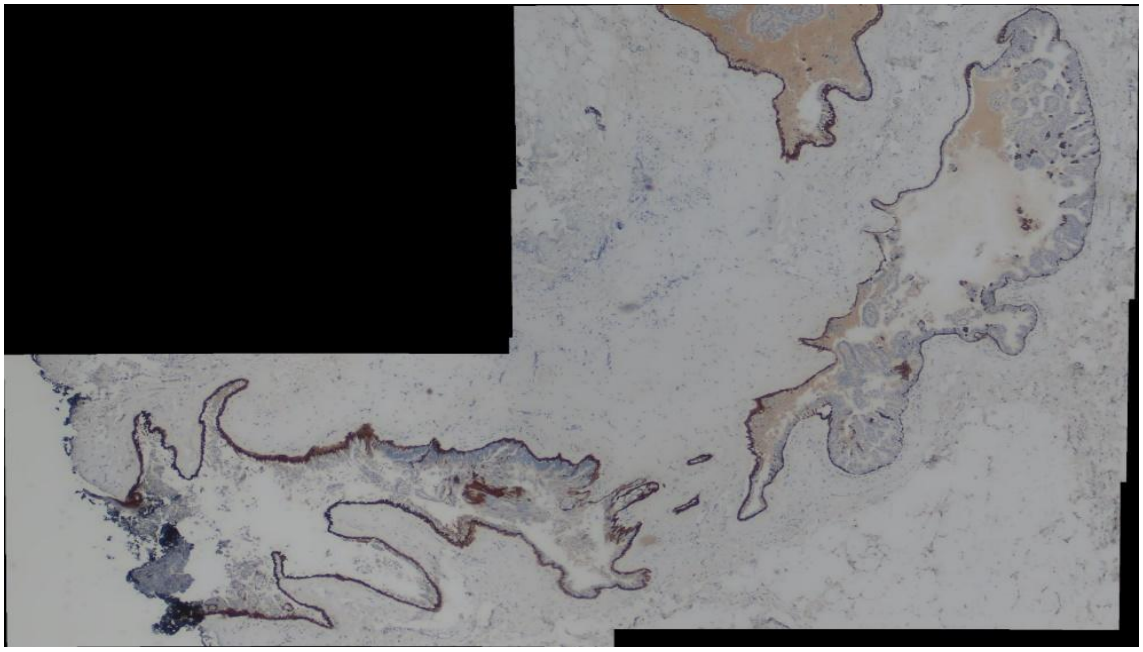
Supplementary figure 27: **Case 26 IHC: ER, (Note decreasing intensity of staining in the cauterized area on the left) (x4 stich)**



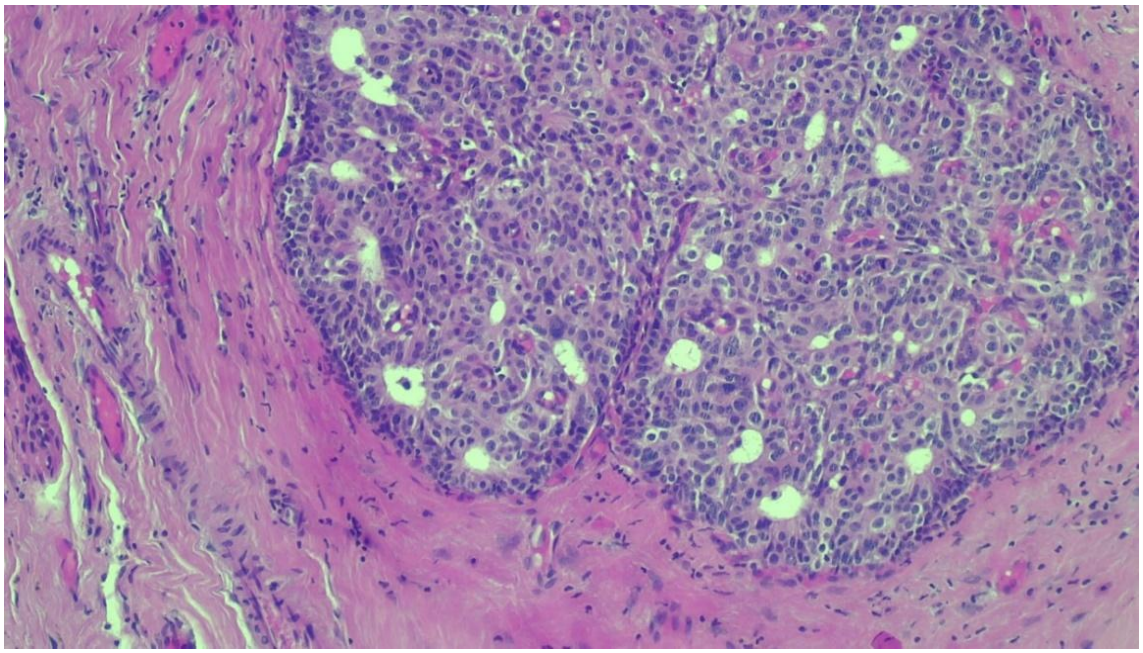
Supplementary figure 28: **Case 26 IHC: PR, (x4 stich)**



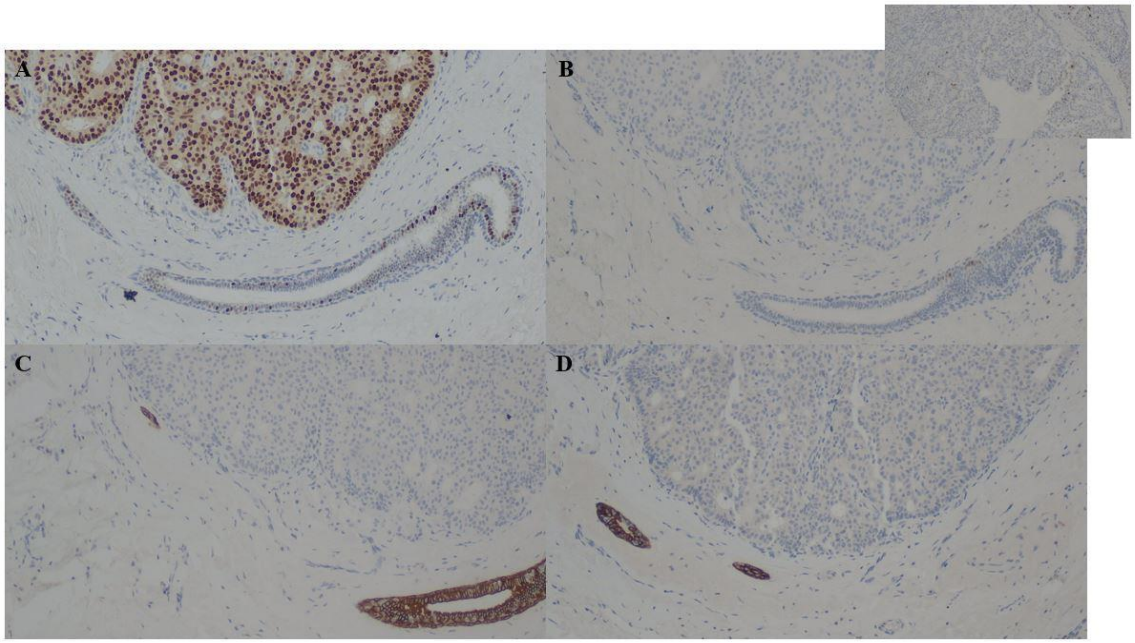
Supplementary figure 29: **Case 26 IHC: CK5, (x4 stich)**



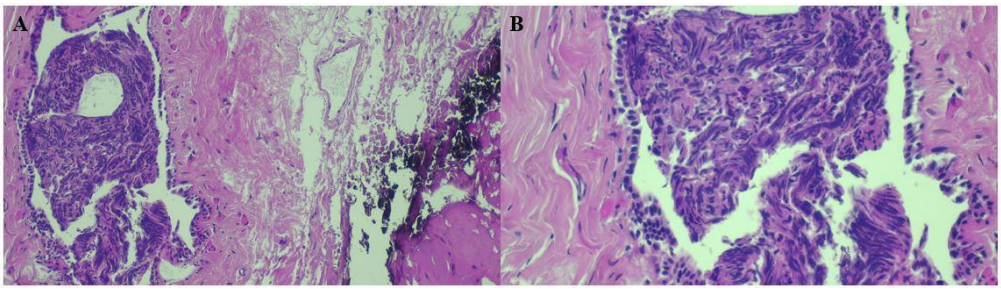
Supplementary figure 30: **Case 26 IHC: CK14 (x4 stich)**



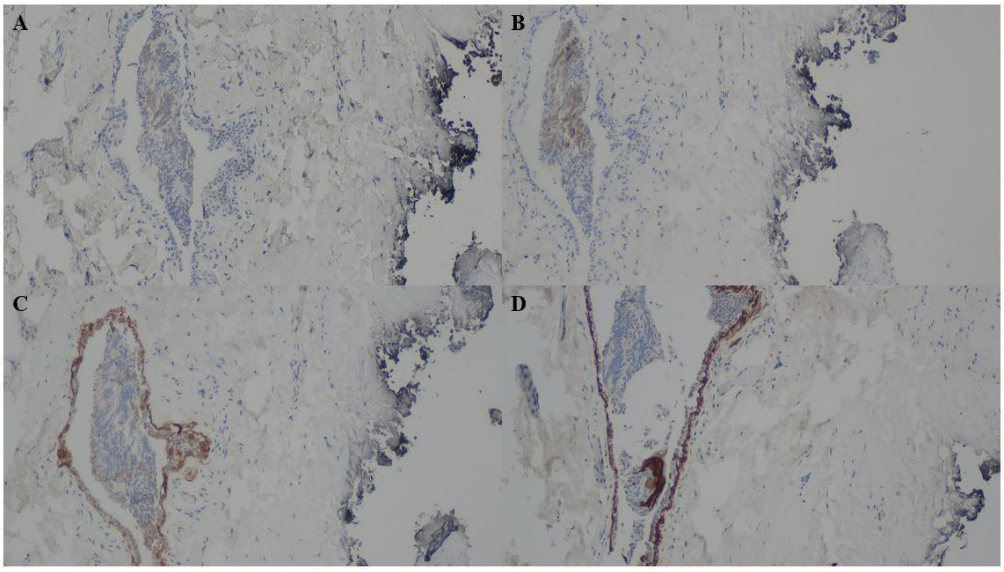
Supplementary figure 31: **Case 30 HE control (ER+PR+ DCIS-like NST) (x10)**



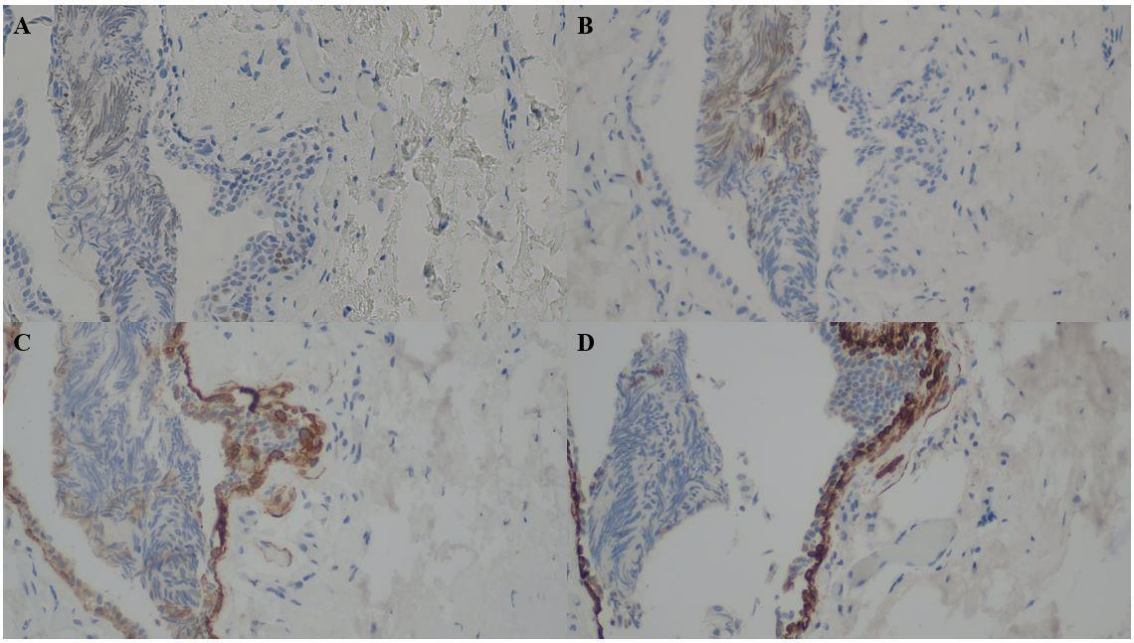
Supplementary figure 32: **Case 30 IHC: A*:* ER (strong, diffuse ER+ cancer and mosaic like normal), B: PR (inset focal PR+ @ other focus), C: CK5 (no staining in cancer, and no myoepithelium), D: CK14 (same as for CK5) (x10)**



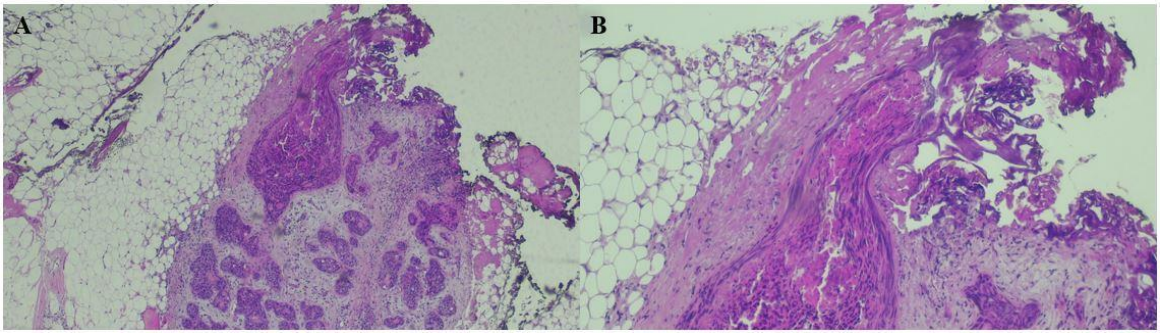
Supplementary figure 33: **Case 30 uncertain A: HE (x10), B: HE (x20)**



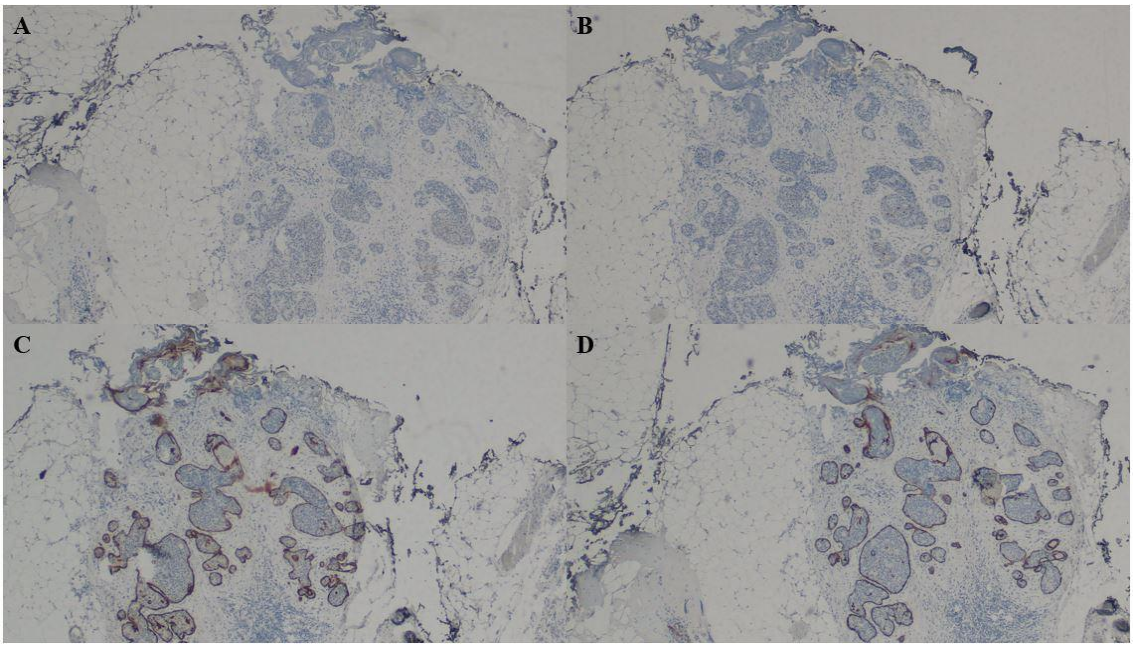
Supplementary figure 34: **Case 30: Variable mosaic staining with all 4 IHCs supporting the non-neoplastic nature, A: ER, B: PR, C: CK5, D: CK14 (x10)**



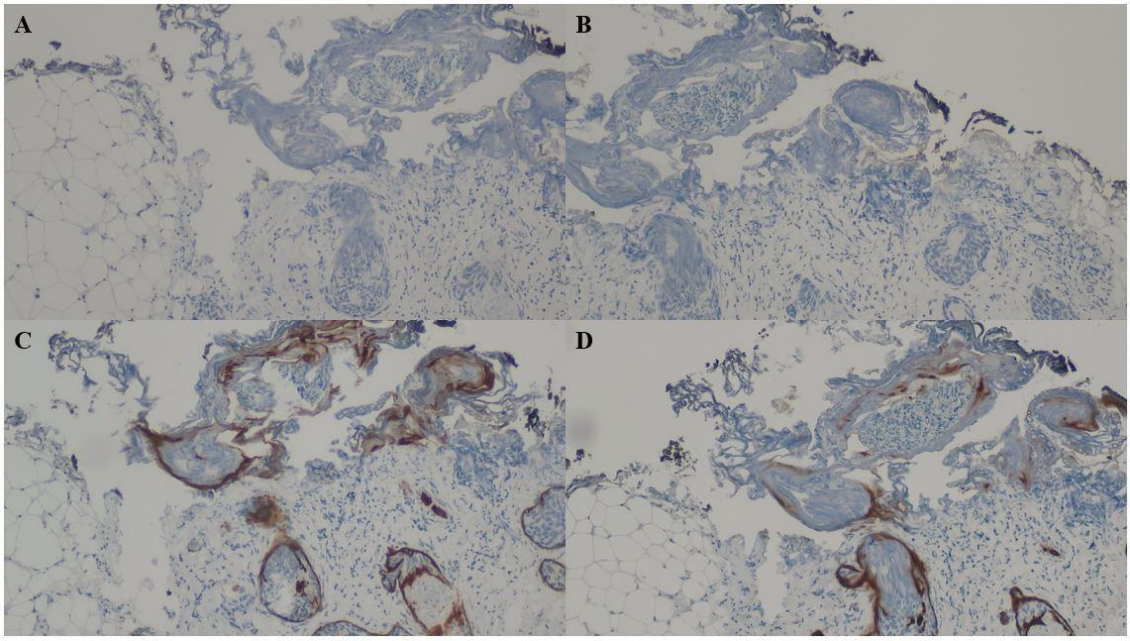
Supplementary figure 35: **Case 30: Variable mosaic staining with all 4 IHCs supporting the non-neoplastic nature, A: ER, B: PR, C: CK5, D: CK14 (x20)**



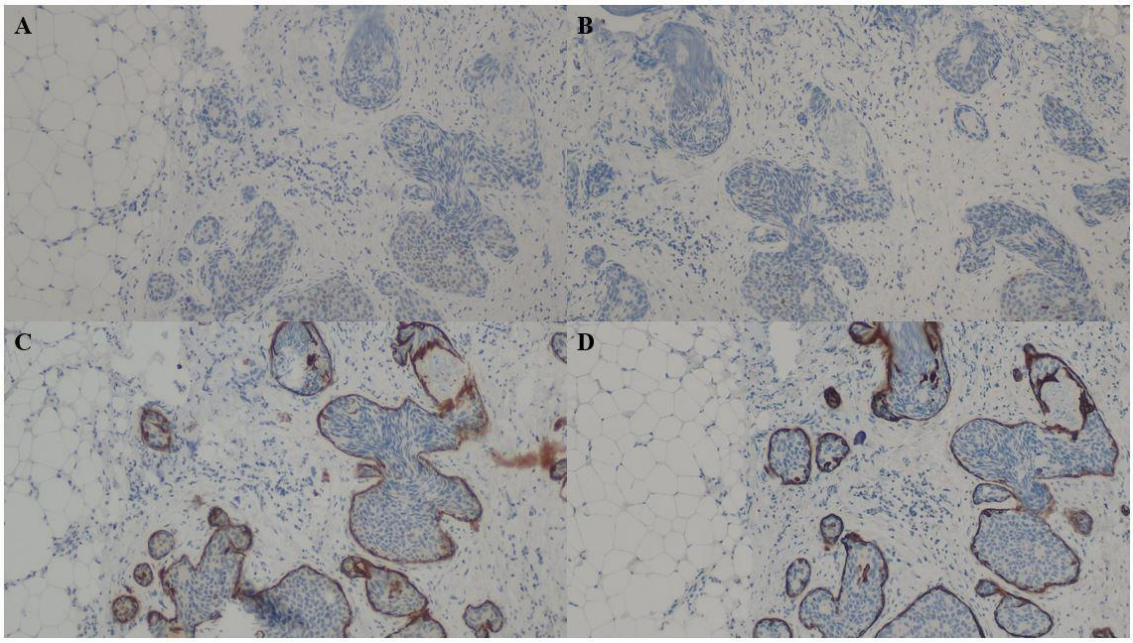
Supplementary figure 36: **Case 34 ER+PR+ DCIS: A: HE (x4), B: HE (x10)**



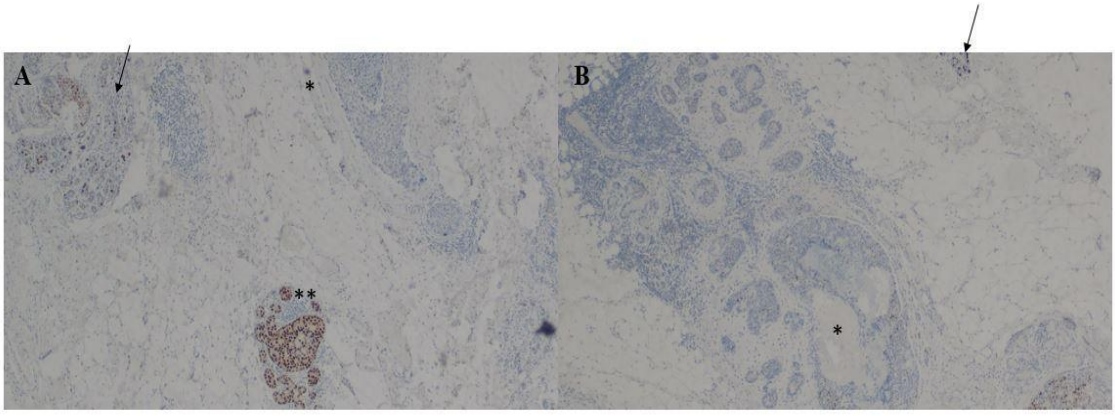
Supplementary figure 37: **Case 34 IHC: A: ER (negative – not supportive of non-neoplastic), B: PR (negative – not supportive of non-neoplastic), C: CK5 (negative – supportive of neoplastic, DCIS), D: CK14 (negative – supportive of neoplastic, DCIS) (x4)**



Supplementary figure 38: **Case 34 IHC: A: ER (negative – not supportive of non-neoplastic), B: PR (negative – not supportive of non-neoplastic), C: CK5 (negative – supportive of neoplastic, DCIS), D: CK14 (negative – supportive of neoplastic, DCIS) (x10)**



Supplementary figure 39: **Case 34 IHC: A*:* ER (negative – not supportive of non-neoplastic), B: PR (negative – not supportive of non-neoplastic), C: CK5 (negative – supportive of neoplastic, DCIS), D: CK14 (negative – supportive of neoplastic, DCIS) (x20)**



Supplementary figure 40: **Case 34 IHC: Control DCIS with focal (*) or diffuse (**) staining and normal (→) (same slide), A: ER, B: PR (x4)**

11. MAGYAR NYELVŰ ÖSSZEFOGLALÓ

Az emlőrák (IBC) a leggyakoribb rosszindulatú daganat és vezető daganatos halálok nőkben [1]. Az emlőrákok molekuláris alcsoportjaira következtethetünk az immunhisztokémiai (IHC) vizsgálatok segítségével, és megkülönböztetjük a következőket: luminális A-szerű (ösztrogén receptor (ER) pozitív, progeszteron receptor (PR) pozitív, humán epidermális növekedés faktor receptor 2 (HER2) negatív alacsony proliferációval), luminális B-szerű (ER pozitív, PR lehet negatív, magas proliferációval és/vagy HER2 pozitivitással), tripla negatív (TNBC: ER, PR és HER2 negatív) és HER2-pozitív (ER és PR negatív) IBC-k [2]. Az ER, PR, HER2 státusz nem csak prognosztikus, de prediktív jelentőségű is. Az IBC-k alacsony molekula-súlyú cytokeratinokkal (CK) pozitívak (CK7, CK8/18, CK1), továbbá expresszálnak különböző emlőmarkereket (pl. GATA3, mammaglobin (MGB), GCDFP-15, SOX10). Az esetek egy hatodában bazális marker pozitivitás jellemző, közöttük a magas molekula-súlyú CK-kal (CK5/6, CK14, CK17), valamint EGFR-rel is. Primer vagy áttéti folyamat esetén a kiindulás meghatározásában nagy jelentősége van az IHC-nak, azonban TNBC-k esetén kihívásba ütközhetünk, a marker expresszió hiánya miatt. Az eddig vizsgált markerek nem bizonyultak kellően specifikusnak. A célunk a TRPS1-nek, mint lehetséges emlőmarkernek a vizsgálata volt.

A már említett markerek közül fontos megjegyezni, hogy az ép emlő epithelium és a közönséges hyperplasia (UDH) mozaik-szerű pozitivitást mutat ER-rel és PR-rel, ezzel szemben az alacsony grádusú neoplasztikus elváltozások, mint az atípusos ductalis hyperplasia (ADH) és az alacsony nuclearis gradusú DCIS diffúzan expresszálnak ER-t és PR-t, mindemellett CK5 és CK14 negatívak. A magas grádusú carcinomák különböző expressziós mintázattal rendelkeznek ER és PR tekintetében; azonban CK5 és CK14 IHC-val az esetek túlnyomó többségében negatívak, bár előfordulhat diffúz pozitivitás is. Ezt a tudást hasznosítottuk a kauter károsodott szövetminták esetén a károsodott reszekciós felszínben elhelyezkedő elváltozások benignus vagy malignus természetének tisztázására.

A galectin-1 egy immunszupresszív fehérje, mely a tumorsejtek túlélésében játszhat szerepet, így a galectin-1 jelenléte rossz kórjóslatot tükrözhet. Más szolid tumorokban már leírták prognosztikai szerepét, viszont TNBC-ben prognosztikai jelentőségét még nem tanulmányozták.

(1)

A TNBC-k esetében az ER és PR, valamint a HER2 expresszió hiánya figyelhető meg definíció szerint. Többségükben agresszív tumorok, magas metasztatikus potenciállal. A GCDFP-15, a GATA3, a MGB és a SOX10 nem kellően specifikusak emlőrákokra. A célunk a trichorhinophalangeal syndrome type 1 (TRPS1) fehérjének, mint lehetséges emlőmarkernek a vizsgálata volt CK5 pozitív TNBC-kben, az értékelés reprodukálhatóságának vizsgálata mellett. Százhúsz TNBC-ből készült szöveti multiblokk (TMA) TRPS1 IHC festődését vizsgálatuk. A pozitivitás küszöbértéke legalább 10%-os sejtmagi festődés volt, ennek reprodukálhatóságát kappa statisztikával vizsgáltuk. A minták GATA3, GCDFP-15, MGB és SOX10 pozitivitására vonatkozó adatokat korábbi vizsgálatainkból nyertük. Száztizenhét mintát tudtunk értékelni. TRPS1 pozitivitás 92 esetben (79%) mutatkozott, ami felülmúlja a korábban tesztelt markereket, melyekben a következőképpen alakult a pozitivitás: SOX10: 82 (70%), GATA3: 11 (9%), MGB: 10 (9%) és GCDFP-15: 7 (6%). A 25 TRPS1 negatív esetből 11 pozitívnak bizonyult SOX10 IHC-val. A TRPS1, illetve SOX10 kettős negatív esetekből 5 bizonyult pozitívnak egyéb markerekkel. A reprodukálhatóság tekintetében a TRPS1 expresszió esetében jelentős véleményegyezést tapasztaltunk (Cohen kappa: 0,67). Az 5 vizsgált markerből a TRPS1 bizonyult legszenzitívebb emlő eredetet igazoló markernek, a CK5 pozitív TNBC-k kapcsán. A TRPS1 negatív esetek túlnyomó része SOX10 pozitivitást mutatott, a fennmaradó dupla negatív esetek egy része viszont pozitívnak bizonyult GCDFP-15 és/vagy MGB markerekkel.

(2)

A mindennapi emlőpatológiai gyakorlatban az emlőmegtartó műtétek következtében gyakrabban fordulnak elő kauterizációs károsodást mutató reszekciós felszínek, melyek megítélése során nehézségekbe ütközünk. Célunk egy olyan IHC panel használata volt, mely segít meghatározni a kauter károsította reszekciós felszínben elhelyezkedő elváltozások neoplasztikus esetleges nem-neoplasztikus voltát. Retrospektíven vizsgáltunk 34 emlő elváltozást, mely 23 betegből származott. Az ER, PR, CK5 és CK14 IHC vizsgálatok hasznát határoztuk meg az elektrotermikusan károsodott szövetek megítélésében. Az összes eltérés közül 27 bizonytalan természetű elváltozást azonosítottunk. Az esetek túlnyomó többsége (18/27) neoplasztikus vagy nem-neoplasztikus kategóriába sorolható volt, 6/27 eset valószínű neoplasztikus vagy nem-neoplasztikus kategóriába került, és 3 eset maradt, melyekről egyértelműen nem lehetett állást foglalni a HE és IHC festések alapján. Mind a négy IHC vizsgálat segítségünkre volt az elváltozások természetének meghatározásában az esetek közel felében. Három vagy négy IHC segített 19/27 esetben a diagnózis megállapításában. A

cytokeratinok (CK5 és CK14) bizonyultak a leghasznosabbnak. Az IHC vizsgálatok segítségével 24 esetet tudtunk kategorizálni. Az eredményeink alapján a CK5, CK14, PR és ER IHC reakciók segítségünkre lehetnek a mindennapi patológiai gyakorlatban a kauter károsodást mutató sebészi reszekciós felszínekben elhelyezkedő emlőelváltozások elemzésében, természetük meghatározásában. Az eredmények interpretációjakor a betegnél ismert emlőelváltozásokat és azok IHC fenotípusát is figyelembe kell vennünk.

(3)

A galectin-1 a lektinek csoportjába tartozó szénhidrátkötő képességgel bíró, a sejten belül a sejtmagra, a cytoplasmára és a sejtmembránra lokalizált, valamint az extracelluláris mátrixba kiválasztott protein [48, 51, 52]. A galectin-1 az emlőrákokban részt vesz az immunszupresszióban, a tumor növekedésben és a metasztázis képzésben. A Bács-Kiskun Megyei Oktató kórházban 2005 és 2016 között sebészetileg kezelt 95 TNBC TMA blokkjainak galectin-1 IHC vizsgálatát végeztük. Az eseteket a tumor stroma festődési intenzitása alapján (negatív, gyenge, közepes, erős, nagyon erős), valamint a tumor stroma ≥50%-os pozitivitása alapján értékeltük ki. A galectin-1 expressziót a túlélési adatokkal (teljes túlélés - OS, progresszió mentes túlélés - PFS) vetettük össze. A Kaplan-Meier görbe elemzéseket, a log-rank analízist és a Cox-regressziós analíziseket SPSS statisztikai szoftverrel készítettünk. A log-rank analízissel szignifikáns összefüggést azonosítottunk a galectin-1 expresszió és a teljes túlélés (OS) között a negatív, gyenge, közepes, erős és nagyon erős festődési erősségű esetek között (szabadság fok: 4, p=0.006). A festődési erősség szerinti 5 csoportot ezek után kettő csoportra osztottuk, az egy csoportra eső alacsony esetszám miatt. Így elkülönítettük a negatív, a gyenge és a közepes intenzitású csoportot, az erősen és nagyon erősen expresszáló, intenzíven festődő esetektől. Ebben a klasszifikációban is szignifikáns eltérést azonosítottunk az OS tekintetében (p=0.008). Az eseteket, a tumort infiltráló lymphocyták aránya (sTIL) alapján, 30%-os küszöbértékkel alacsony-sTIL és magas-sTIL csoportokra osztottuk. Az alacsony-sTIL csoportban szintén szignifikáns volt a különbség az OS tekintetében a két alcsoportban (p=0.005).

Az 50%-os határértéket alkalmazó kategorizálás esetén nem csak az OS (p=0.020), de a PFS (p=0.007) tekintetében is jelentős eltérést mutatkozott a csoportok között. Az alacsony-sTIL csoportban szintén szignifikáns volt a különbség az PFS tekintetében a két alcsoportban (p=0.025). A galectin-1 expressziót és az emlőrák ismert prognosztikai faktorait (pT, pN kategória, grádus, nyirokérinvázió, sTIL) Cox regressziós analízissel elemeztük. Az

egyváltozós Cox regressziós elemzésben a pT (p=0.036) és a pN kategória (p=0.003), az erős galectin-1 stromális expresszió (p=0.010) és ≥50% stromális pozitivitás (p=0.024) az OS tekintetében prognosztikai jelentőségűnek bizonyult. A többváltozós analízisben, amely csak az egyváltozós analízisben szignifikáns változókat tartalmazta a pN kategória és a galectin-1 stromális intenzitásnak volt független prognosztikai szerepe. Az alacsony sTIL csoportban (43 eset) az egyváltozós Cox regressziós analízisben a stroma inenzitás (p=0.008), a ≥50% galectin-1 peritumorális expresszió (p=0.011) és a pN kategória (p=0.046) bizonyult szignifikánsnak OS tekintetében. A többváltozós analízissel csak az erős galectin-1 pozitivitás bizonyult független prognosztikai változónak (p=0.009).

PFS tekintetében az egyváltozós Cox regressziós analízisben a pT kategória (p=0.004), a pN kategória (p=0.001), a ≥50% stromális galectin-1 expresszió bizonyult sziginifikánsnak. A többváltozós analízisben a pN kategória (p=0.004) a stromális galectin-1 festődési százalék (p=0.008) bizonyult független prognosztikai tényezőnek. Az alacsony-sTIL csoportban egyváltozós és többváltozós analízisben is a pN kategória és a galectin-1 stromális festődési százaléknak van független prognosztikai jelentősége.

Az eredményeink alapján a galectin-1 expresszió intenzitása és részleges vagy diffúz jellege TNBCk-ben prognosztikai jelentőséggel bír.

12. APPENDIX

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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