Evaluation of diagnostic and prognostic features of breast cancer

Ph. D. Thesis

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Szeged, Hungary

2021

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

I. **Anita Sejben**, András Vörös, Arbel Golan, Tamás Zombori, Gábor Cserni. The added value of SOX10 immunohistochemistry to other breast markers in identifying cytokeratin 5 positive triple negative breast cancers as of mammary origin. Pathobiology. E-Pub ahead of print 2021 Feb 10; 1-6. doi: 10.1159/000512006

IF (2019/2020): 1.985

II. **Anita Sejben**, Tibor Nyári, Tamás Zombori, Gábor Cserni: Comparison of Nottingham Prognostic Index, PREDICT and PrognosTILs in triple negative breast cancer –a retrospective cohort study. Pathol Oncol Res. 2020; 26: 2443-2450. doi: 10.1007/s12253-020-00846-8

IF (2019/2020): 2.826

III. **Anita Sejben**, Renáta Kószó, Zsuzsanna Kahán, Gábor Cserni, Tamás Zombori: Examination of tumor regression grading systems in breast cancer patients who received neoadjuvant therapy. Pathol Oncol Res. 2020; 26: 2747-2754. doi: 10.1007/s12253-020-00867-3

IF (2019/2020): 2.826

OTHER PUBLICATIONS

IV. Klaudia Farkas, Zita Reisz, **Anita Sejben**, László Tiszlavicz, Mónika Szűcs, Tibor Nyári, Zoltán Szepes, Ferenc Nagy, Mariann Rutka, Anita Bálint, Renáta Bor, Ágnes Milassin, Tamás Molnár: Histological activity and basal plasmacytosis are nonpredictive markers for subsequent relapse in ulcerative colitis patients with mucosal healing. J Gastroenterol Pancreatol Liver Disord. 2016; 3: 1-4. doi: 10.15226/2374-815X/3/4/00163

IF: 0

V. Ágnes Milassin, **Anita Sejben**, László Tiszlavicz, Zita Reisz, György Lázár, Mónika Szűcs, Renáta Bor, Anita Bálint, Mariann Rutka, Zoltán Szepes, Ferenc Nagy, Klaudia Farkas, Tamás Molnár: Analysis of risk factors – especially different types of plexitis – for postoperative relapse in Crohn’s disease. World J Gastrointest Surg. 2017; 27: 167-173. doi: 10.4240/wjgs.v9.i7.167

IF (2017): 2.813

VI. **Sejben Anita**, Tiszlavicz László, Polyák Kornélia, Kovács László, Maráz Anikó, Török Dóra, Leprán Ádám, Ottlakán Aurél, Furák József: Li-Fraumeni-szindróma. Orv Hetil. 2019; 160: 228-234. doi: 10.1556/650.2019.31290

IF (2019): 0.497

VII. Gábor Cserni, **Anita Sejben**: Grading ductal carcinoma in situ (DCIS) of the breast – What's wrong with it? Pathol Oncol Res. 2020; 26: 665-671. doi: 10.1007/s12253-019-00760-8

IF (2019/2020): 2.826

VIII. **Sejben Anita**, Tiszlavicz László, Furák József, Boros Krisztina, Sápi Zoltán, Zombori Tamás: A tüdőartériák intimájából kiinduló sarcoma. Orv Hetil. 2020; 161: 232-236. doi: 10.1556/650.2020.31642.

IF (2019/2020): 0.497

IX. Tamás Zombori, **Anita Sejben**, László Tiszlavicz, Gábor Cserni, Regina Pálföldi, Edit Csada, József Furák: Architectural grade combined with spread through air spaces (STAS) predicts recurrence and is suitable for stratifying patients who might be eligible for lung sparing surgery for stage I adenocarcinomas. Pathol Oncol Res. 2020; 26: 2451-2458. doi: 10.1007/s12253-020-00855-7

IF (2019/2020): 2.826

X. Tamás Zombori, Sándor Turkevi-Nagy, **Anita Sejben**, Gréta Nagy-Juhász, Gábor Cserni, József Furák, László Tiszlavicz, László Krenács, Bence Kővári: The panel of Syntaxin 1 and Insulinoma-Associated Protein 1 outperforms classic neuroendocrine markers in pulmonary neuroendocrine neoplasms. APMIS. E-pub ahead of print 2021 Jan 8 doi: 10.1111/apm.1311

IF (2019/2020): 2.02

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LIST OF ABBREVATIONS:

AJCC: American Joint Committee on Cancer

ALND: axillary lymph node dissection

AR: androgen receptor

BC: breast cancer

BRCA-1: Breast Cancer Gene-1

CK: cytokeratin

CMF: cyclophosphamide, methotrexate and 5-fluorouracil

DCIS: ductal carcinoma in situ

DFS: disease-free survival

DOD: dead of disease

EGFR: epidermal growth factor receptor

ER: estrogen receptor

EWGBSP: European Working Group for Breast Screening Pathology

GATA3: GATA binding protein 3

GCDFP-15: gross cystic disease fluid protein-15

GPG: good prognostic group

HE: hematoxylin and eosin staining

HER2: human epidermal growth factor receptor 2

HR: hormone receptor

IHC: immunohistochemistry

IIOBWG: International Immuno-Oncology Biomarker Working Group

ILC: invasive lobular carcinoma

Ki-67: Ki-67 proliferation marker

L: lymphovascular invasion

LABC: locally advanced breast cancer

LCIS: lobular carcinoma in situ

LHRH: luteinizing hormone-releasing hormone

MG: mammaglobin A

MPG: moderate prognostic group

NAT: neoadjuvant therapy

NPI: Nottingham Prognostic Index

NR: nodal regression

NSABP: National Surgical Adjuvant Breast and Bowel Project

NST: breast cancer of no special type

NY-BR-1: New York-Breast-1

OS: overall survival

pCR: pathological complete regression

PN: perineural invasion

PPG: poor prognostic group

PR: progesterone receptor

PST: primary systemic treatment

R: resection (completeness of surgical resection)

RCB: residual cancer burden

RDBN: Residual disease in breast and nodes

ROC: receiver operating characteristic

SNB: sentinel node biopsy

SOX10: SRY-related HMG-box 10

TIL: tumor infiltrating lymphocytes

TMA: tissue microarray

TNBC: triple negative breast cancer

TR: tumor regression

V: vascular invasion

VGPG: very good prognostic group

VPPG: very poor prognostic group

WT1: Wilms' tumor-1

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1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Breast cancer (BC) is the most common cancer in females and is also a significant cause of cancer related mortality all over the world [1; 2]. As such, it is one of the most investigated fields of medicine with research including diagnostics in general, surgical and oncological treatments, patient follow-up, prevention and screening programs. The importance of BC is accentuated by mainstream media, Breast Cancer Awareness Month in October, many educational videos and lectures that exist in the online space and self-help groups for patients.

BC does not represent one specific disease, but should be considered a group of malignant lesions of the breast. Several classifications of BC have been developed, making an attempt to reflect the predictive and prognostic features of each category. Most commonly used classifications are according to histological features, grade, the TNM system and the staging system that is based on it [3].

The first molecular, gene expression profile-based classification of BC was published by Perou and coauthors in 2000 in Nature [4]. The system has been adapted for immunohistochemistry (IHC) with examinations of estrogen (ER) and progesterone receptors (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 proliferation marker, in this manner, the surrogate molecular classification system can easily be used in all parts of the world. Luminal A-like subtype of BC (more or less matching the luminal A class of the gene expression profile-based subtypes) integrates hormone receptor (HR) positive, HER2 negative tumors with low expression of Ki-67. Tumors that fall into this category are usually slowly growing and have a generally favorable prognosis. The luminal B-like subtype includes HR positive cases, too with HER2 overexpression/amplification and/or a high proliferation index; these BCs are often further divided on the basis of their HER2 status. As reflected by their higher proliferation rates, these cancers tend to grow faster and are associated with a higher incidence of metastasis therefore this group has less favorable prognosis than Luminal A-like cases. Cases that lack HR expression but are positive for HER2 either by IHC or in situ hybridization, are called HER2-type tumors (reflecting the overlap with the HER2-enriched class of the molecular classification). These neoplasms are generally more aggressive, due to more rapid proliferation compared to luminal cancers. After the introduction of targeted anti-HER2 therapies, their prognosis has significantly improved. Tumors that lack the expression

of ER, PR and HER2 are the so called triple negative BCs (TNBC) [5; 6]. Even though the complete pathogenesis of this subtype is yet unknown, as a group, it is more common in young patients, women of African-American ancestry and with Breast Cancer Gene-1 (BRCA-1) mutations [7]. TNBCs show the worst prognosis of all types, although some rare special type BCs (e.g. tall cell carcinoma with reverse polarity, secretory carcinoma, low grade adenosquamous carcinoma) belonging into this category have admittedly good prognosis [8].

TNBCs represent a heterogeneous group of BCs characterized by variable though distinct molecular profiles [8; 9]. About 15% of breast carcinomas belong to the TNBC category. Distant hematogenous metastasis formation and local recurrences are frequent and the treatment efficiency of TNBC is lower than that of other types of BC. While TNBCs lack the expression of the previously mentioned receptors, they also commonly lack the expression of so called “classic” breast markers. In cases when the first sign of BC is a metastatic lymph node or a distant metastasis, it remains a challenging task to prove that the primary tumor is a TNBC.

The treatment of TNBC is of primary importance for clinicians due to its poor overall prognosis [9; 10; 11]. By taking molecular profiles and BRCA deficiency into account, more personalized treatment methods are currently available [12]. Besides chemo- and radiotherapy, the role of immuno- and targeted therapy is increasing, both being currently under investigation with promising results [13; 14; 15]. Besides all recent discoveries, the variability of TNBC causes challenges in both diagnosis and prognosis. The purpose of multivariable analysis-derived risk stratification systems is to reflect the prognosis appropriately. The firstly introduced such system, the Nottingham Prognostic Index (NPI), includes tumor size, nodal stage and tumor histological grade; moreover, originally it divides tumors by prognosis into 3 categories [16]. PREDICT is more a complex prognostic tool that takes into account the following: the presence of ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), age at diagnosis, menopausal state, ER, PR, HER2 and Ki-67 status, invasive tumor size, tumor grade, method of tumor detection and number of positive lymph nodes [17]. The presence and amount of tumor infiltrating lymphocytes (TILs) has been proven to have prognostic value; therefore, a so called PrognosTILs tool has been developed for similar purposes. Percentage of stromal TILs, age, tumor size, number of positive lymph

nodes, histological grade and treatment are parameters of entry into this prognostic device [18].

The treatment of BC includes considerable varieties of options because of heterogeneity and the above mentioned features. Generally, the type of treatment is chosen according to molecular subtypes as reflected by predictive markers. In cases of locally advanced BC (LABC), primary systemic treatment (PST, including endocrine, targeted or chemotherapy) is often used. HER2-positive and TNBC cases show the greatest response to PST [19]. The interpretation of regression is partly a radiological, but partly also a pathological task. The latter is performed by the application of different tumor regression grading systems. However, several regression grading systems have been implemented, and there is no international consensus about their utilization in the routine histopathology reports.

We focused on the immunophenotyping of TNBCs with breast markers, on the prognostic subclassification of TNBCs and on the comparison of different tumor regression grading systems in patients having LABC treated with PST.

1.2. EXPRESSION OF BREAST MARKERS IN TRIPLE NEGATIVE BREAST CARCINOMAS

The expression of ER, PR, sometimes even HER2, or “breast markers” like GATA3, mammaglobin (MG), GCDFP-15 (growth cystic disease fluid protein-15) and NY-BR-1 (New York-Breast-1) can point to the breast origin of a metastasis, but TNBCs - by definition - lack the first three and might also lack the others [20; 21]. In a previous work, we identified GATA3 as the most gratifying breast marker, which could still be complemented by MG and GCDFP-15, with practically no added value of NY-BR-1. Acknowledging that neither of these markers are absolutely specific, we also suggested that only about half of cytokeratin 5 (CK5) expressing TNBCs could be proven to be of mammary origin with their help, therefore better or alternative markers would be useful in clinical practice [21].

1.3. EVALUATION OF PROGNOSIS IN TRIPLE NEGATIVE BREAST CANCER CASES

Prediction of prognosis remains essential to clinicians in their decision-making process, helps stratifying patients by risk and better allows preparing individual treatment plans [22]. Various prognostic factors have already been presented in TNBC. Ovcaricek and coauthors described nodal status and age as independent prognostic factors for disease-free survival (DFS), whereas for overall survival (OS), only nodal status proved to be an independent factor [23]. Urru et al. have demonstrated that tumor stage at diagnosis and positive lymph node ratio are relevant predictors of survival and tumor recurrence, with the addition of Ki-67 status for recurrence prediction [24]. Asaga and coworkers have used a different approach, and analyzed clinical response to preoperative systemic chemotherapy [25].

The NPI was described by Haybittle and coauthors in 1982, and it was originally designed for primary operable BC. It takes tumor size, nodal stage and tumor histological grade into consideration [16]. On the basis of its equation and the values of the NPI, patients could be divided into three prognostic categories according to the original article: Category I (good prognosis); Category II (moderate prognosis) and Category III (poor prognosis) [16; 26]. Later the prognostic groups were subdivided to form the excellent / very good (VGPG), the good (GPG), the moderate 1 (MPG1), the moderate 2 (MPG2), the poor (PPG) and the very poor prognostic groups (VPPG) [27]. Different cut-off values and diverse definitions of NPI-based groups (ranging from three to ten classes) have been used by some research groups [22]. The NPI has proven to be a valid prognostic tool in BC risk stratification and treatment [28].

A more complex prognostic model, PREDICT was published by Wishart and coauthors in 2010. The algorithm was developed from 5694 patients' data from the Eastern Cancer Registration and Information Centre. The selected patients were operated on for invasive BC. Based on the factors that were found to hold independent prognostic value, an algorithm was established that includes the presence of DCIS or LCIS, age at diagnosis, menopausal state, ER, PR, HER2 and Ki-67 status, invasive tumor size, tumor grade, method of tumor detection and number of positive lymph nodes [17]. PREDICT is also endorsed by the American Joint Committee of Cancer (AJCC) [29]. The on-line calculator estimates OS for 5, 10 and 15 years. Although the tool generally received good ratings for validity, Maishman and coauthors' results showed that PREDICT was a great tool only in long-term survival

estimates, and overestimated short-time survivals, especially in ER-positive tumors [28; 30; 31].

TILs reflect prognosis in TNBC, since their higher proportion correlates with better outcome in this subset of breast tumors, and indicates the prominent role the immune system plays in TNBC. While TNBCs lack targeted therapy, the interest for immune modulators has increased [32; 33]. Loi and coworkers conducted a pooled analysis of 2148 patients and identified the following factors that independently influence the prognosis of primary TNBCs: percentage of stromal TILs, age, tumor size, number of positive lymph nodes, histological grade and treatment. Invasive disease-free survival (i-DFS), distant disease-free survival (d-DFS) and OS results were examined in 3- and 5-year-intervals [18]. Based on the results, an equation was developed for survival estimates. For easier utilization, an online tool named PrognosTILs was developed for early stage TNBCs [34]. With this application, the 5-year and 10-year OS and DFS estimates can be calculated.

1.4. NEOADJUVANT THERAPY IN BREAST CANCER - THE PATHOLOGISTS' PERSPECTIVE

Treatment of LABC patients has been one of the great challenges of breast oncology for a long time. Patients with such advanced disease benefit from treatment devised by a multidisciplinary team of specialists: oncologists, surgeons, pathologists and radiologists [35]. Neoadjuvant therapy (NAT) has changed the management of LABC, since it can achieve reduction or even complete regression of the primary tumor and its metastases [36; 37]. This downstaging can allow some patients who would have had mastectomy as surgical treatment to be treated with breast conservation [38]. While receiving NAT, patients have to be under constant oncological and radiological follow-up [39].

The effectiveness of NAT completed with surgical and if needed postoperative endocrine treatment seems to be equivalent with adjuvant therapy on the basis of DFS and OS [40; 41]. Pathological complete regression (pCR) occurs more frequently in triple negative or HER2-positive cancers than in ER-positive ones [42; 43]. The work-up of surgical specimen after NAT requires the undivided attention of the pathologist. The identification of the primary tumor bed can be challenging because of its resemblance to fibrotic breast tissue. Insertion of metal clips into the tumor and/or specimen mammography can simplify the identification process. Specimen sampling requires adequate radio-pathological correlation [44; 45]. The

evaluation of tumor regression after NAT has to be established with full consideration given to radiology, gross morphology and microscopy.

The characterization of regression differs from country to country due to lack of international consensus on definitions. PCR implies no residual tumor in the surgical specimen, but the meaning is interpreted variously. In some European countries, pCR generally means the absence of in situ or invasive tumor tissue in the specimen. A significant difference in DFS between ypT0ypN0M0 and ypTisypN0M0 was demonstrated by the German and Austrian Breast Groups [46]. The United States Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research and the American Joint Committee on Cancer define pCR as the absence of residual invasive cancer in the surgical specimen [47; 48].

The histology of post-NAT tumors represents a spectrum from pCR to tumor growth and progression [49]. Regression can be reflected by the changes in tumor size,
the cellularity of the tumor bed, the presence of lymph node metastases and of DCIS. Since all of these factors may affect prognosis, it is essential that all are represented in the histopathological findings [50]. One of the most essential prognostic factors in BC after NAT continues to be the size of the invasive cancer. In case of unifocal BCs, the largest dimension of the invasive tumor will produce the ypT category, while in cases of multifocal ones, the largest dimension of the largest focus defines the ypT category.

1.5. TUMOR REGRESSION GRADING SYSTEMS IN NEOADJUVANT BREAST CANCER CASES

The evaluation of regression remains a complicated and versatile task especially due to worldwide application of numerous grading systems. The firstly described National Surgical Adjuvant Breast and Bowel Project (NSABP) B18 trial classified all NAT cases into two groups. The first group contains pCR cases (including ypT0 and ypTis), whereas the second group refers to all residual invasive tumor cases [51]. Further regression grading systems, namely Chevallier, Sataloff, Miller-Payne, Denkert-Sinn, Residual Cancer Burden (RCB), TR/NR (suggested system in the European guidelines for reflecting tumor regression and nodal regression) and Residual disease in breast and nodes (RDBN) define the presence or absence of complete pathological regression with one or more categories for tumors with some regression [52-58]. The TR/NR, Sataloff and RCB systems take residual tumor burden into account, the Chevallier grade considers the presence of some regression, while the

Denkert-Sinn grade includes tumor size, and the Miller-Payne system integrates change of cellularity between the biopsy and the resection specimen. The Sataloff, TR/NR and RCB grading systems include lymph node status as well [45; 53; 56]. The RDBN score can be calculated by the following equation RDBN = 0,2 x tumor size (mm) + Nottingham histologic grade (1–3) + lymph node involvement (0–3). According to the RDBN score a good (≤ 3.4), a moderate (3.4 < and ≤ 5.4), and a poor (>5.4) prognostic group were identified [58]. The quantification of residual tumor can be performed by using the RCB calculation. The algorithm was developed by Symmans and coworkers and takes notice of the two largest diameters of the residual tumor, its cellularity, the presence and proportion of DCIS and the number of metastatic lymph nodes with the size of the largest nodal metastasis [56]. The evaluation of RCB is supported by the online RCB calculator available at: http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3.

Table 1 represents tumor regression grading systems evaluated in our study and defines the differences among them. Although these grading systems are validated, none of them are accepted internationally. The Hungarian protocol in regression grading was recommended by the 3rd Hungarian Consensus Conference on Breast Cancer in 2016 and is practically identical with the recommendation of the European Working Group for Breast Screening Pathology (EWGBSP) [45; 57]. In Germany, the Denkert-Sinn grade is utilized, while in the USA and many other countries the RCB becomes increasingly adopted.

TR/NR [57]	Chevallier [52]	Sataloff [53]	Denkert-Sinn [55]	Miller-Payne [54]	RCB [56]
TR1a: No residual carcinoma.	G1: Disappearance of all tumor either on macroscopic or microscopic assessment.	TA: Total or nearly total therapeutic effect (i.e. isolated tumor cells).	TRG0: No signs of regression.	G1: No change or some alteration to individual malignant cells but no reduction in overall cellularity.	pCR: ypT0 and ypTis: 0 (RCB index score)
TR1b: No residual invasive tumor but DCIS present.	G2: Presence of in situ carcinoma only in the breast, without invasive tumor and tumor cells in the lymph nodes.	TB: Therapeutic effect subjectively superior to 50%.	TRG1: Tumor sclerosis with focal inflammation and/or minimal cytopathic changes (>5 mm).	G2: A minor loss of tumor cells but overall cellularity still high; up to 30% loss.	RCB-I: 0.1-1.35 (RCB index score)
TR2a: Minimal residual disease/near total effect (e.g. < 10% of tumor remaining).	G3: Presence of invasive carcinoma with stromal alteration, such as sclerosis or fibrosis.	TC: Therapeutic effect less than 50%, but evident effect.	TRG2: Great amount of tumor sclerosis. May be multifocal, presence of minimally invasive tumor (Not more than 5 mm, usually with intraductal spread).	G3: Between an estimated 30% and 90% reduction in tumor cells.	RCB-II: 1.36-3.27 (RCB index score)
TR2b: Evidence of response to therapy but with 10–50% of tumor remaining.	G4: No or few modifications of the tumoral appearance.	TD: No therapeutic effect.	TRG3: No signs of residual invasive tumor.	G4: A marked disappearance of tumor cells such that only small clusters or widely dispersed individual cells remain; more than 90% loss of tumor cells.	RCB-III: >3.28 (RCB index score)
TR2c: > 50% of tumor cellularity remains evident, when compared with the previous core biopsy sample, although some features of response to therapy present.			TRG4: No signs of invasive or in situ tumor.	G5: No malignant cells identifiable in sections from the site of the tumor; only vascular fibroelastotic stroma remains often containing macrophages. However, ductal carcinoma in situ (DCIS) may be present.	
TR3: No evidence of response to therapy					
NR1: No evidence of metastatic disease and no evidence of changes in the lymph nodes.		NA: Therapeutic effect, but no metastasis.			
NR2: Metastatic tumor not detected but evidence of response/down-staging, e.g. fibrosis.		NB: No metastasis, no therapeutic effect.			
NR3: Metastatic disease present but also evidence of response, such as nodal fibrosis.		NC: Therapeutic effect, but metastasis.			
NR4: Metastatic disease present with no evidence of response to therapy.		ND: Metastasis, no therapeutic effect.			

Table 1 Tumor regression grading systems for breast cancer specimens after NAT

2. AIMS

The aims of the thesis are as follows:

1. To assess the added value of SOX10 IHC to known GATA3, MG, GCDFP-15 and NY-BR-1 statuses in a series of CK5 positive primary TNBCs;

2. To compare the validity of three multivariable analysis derived prognostic systems, the NPI, PREDICT and PrognosTILs (a prognosticator including tumor infiltrating lymphocytes, TILs) in a series of TNBCs;

3. To compare the prognostic impact of different regression grading systems on DFS and OS, namely the TR/NR, Chevallier, Sataloff, Denkert-Sinn, Miller-Payne, NSABP-B18, Residual Disease in Breast and Nodes and Residual Cancer Burden in BC patients receiving PST.

3. MATERIALS AND METHODS

3.1. IMMUNOHISTOLOGICAL EVALUATION OF TRIPLE NEGNATIVE BREAST CANCERS

A series of CK5 positive TNBCs, characterized by GATA3, MG, GCDFP-15 and NY-BR-1 IHC in a previous analysis, was used for SOX10 IHC. The tumors were assessed in tumor microarrays (TMAs); each cancer was represented by dual 2-mm-diameter tissue cores.

Tumors were derived randomly from patients operated on and diagnosed with TNBC at the Bács-Kiskun County Teaching Hospital, Kecskemét between August 2005 and August 2015. The surgical specimens were fixed in 10% neutral buffered formalin for at least 24 hours. The TMAs had been constructed from archived paraffin-embedded blocks using a TMA builder device (Histopathology Ltd, Pécs, Hungary), with each TMA incorporating 20 tumor tissue cores. The TMA blocks were stored at room temperature, similarly to other paraffin blocks. Three-four-micrometer-thick sections were cut for SOX10 IHC using a monoclonal mouse antibody specific for an epitope mapping between amino acids 2-29 at the N terminus of SOX10 of human origin (Santa Cruz Biotechnology, Inc., Dallas, TX). The antibody was used with 1:500 dilution for 30 minutes incubation period and pretreatment was performed at pH 9.

TMAs were scanned and the proportion of positive cells was independently evaluated on the digital slides by the authors, and the few discrepant cases were reassessed by consensus on the original slides. Rate, localization and intensity were registered in all cases.

The data for GATA3, MG, GCDFP-15, NY-BR-1 were taken from the previous analysis [21].

The institutional ethical committee of the Bács-Kiskun County Teaching Hospital and of the University of Szeged was consulted and approved this non-interventional retrospective study. The institutional data safety manager also gave approval for this study not requiring patients' identity related data. The study was finally approved by the ethical committee of the Albert Szent-Györgyi Medical Center of the University of Szeged.

3.2. EVALUATION OF NOTTINGHAM PROGNOSTIC INDEX, PREDICT AND PROGNOSTILS IN TRIPLE NEGATIVE BREAST CANCER

Patients operated on for histologically verified triple negative, invasive breast carcinoma at the Department of Surgery, Bács-Kiskun County Teaching Hospital, Kecskemét between 2005-2016 were included in our consecutive and retrospective study. Follow-up data (OS and DFS) were collected from medical charts. For these outcomes, patients were followed from the date of surgical treatment until the time of recurrence or tumor-related death; those alive without recurrence and those dying from other causes were censored at the time of the last follow-up and death, respectively.

The following clinical and pathological variables were obtained for analysis: age, gender, localization, type of surgical and adjuvant treatments, histological type and grade of cancer, vascular invasion, tumor size, pT and pN categories, and stage. The NPI was calculated with the following equation: NPI = tumor size (cm) x 0.2 + nodal score (1 for pN0, 2 for pN1, 3 for pN2 or pN3) + number value from the histological grade [16]. The Nottingham Prognostic Groups were classified as VGPG: ≤2.4; GPG: 2.41-3.4; MPG1: 3.41-4.4; MPG2: 4.41-5.4; PGP: 5.41-6.4 and VPPG: ≥6.41 [27].

The predicted OS and DFS estimates of PrognosTILs were obtained from an online calculator (https://cesp-proxy2.vjf.inserm.fr/shiny/prognosTILs/). The estimations were based on the following parameters: age, number of positive lymph nodes, tumor size, histological grade, type of chemotherapy and proportion of stromal TILs. For the determination of the latter, the International TILs Working Group (later acting as International Immuno-Oncology Biomarker Working Group - IIOBWG) recommendations and rules were used [18; 35]. To

help in the estimation of stromal TILs, the online calibration system described by the IIOBWG [59] and found at http://virtuelle-mikroskopie.de/TIL-training was also used. After getting accustomed with the scoring system with a hundred cases evaluated in a study by the EWGBSP, the calibration (etalon) pictures for different rates of stromal TILs were screensaved and printed, and these printed pictures were compared with the microscopic images displayed on a monitor for at least three areas. The mean of these estimates was rounded to the closest 10% value also allowing for 5% and 1%, with the help of the calibration picture published in the first article of the IIOBWG for the latter value [60].

The anticipated OS evaluations of PREDICT were determined with the online calculator (https://breast.predict.nhs.uk/predict_v2.0.html), that required the following data: age, menopausal state, ER status, HER2 status, Ki67 status, invasive tumor size, grade of tumor, type of detection, number of positive lymph nodes and presence of micrometastasis in the lymph nodes [17, 29].

The Wilcoxon rank sum test was applied to analyze the correlation between recurrence or tumor-specific death and DFS or OS prediction rate of PrognosTILs and OS prediction rate of PREDICT. The OS and DFS data could not be correlated directly with the survival predictions of PrognosTILs and PREDICT, therefore the patients were classified in the following four categories: patients alive, patients who died of disease (DOD), patients alive with and without recurrence. The calculated OS and DFS survival predictions of PrognosTILs, the OS survival estimates of PREDICT and NPI scores were correlated with the 4 categories by receiver operating characteristic (ROC) curve analysis aiming to compare them and to find cut-off points. Patients DOD and patients alive categories were utilized in ROC curve analysis focusing on 5-year OS prediction of PrognosTILs, PREDICT and NPI scores, while patients with recurrence and patients without recurrence categories were used in a ROC curve of 5-year DFS estimates of PREDICT and NPI scores. The cut-off points identified by ROC curve analysis could show which OS and DFS rates of PrognosTILs, OS estimates of PREDICT and NPI scores are related to more frequent recurrence and tumor-specific death, respectively.

NPI was analyzed with the Kaplan-Meier method and the subgroups were compared with the log rank test. Cox-regression was utilized as univariate analysis. The parameters found significant in the univariate models were entered in a multivariable Cox proportional hazard model to identify factors of independent prognostic significance. PrognosTILs and PREDICT

survival estimates could not be included in the multivariate analysis due to statistical reasons. Statistical models were fitted using SPSS Statistics V.22.0 software (IBM, SSPS 22.0, Armonk, NY USA). All statistical tests were two-sided and $p<0.05$ values were considered statistically significant.

This retrospective study was approved by the institutional ethical committee of the Albert Szent-Györgyi Clinical Centre of the University of Szeged and the ethical committee of the Bács-Kiskun County Teaching Hospital also gave consent for the study.

3.3. EXAMINATION OF TUMOR REGRESSION GRADING SYSTEMS IN BREAST CANCER PATIENTS WHO RECEIVED NEOADJUVANT THERAPY

NAT receiving, consecutive patients operated on for histologically verified invasive breast carcinoma at the Department of Surgery, University of Szeged or Bács-Kiskun County Teaching Hospital, Kecskemét between 1999-2019 were included in our retrospective study. Follow-up data were collected from medical charts.

The following clinical and pathological variables were obtained for analysis: age and gender of the patient, laterality of the disease, type of neoadjuvant and surgical treatments, DFS and OS; histological type and grade of cancer in previous core biopsy and surgical specimen, completeness of the resection, vascular invasion, size - possibly in 2 dimensions, ypT, ypN, ystage, tumor cell density, tumor cellularity in biopsy and resection specimens, presence and proportion of DCIS, presence of metastasis and/or regression in lymph nodes, size of metastatic deposits and receptor status (ER, PR and HER2). Tumor cell density was defined as the proportion of viable tumor cells in the complete tumor bed.

Regression grades (NSABP-B18, TR/NR, Chevallier, Sataloff, Denkert-Sinn, Miller-Payne, and RCB) and morphological variables were correlated with DFS and OS data using Kaplan-Meier estimates. Patients were followed from the date of initiation of NAT until the time of recurrence or tumor-related death. Patients alive without recurrence and patients dying from other causes were censored at the time of the last follow-up and death, respectively. The log rank test was used for pairwise comparisons. All statistical tests were two-sided and $p<0.05$ values were considered statistically significant. The parameters found significant in the univariable models were entered in multivariable Cox proportional hazard model to identify factors of independent prognostic significance. Statistical models were fitted using SPSS Statistics V.22.0 software (IBM, SSPS 22.0, Armonk, NY USA).

This retrospective study was approved by the regional ethical committee of the Albert Szent-Györgyi Clinical Centre of the University of Szeged.

4. RESULTS

4.1. IMMUNOHISTOLOGICAL EVALUATION OF TRIPLE NEGATIVE BREAST CANCERS

Of the 120 TNBCs represented in the TMA cores, 119 could be assessed for SOX10 staining. SOX10 staining was generally a nuclear staining occurring in <1% to 100% of tumor cells (***Figure 1***), therefore two different cut-offs for positive staining were evaluated. With a positivity threshold of >1 % and ≥10%, 93 and 82 were defined as positive. Because the proportion of cases with 1-10% staining was relatively low, and the cases are less easy to pick up, the greater threshold was used for further analysis.

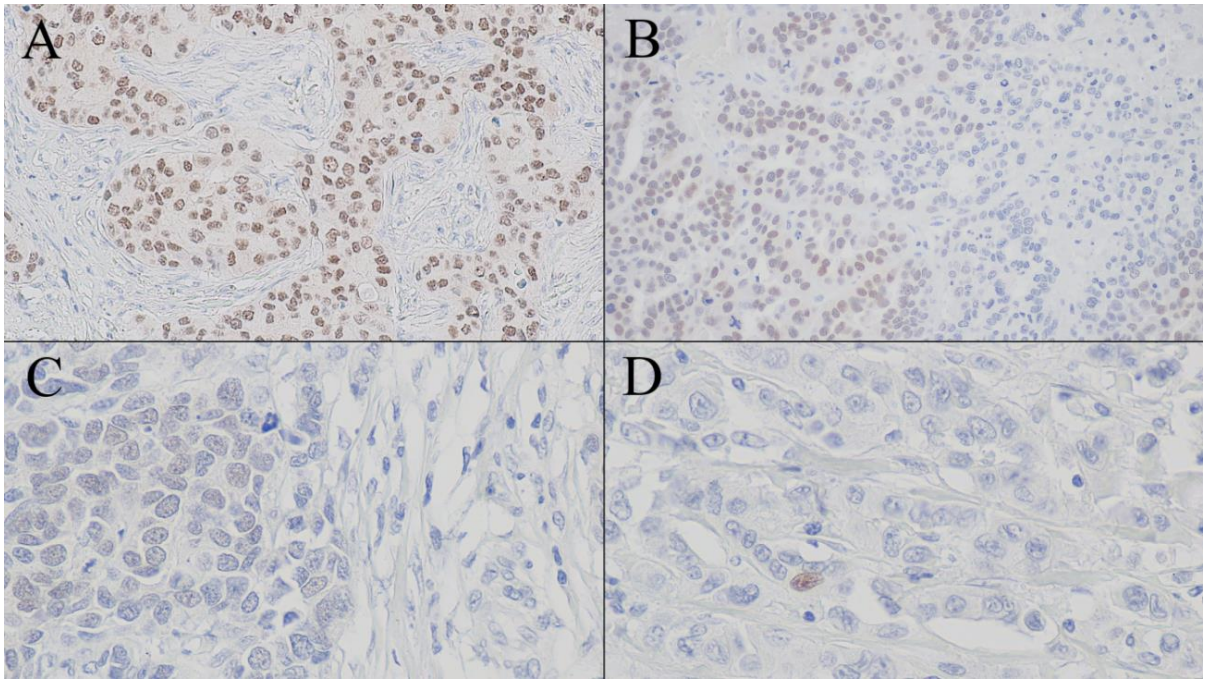
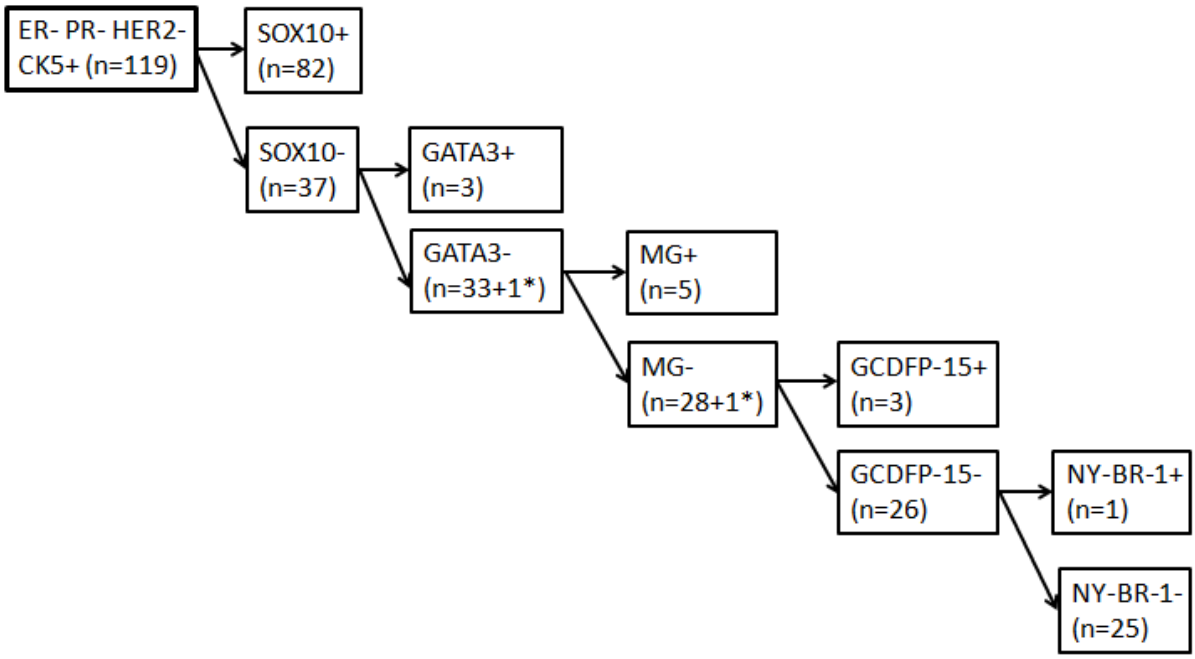


Figure 1 Examples of SOX10 immunostaining. A: diffuse strong nuclear staining (x20); B: partial staining in more than 10% of the cells with moderate intensity (x20); C: Over 10% of nuclei staining weakly (x40); D: Less than 1% of nuclei staining (x40)

Of the 94 GATA3 negative cases, 61 cases were positive with SOX10. Similar results were observed with the other breast markers. Seventy-four out of 104 MG negative cases, 76 out of 109 GCDFP-15 negative cases and 82 out of 117 NY-BR-1 negative cases stained positive

with SOX10. Our series included 78 cases that were negative with all the previously tested markers, 53 of which were identified as positive with SOX10, still leaving 25 (21%) as breast marker negative. The sequential hierarchical staining for breast markers from the most commonly positive to the least commonly positive is shown in ***Figure 2***. Mutual staining figures of pairs of breast markers are shown in ***Table 2***.



* 1 not assessable for GATA3 and MG

Figure 2 Hierarchical labeling of the tumors with SOX10, GATA3, MG, GCDFP-15 and NYBR1 as "breast specific" markers

Any staining	SOX10+	SOX10-	GATA3+	GATA3-	MG+	MG-	GCDFP-15+	GCDFP-15-
GATA3+	20	3						
GATA3-	61	33						
MG+	7	6	2	11				
MG-	74	30	21	83				
GCDFP-15+	5	4	2	6	2	6		
GCDFP-15-	76	33	21	88	11	98		
NY-BR-1+	0	2	0	2	0	1	1	1
NY-BR-1-	82	35	23	93	13	103	7	108

Table 2 Pairs of breast markers and their expressions in the tumors investigated

4. 2. EVALUATION OF NOTTINGHAM PROGNOSTIC INDEX, PREDICT AND PROGNOSTILS IN TRIPLE NEGATIVE BREAST CANCER

Altogether, 136 patients who underwent surgical resection were included in our study. Ten patients (7.4%) were censored due to non-tumor related death. Tumor-specific death was found in 23 cases (16.9%), while 103 patients (75.7%) were alive at the last follow-up, including 20 patients with recurrence (14.7%). The mean and median OS and DFS were 66.8 months and 57.5 months, 59.9 months and 41 months, respectively (range for OS: 7-170 months; range for DFS: 2-170 months). Recurrence was observed in 43 cases, including 11 cases (25.6%) with local or regional recurrence, 23 cases (53.5%) with distant metastasis and two cases with both local and distant types of recurrence. The median time to recurrence was 41 months (range: 2-170 months). Novel malignancies were found in 3 cases (7.0%; ovarian [n=1] and lung cancer [n=2]). The median follow-up was 56 months (range: 7-170 months).

The basic clinical and pathological characteristics are displayed in ***Table 3*** [48]. The mean and median age of the patients were 59.6 and 59 years, respectively (range: 32-91). In univariate Cox-regression, the type of surgery, the pT and pN categories, the stage of the disease and the type of adjuvant therapy were found to be significant variables.

The predictions from PrognosTILs and PREDICT and the NPI scores were established in 93, 126 and 125 cases, respectively. Concerning the 5-year OS and DFS predictions of PrognosTILs, the mean, the median and the range of estimates are presented in ***Table 4***. The comparison of predicted survival estimates and outcomes revealed that the predicted OS estimates of the patients DOD were significantly lower than those of patients who were alive ($p=0.015$); similarly, the predicted DFS estimates of patients with recurrence were significantly lower, than those of patients without recurrence ($p<0.001$). ***Table 5*** highlights the mean, the median and the range of the 5-year OS estimates of PREDICT. The statistical analysis strengthened, that the predicted OS estimates of patients DOD were significantly lower, than those of patients who were alive ($p=0.020$).

			pOS	pDFS
Age (years)	**n**	**%**	p=0.102	p=0.207
30-39	12	9.5		
40-49	15	11.9		
50-59	37	29.3		
60-69	35	27.8		
70-79	21	16.7		
80-91	6	4.8		
Laterality			p=0.645	p=0.958
right	58	46.0		
left	68	54.0		
Type of surgery			p=0.354	**p=0.017**
mastectomy	24	19.0		
breast conserving surgery	102	81.0		
Histology diagnosis			p=0.626	p=0.566
NST carcinoma	112	88.8		
medullary carcinoma	7	5.6		
other	7	5.6		
Grade			p=0.967	p=0.88
2	5	4.0		
3	121	96.0		
pT			p=0.222	**p=0.009**
pT1	67	53.1		
pT2	55	43.7		
pT3	1	0.8		
pT4	3	2.4		
pN			**p=0.006**	**p<0.001**
pN0	75	59.6		
pN1mi	8	6.3		
pN1	31	24.6		
pN2	9	7.1		
pN3	2	1.6		
pNx	1	0.8		
Vascular invasion			p=0.573	p=0.400
absent	100	79.4		
present	26	20.6		
Anatomic stage			**p=0.05**	**p<0.001**
I	47	37.3		
II	51	40.5		
III	27	21.4		
no data	1	0.8		
Adjuvant therapy			p=0.151	**p=0.003**
chemotherapy	10	7.9		
radiotherapy	15	11.9		
both	85	67.5		
neither	16	12.7		
Generation of chemotherapy			p=0.092	p=0.303
second generation	16	12.7		
third generation	73	57.9		
other (CMF)	6	4.8		
no data	31	24.6		

Table 3 Clinical and pathological characteristics of patients evaluated and the results of univariate Cox-regression [pT, pN categories defined by AJCC [61], CMF: cyclophosphamide, methotrexate and 5-fluorouracil; second generation systemic treatment refers to anthracycline based regimens without taxanes; third generation refers to taxane containing regimens, NST: No special type carcinoma].

PrognosTILs predictions			**average**		**median**		**range**		Wilcoxon-test
	n	**%**	**OS**	**DFS**	**OS**	**DFS**	**OS**	**DFS**	**pOS=0.015**
patients deceased due to tumor	14	15.0	80.1	80.6	80	76	74-92%	69-92%	
patients alive	79	85.0	85	82	85	83	49-95%	44-95%	
patients with recurrence	27	29.0	80.3	77.3	80	77	49-93%	44-93%	**pDFS<0.001**
patients alive with recurrence	13	14.0	80.6	77.7	83	80	49-93%	44-93%	
patients alive without recurrence	66	71.0	85.8	84	86	83	71-95%	67-95%	
all (where PrognosTILs was evaluated)	93	100.0	84.2	81.7	84	82	49-95%	44-95%	

Table 4 The 5-year OS and DFS predictions of PrognosTILs according to outcome. Significant differences were detected between OS predictions of patients who died of disease and patients alive, and DFS predictions of patients with and without recurrence.

PREDICT estimates						**Wilcoxon-test**
	n	**%**	**mean**	**median**	**range**	**pOS=0.020**
patients deceased due to tumor	23	18.3	62.9	65.5	9.2-85.1%	
patients alive	103	81.7	71.8	78.1	7.1-86.5%	
all (where PREDICT was evaluated)	126	100	70.1	75.3	7.1-86.5%	

Table 5 The basic characteristics of 5-year OS predictions of PREDICT according to outcome. The survival estimates of patients dying of tumor progression were lower than those of patients who were alive at last follow-up.

The NPI-based GPG included only 3 cases, therefore this group was excluded from further evaluation. ***Figure 3*** demonstrates the results of Kaplan-Meier analysis of the NPI subgroups. Significant differences were detected between OS and DFS estimations of different prognostic groups, namely the OS estimates of MPG1 vs. PPG (p=0.017), MPG1 vs. VPPG (p=0.049), MPG2 vs. PPG (p=0.026); and the DFS estimates of PPG vs. MPG1 (p=0.002), PPG vs. MPG2 (p=0.035), PPG vs. VPPG (p=0.013), VPPG vs. MPG1 (p<0.001) and VPPG vs. MPG2. (p=0.001). In the univariate Cox-regression, NPI was found to be a significant prognostic variable (pOS=0.022; HR: 1.71, 95% CI: 1.08-2.72; pDFS<0.001; HR: 2.02, 95% CI: 1.43-2.86).

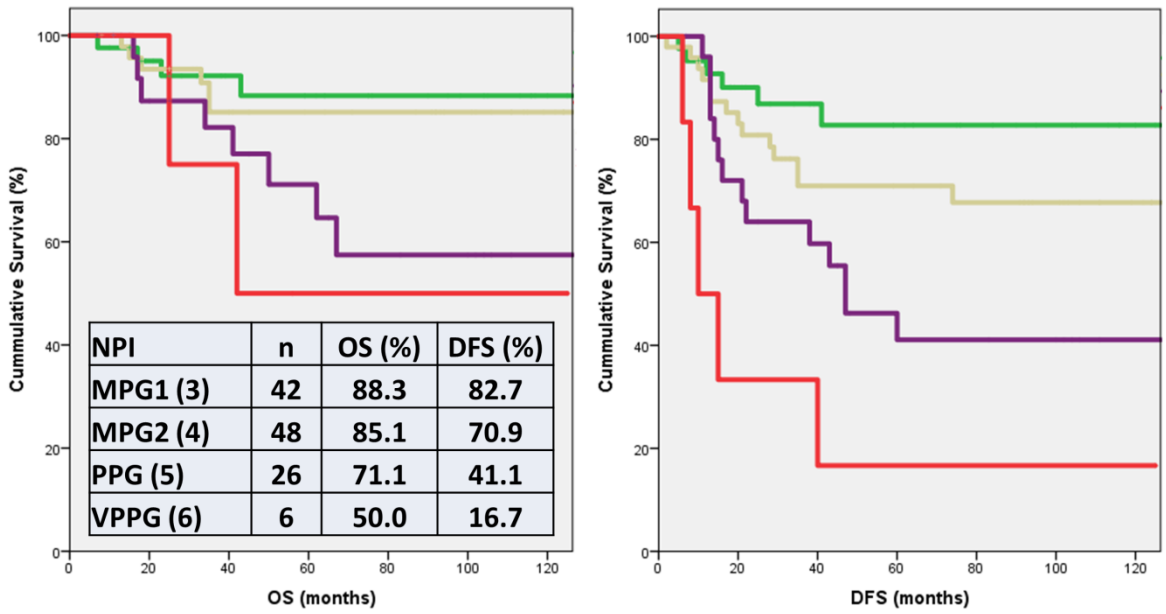


Figure 3 Kaplan-Meier analysis of NPI. According to the log rank test significant differences were observed between the OS estimates of MPG1 vs. PPG (p=0.017), MPG1 vs. VPPG (p=0.049) andMPG2 vs. PPG (p=0.026); and the DFS estimates of PPG vs. MPG1 (p=0.002), PPG vs. MPG2 (p=0.035), PPG vs. VPPG (p=0.013), VPPG vs. MPG1 (p<0.001) and VPPG vs. MPG2 (p=0.001)

Figure 4 displays the results of ROC curve analysis focusing on 5-year OS estimates of PrognosTILs, PREDICT and NPI scores. The area under the curve (AUC) of PrognosTILs, PREDICT and NPI were 0.759, 0.762 and 0.792, respectively. ***Figure 5*** demonstrates the ROC curve analysis of 5-year DFS estimates of PrognosTILs and NPI scores. The AUC values of PrognosTILs and NPI were 0.713 and 0.781, respectively. The findings of ROC curve analyses drew attention to the similarities of these predictive systems concerning sensitivity and specificity and to the fact that they are not ideal for defining cut-off values.

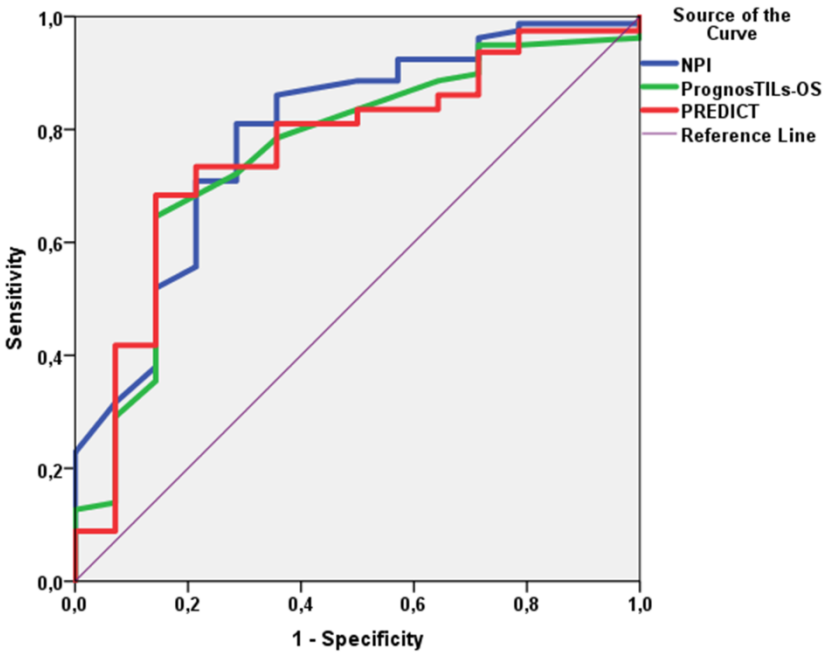


Figure 4 ROC curve analysis of 5-year OS predictions of PrognosTILs, PREDICT and NPI scores (area under the curve values for PrognosTILs, PREDICT and NPI were 0.759, 0.762 and 0.792, respectively)

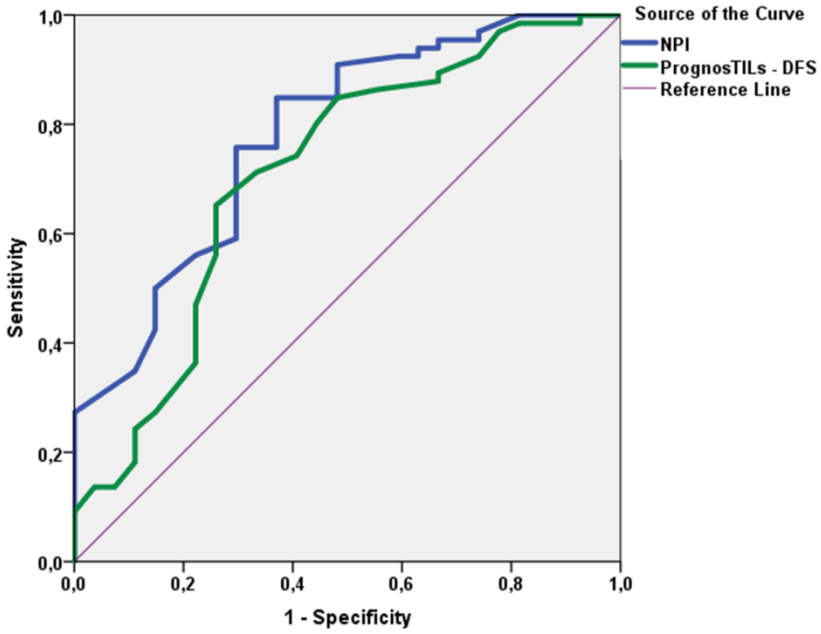


Figure 5 ROC curve analysis of 5-year DFS predictions of PrognosTILs and NPI scores (area under the curve values for PrognosTILs and NPI were 0.713 and 0.781, respectively)

The multivariate Cox proportional hazard model revealed that among the variables found significant in univariate models (type of surgery, pT, pN, stage, adjuvant therapy and NPI), only NPI was an independent prognostic marker for TNBC (pOS=0.006; HR: 1.66, 95% CI: 1.16-2.37; pDFS<0.001; HR: 1.92, 95% CI: 1.46-2.53).

4.3. EXAMINATION OF TUMOR REGRESSION GRADING SYSTEMS IN BREAST CANCER PATIENTS WHO RECEIVED NEOADJUVANT THERAPY

Data of 746 patients who underwent NAT and surgical resection were collected. The median patient age was 55 years (range: 26-91) and 2 of them were males. ***Table 6*** summarizes the oncological and surgical treatments of all patients in the examined population. The majority of patients received primary chemotherapies, whereas 16.4% got primary endocrine therapy. Regarding primary systemic chemotherapy, the majority of patients were given third generation (taxane containing) regimens. Eleven percent of the patients had been given second generation (anthracycline based) chemotherapeutics. Patients who received a combination of platinum compounds with cyclophosphamide fell into the “others” category. Anti-HER2 treatment was essentially given in combination with chemotherapy for HER2-positive tumors. Concerning primary endocrine therapy, the most frequent agents used were aromatase inhibitors and the average hormonal therapy treatment period was 1 year.

Neoadjuvant therapy		
Primary hormonal therapy (n=123=100%)	**n**	**%**
Tamoxifen	4	3.25
Aromatase inhibitor	102	82.93
Tamoxifen and LHRH-analogue	3	2.44
Aromatase inhibitor and LHRH-analogue	14	11.38
Primary systemic therapy (n=623=100%)	**n**	**%**
Second generation chemotherapy	70	11.24
Third generation chemotherapy	550	88.28
Others	3	0.48
Anti-HER2 (in combination therapy)	91	14.60
Surgical treatment (n=746=100%)	**n**	**%**
Breast conserving excision	249	33.38
Mastectomy	497	66.62
Re-excision	17	2.28
SNB	72	9,65
ALND	593	79,49
SNB+ALND	60	8.04

Table 6 Types of NAT and surgical treatment in the examined population (ALND: axillary lymph node dissection, SNB: sentinel node biopsy)

Two thirds of the patients underwent mastectomy. Re-excisions were rarely performed and were done because of positive or close resection margins. Regional lymph nodes were examined in almost all cases, most commonly by means of ALND. As ***Table 7*** demonstrates, with histological examination, 87.8% of patients had invasive NST carcinoma in surgical specimens. Invasive tubular, mucinous, medullary and metaplastic BCs were grouped into the others category. The presence of residual DCIS was described in 212 cases. One fifth of the patients achieved pCR. The most frequent pathological tumor category was ypT2 (20.2%), while 38.9% of the patients fell in ypN0 category. Most cases expressed ER and PR, while HER2 positivity was observed in 126 cases (17%). Median patient follow-up was 53.8 months (range: 4-238 months; average: 65.1 months). Relapses occurred in 34.85% of all cases during the follow-up period and tumor specific death was described in 122 (16.3%) cases.

According to the original histopathology reports and previous databases, the numbers of patients evaluated with the different regression grading systems are as follows: NSABP-18 grade: 746, Chevallier-grade: 717, Sataloff (T) grade: 494, Miller-Payne grade: 386, TR grade: 392, Denkert-Sinn grade: 348, RDBN grade: 405 and RCB: 212. ***Figure 6*** and ***Supplementary Figure 1-8*** show the DFS and OS estimates of the different grading systems, respectively. The DFS and OS estimates of pCR (ypT0) and residual in situ carcinoma (ypTis) together were significantly different from the survivals of tumors without regression and moderate regression categories in all grading systems ($p<0.001$). There was no significant DFS and OS difference observed between the ypT0 and ypTis categories. Survival values associated with different partial or no response categories showed no significant differences between each other, with the exceptions of DFS for the RCB-I vs III and II vs III categories.

As all regression grading systems showed a significant effect on survival in the univariable models, they were all entered in the multivariable Cox-regression analysis. According to our results the RCB ($p=0.019$) proved to be an independent prognostic marker for DFS, whereas the ystage ($p=0.011$) and lymph node status ($p=0.045$) showed similar results for OS.

Histological subtype (core)	**n**	**%**
NST	655	87.80
ILC	55	7.37
others	36	4.83
grade	**n**	**%**
1	35	4.69
2	246	32.98
3	420	56.30
No data	45	6.03
DCIS (present)	212	28.41
R (R1/R0)	130/616	17.42
L (L1/L0)	151/560	21.23
Pn (Pn1/Pn0)	10/324	2.99
Hormonal state	**n**	**%**
HR +, HER2 -	439	58.85
HER2 +, HR +/-	126	16.89
Triple negative	181	24.26
ypT	**n**	**%**
ypT0	106	14.21
ypTis	28	3.75
ypT1a	48	6.43
ypT1b	25	3.35
ypT1c	110	14.75
ypT2	151	20.24
ypT3	55	7.37
ypT4	29	3.90
No data	194	26.00
ypN	**n**	**%**
ypN0	290	38.87
ypN1	227	30.43
ypN2	127	17,02
ypN3	61	8.18
No data	41	5.50
ystage	**n**	**%**
0	9	1.21
I	75	10.05
II	209	28.02
III	207	27.75
IV	6	0.80
No data	240	32.17

Table 7 Morphological features of BC in the examined population (R: Resection, L: (Lympho)vascular invasion, Pn: Perineural invasion; ypT and ypN categories are defined by AJCC. Not all evaluated features were available for all cases, hence the differences in the sums of some rows

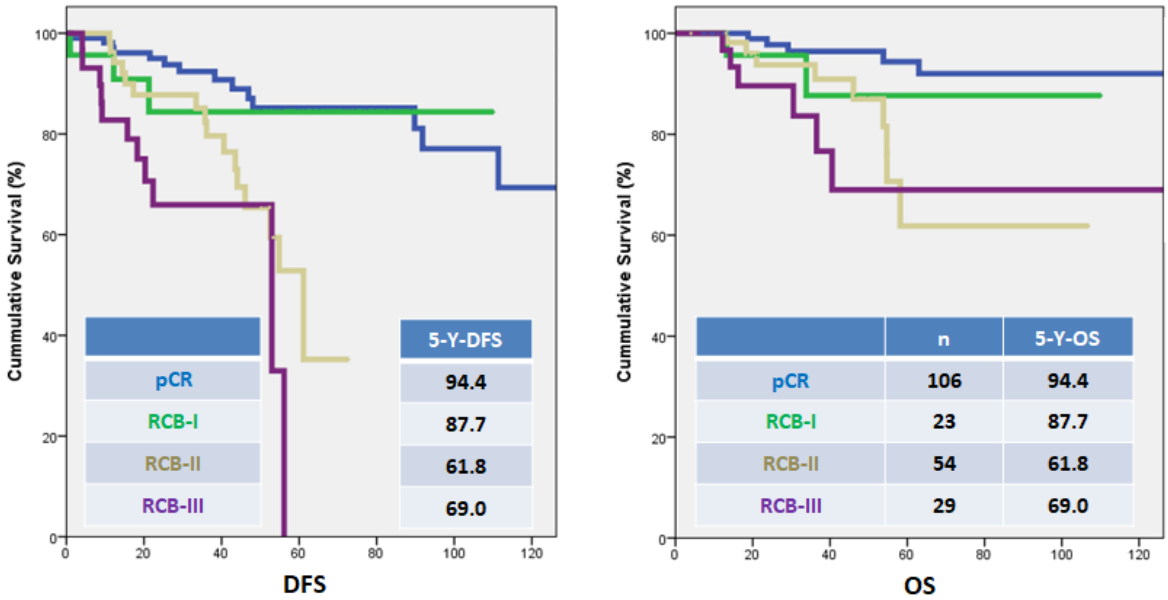


Figure 6 Kaplan-Meier evaluation of the RCB grading system for DFS and OS. Significant differences were found between DFS estimates of pCR vs. RCB-II ($p<0.001$), pCR vs. RCB-III ($p<0.001$), RCB-I vs. RCB-III ($p=0.035$), RCB-II vs. RCB-III ($p=0.05$). Regarding OS, significant differences were observed between estimates of pCR vs. RCB-II ($p=0.005$) and pCR vs. RCB-III ($p<0.001$), respectively

5. DISCUSSION

5.1. IMMUNOHISTOLOGICAL EVALUATION OF TRIPLE NEGATIVE BREAST CANCERS

The expected lifetime risk of developing cancer in women is around 1 out of 3, making the possibility of developing multiple primary cancers a real possibility and giving emphasis to the distinction between metastasis of a known primary tumor and an independent second primary cancer [62]. TNBCs represent a minority of BCs, but as triple negative NST carcinomas are often of poor prognosis, metastases may occur at a relatively higher rate. Owing to their phenotype overlapping with myoepithelial differentiation, TNBCs may show variable histologies, including spindle cells (sarcomatoid appearance), squamous metaplasia and other rarer (e.g. sebaceous, chondroid or osseous) metaplasias, making their recognition as metastatic breast carcinomas at a metastatic site more difficult. This is why IHC markers supporting the breast origin of TNBCs is important.

In our previous study, by using GATA3, MG, GCDFP-15 and NY-BR-1 we came out with an algorithmic value of these “breast markers” in CK5 expressing TNBCs believed to represent basal-like breast carcinomas, where GATA3 was the mostly expressed marker. Our results suggested that only about half of these cancers could be classified as of mammary origin on the basis of these four markers [21].

In 2013 Cimino-Mathews et al. published the first study investigating the utility of SOX10 IHC labeling in TNBC and metaplastic breast carcinoma cases. TMA blocks from 168 primary BCs were investigated; 40% showed positivity with SOX10, these were primarily basal-like unclassified TNBCs and metaplastic carcinomas. Sixty-six percent of TNBCs but only 5% of luminal and HER2 positive cases were positive with SOX10 [63].

Nelson and coauthors reported about promising SOX10 results. TMA blocks were made from 26 patients’ samples. Thirty-eight percent stained positively with SOX10, while no cases were positive with ER and HER2. A retrospective study was also performed in metastatic carcinomas of possible breast origin, and 57% of cases were labeled with SOX10. All SOX10 positive cases were confirmed to be negative with ER. Nelson and coauthors recommended the use of SOX10 in metastatic cases from unknown primary tumors to prove their melanoma or TNBC origin [64].

Since 2017, several research groups started to investigate SOX10. Al-Zahrani and coauthors compared its application with androgen receptor (AR). AR staining was positive in 95% of cases, mostly along with HER2 or ER and PR positivity, but no special BC subtype could be identified on the basis of AR staining alone. SOX10 proved to be positive in one third of cases that were triple-negative [65].

Tozbikian examined the IHC profile of 57 TNBC cases. Eighty-two percent showed positivity with GATA3, 58% with SOX10 and 25% with AR. Ninety-five percent proved to be positive with either GATA3 or SOX10, and 46% showed dual positivity; 80% of GATA3 negative cases were SOX10 positive. Their study concluded that while GATA3 was a more sensitive marker for TNBC cases, it is useful to add SOX10 to the IHC panel [66].

Harbhajanka et al. investigated 48 TNBC cases in TMAs, and SOX10 showed positivity in 37.5% of the cases. A negative correlation with AR positive molecular subtypes of TNBCs was observed, and a positive correlation was proved with WT1 (Wilms’ tumor-1). However,

no correlation was seen with the breast markers GATA3, MG, GCDFP-15 and basal-like subtype markers EGFR (epidermal growth factor receptor) and CK5/6 [67].

The most comprehensive study is the one by Laurent et al., who reported their results about SOX10, GATA3, GCDFP-15, AR and MG in 207 metastatic TNBC cases and compared them with 152 primary lung adenocarcinomas. SOX10 showed the best sensitivity (62.3%) and specificity (100%) in comparison with GATA3 (30.4% sensitivity and 98.7% specificity), GCDFP-15 (20.8% sensitivity and 98% specificity), MG (38.2% sensitivity and 81.6% specificity) and AR (30% sensitivity and 86% specificity). 6.3% of TNBC cases had no reaction with any of the above mentioned markers [20].

In 2020, Qazi et al. suggested the combined use of SOX10 and GATA3 in cases of low ER expression and reduced GATA3 intensity. The study included 246 patients' samples as TMA blocks containing both ER positive and negative cases. Overall, 93%, and of TNBC cases, 63% showed positivity with GATA3; in parallel 15% of all and 74% of TNBCs were positive with SOX10. Less than 1% had no reaction with either antibody and only 3% of ER positive cases were SOX10 positive [68].

In our current study, by adding SOX10 IHC to our previous "breast marker" panel, there was an improvement in identifying CK5 expressing TNBCs as of mammary origin, and the algorithm could be changed substantially (***Figure 2***). Using the 10% cut-off for SOX10 positivity, 68.9% (95% CI: 59.8-77.1) of the cases were found to be SOX10 positive and 9.3% of cases were positive only with the previously used markers, which proves the added value of GATA3, MG, GCDFP-15 and NYBR1. Twenty-one percent of the cases remained negative with all "breast markers", suggesting that negativity of all the examined markers doesn't securely exclude mammary origin. These results are in keeping with former reports, and suggest that SOX10 is probably the best "breast marker" of TNBCs, followed by GATA3 [20; 68]. Minor discrepancies in the proportion of cases staining and the value of MG versus GCDPF15 may stem from our cohort being restricted to CK5 expressing (and most likely basal-like) TNBCs, whereas others also included apocrine TNBCs which are expected to be positive with GCDFP-15 (also an apocrine marker) more commonly. In 41% of the cases in our study, the staining observed was focal, in these, the application of the TMA technique can be a limitation.

5.2. EVALUATION OF PROGNOSIS IN TRIPLE NEGATIVE BREAST CANCER CASES

TNBCs are generally considered as the worst IHC based molecular subtype of BC, owing to their poor prognosis and the limited therapeutic success associated with them. Despite the overall bad prognosis of TNBC, there are some tumors that by definition fall into this category, but belong to a better prognostic group. These include rare tumors like tall cell carcinoma with reversed polarity, secretory carcinoma, non-high grade, i.e. classical adenoid cystic carcinoma [69-71]. Even without these low grade special type carcinomas, the prognosis of TNBC is heterogeneous and depends on a number of prognostic factors.

The presence of distant metastasis, nodal status, tumor size and histological grade are established prognostic factors of BCs, and have their role in predicting the outcome of TNBCs as well. More recently the proportion of stromal TILs has also been recognized as an independent prognosticator of TNBCs, and the prognostic value of TILs was also found in a more recent meta-analysis [18; 72]. When prognostic factors show divergent features, i.e. clinicians are faced with a combination of factors toward good and bad prognosis, predictive models based on multivariable analysis of multiple prognostic factors are much more valuable than isolated factors. The NPI is one such factor and was derived from the multivariable analysis of 387 patients with different molecular subtypes of BC and was later validated in a series of 320 independent consecutive cases [73]. Several external studies have demonstrated its ability to give a prognostic classification of BCs [74-76]. Although the improvements in treatment have significantly altered the outcomes of BC, and this improvement is also reflected in the NPI prognostic group-specific survivals, the prognostic separation of BCs on the basis of the NPI was still found to be valid [77]. The PREDICT tool was derived from a much greater population and was also independently validated in a number of reports [28; 78]. PrognosTILs is a novel multivariable prognosticator model and calculator derived from the pooled analysis of 2148 individual patients' data from 9 studies on TNBCs proving the prognostic value of stromal TILs in the adjuvant setting [18]. This distinguishes it from NPI and PREDICT which were built on data from ER-positive and ER-negative tumors together, and theoretically could mean that it is better fitted to predict the prognosis of TNBCs.

The significance of the NPI in TNBC was first examined by Albergaria and coauthors in 2011 with reassuring results. NPI results correlated well with real survival data due to the facts that TNBCs are frequently high grade and large tumors [79]. PREDICT, to our knowledge has not

yet been evaluated for TNBCs alone, whereas PrognosTILs is relatively recent for larger validation on comparison studies.

In univariate Cox-analysis, type of surgery, pT, pN, stage, NPI and adjuvant therapy were found significant prognostic variables. We also found that lower 5-year OS and DFS predictions of PognosTILs are related with more frequent tumor specific death and recurrence (pOS=0.015, pDFS<0.001), while the lower 5-year OS predictions of PREDICT are associated with higher rate of tumor specific death (p=0.02). Concerning the NPI, we demonstrated that there are significant differences among OS and DFS estimates of certain prognostic groups (***Figure 3***). PrognosTILs and PREDICT derived estimates of survival, as scale variables could not enter the Kaplan-Meier analysis. The direct comparison of the multivariable prognosticators was performed with ROC curve analysis. Regarding the OS follow-up data, PrognosTILs, PREDICT and NPI, while regarding the DFS follow-up data, PrognosTILs and NPI were compared. All three predictors of outcome reflect fair performance with areas under the ROC curves falling between 0.7 and 0.8. The sensitivity and specificity of these predicting systems are rather similar, although there seems to be a tendency for NPI values to better predict outcome on the basis of the somewhat greater AUC values. In keeping with the results of Albergaria et al., the multivariate Cox-regression strengthened that NPI is an independent predictor of OS and DFS in TNBCs (pOS=0.006; HR: 1.66, 95% CI: 1.16-2.37; pDFS<0.001; HR: 1.92, 95% CI: 1.46-2.53) [79]. Considering that the ROC curve analysis yielded similar results for the three multivariable prognosticators studied, it can be inferred that any of these is suitable to predict the outcome of TNBCs, and none of these is inferior to the others.

The results also show that TNBCs are prognostically heterogeneous. No case was classified as of very good prognosis on the basis of the NPI, and only 3 cases fell into the GPG. This is due to the fact that only 5 tumors were of histologic grade 2, whereas the remaining were high grade, and with this combination, their NPI value was immediately >4.

The lack of all prognostic markers for all cases and the fact that this was a single institution study of retrospective nature with limited number of cases are possible limitations of this work. A further limitation may be that values predicted by PrognosTILs and PREDICT, due to statistical reasons, could not be entered into the multivariate Cox-regression analysis, and could not be compared to NPI in this setting; but this drawback was compensated by the ROC curve analysis of the three prognosticators. Our study has strengths, as well. To our

knowledge, this study is the first to evaluate the value of PREDICT in TNBCs, and these multivariable prognostic tools have never been compared in a single study. Another advantage of the study design was the uniform evaluation of TILs with rigorous adherence to internationally agreed guidelines.

5.3. EXAMINATION OF TUMOR REGRESSION GRADING SYSTEMS IN BREAST CANCER PATIENTS WHO RECEIVED NEOADJUVANT THERAPY

Due to the increasing use of NAT in patients having LABC, an increasing number of articles about its effectiveness have been published [43]. Although imaging techniques serve as great options to monitor regression during and after NAT, histopathological review remains the gold standard in the evaluation procedure [80]. Although several national guidelines aiming at the standardization of specimen cut-up and reporting have been introduced, for example in Australia, Belgium, Germany, the UK, Netherlands, the USA and Hungary, there is no international agreement in the interpretation of tumor regression, in the definition of pCR, and in the measurement of tumor size in cases where fibrosis develops as a result of NAT or multifocality is present [45; 81-86].

Several regression grading systems have been introduced which are based on prognostic markers such as tumor size (in one or more dimensions), change in cellularity, presence of DCIS, presence of regression or metastasis in lymph nodes and the size of lymph node metastasis [51-57]. The definition of pCR and the complete lack of regression -as the extreme ends of the regression spectrum- are common features of these systems which also define one or more subgroups for partial regression categories. Despite of the relative abundance of regression grading systems, there is a lack of international consensus on their application. All grading systems attempt to quantify the degree of regression or the amount of residual tumor, and there is agreement that a quantitative characterization of tumor regression is necessary for the evaluation of the effectiveness of NAT, and may have further role in therapeutic decisions (e.g. alternative treatments if no regression is present).

Although the presence of residual DCIS has been reported to convey a worse prognosis than complete absence of in situ and invasive carcinoma, there was no significant difference between OS and DFS estimates of ypT0ypN0 and ypTisypN0. Our results are therefore supporting the more permissive definition of pCR (including ypTis) defined by the United States' FDA and endorsed by the AJCC [48; 49] and the European Guidelines [57]. Our findings regarding the prognostic impact of pCR are in keeping with those of others, since

patients with pCR had a favorable prognosis (both in DFS and OS) compared to patients having partial regression. Concerning the subcategories of partial regression, we observed significant differences only between DFS estimates of certain RCB classes, namely between RCB-I vs. RCB-III and RCB-II vs. RCB-III classes. No other regression classification system showed subgroups of partial response with significant differences between each other.

RCB was developed by Symmans and coworkers in 2007. In their study, the prognostic role of morphological variables was evaluated by Cox-regression, and from the variables found statistically significant, a complex equation was produced to determine the RCB index score. The RCB index score was correlated with survival data and cut-off scores were assigned to identify the RCB classes. In concordance with the original results by Symmans et al., there were no significant differences in DFS and OS estimates between RCB-0 (pCR) and RCB-I (nearly pCR) classes. Furthermore, the multivariable Cox-regression models for DFS suggest that the RCB system is the only significant prognosticator among regression grades (p=0.019) [56].

In a subsequent publication, Symmans and co-authors have demonstrated that the RCB is a prognostic marker independent from the type of primary chemotherapeutic regime and significant differences have been described between RCB classes among HR positive (ER+ and/or PR+, HER2-), HER2 positive (HR positive or negative) and triple negative (ER-, PR, HER2-) BC cases [87]. Our results support these conclusions, and moreover, by adding primary endocrine therapy to our calculations, RCB remained an independent prognostic marker.

Considering literature data and our results, RCB is highly recommended to be included in routine histopathological reports of BCs treated with NAT. Although most elements of RCB are routinely part of histopathological reports, the characterization of some others, namely the second largest dimension of tumor size, the cellularity and the proportion of DCIS, require experience in practice. The standardization of reporting these markers are supported by the concise guidance at the RCB calculator website [87].

Corben and co-authors emphasized the role of the presence and size of lymph node metastasis. Those grading systems that include lymph node status (RCB, Sataloff, TR-NR, RDBN) show better correlation with long term survival than those including only invasive tumor size and cellularity [40]. In keeping with Corben's results, we found the ypN category as a significant prognostic marker according to OS estimates. The presence of nodal

metastasis was associated with poor prognosis regardless of the presence or absence of nodal regression. Corben and co-workers suggested the RDBN grade to be the most optimal regression grading system among the 5 investigated [40]. However, we found no significant differences in DFS or OS between the RDBN groups with Cox-regression. This contrast may be due to different factors, like the differences in patients and in cohort sizes (62 vs 746) and the inclusion of primary endocrine therapy in the present analysis.

Concerning the limitations of our study, it has to be mentioned that not all grading systems were assessed in all cases. Several patients had gone through lymphadenectomy prior to NAT and this could influence the prognostic value of a given grading system. Furthermore, the institution where the core needle biopsy was taken differed from the place of surgery in many cases, therefore the comparison of these samples was not always possible. On the other hand, the strengths of our evaluation include a large cohort of patients having primary endocrine treatment or chemotherapy with relatively long follow-up data. Our multicenter study was based on two Hungarian departments with similar cut-up and reporting protocol, following the recommendations of 3rd Hungarian Consensus Conference on Breast Cancer. Although not all grading systems were evaluated in all cases, even the smallest group included more than 200 patients, and this proved sufficient for statistical analysis.

6. CONCLUSIONS

1. Based on our data, SOX10 proved to be the most sensitive breast marker in CK5 expressing TNBCs, likely to correspond to basal-like TNBCs on the basis of the IHC based surrogate classification. With the additive value of GATA3, MG, GCDFP-15 and NY-BR-1, more than three quarters of the investigated 119 cases could be identified as BCs. With the joint use of SOX10, GATA3, MG and GCDFP-15 78.2% (95% CI: 69.7-82.2) sensitivity was achieved. We propose SOX10 as first line approach to identify TNBCs, with the addition of GATA3, MG and GCDFP-15 for the negative cases; NY-BR-1 has little added value in this context.

2. Our further findings reflect the diverse nature of TNBC and highlight the difficulties of predicting the outcome of this disease. Although the NPI seemed to give somewhat higher AUC values in the direct comparisons with PREDICT and PrognosTILs, none of the multivariable prognosticators is inferior to the others according to our data.

3. In our retrospective study involving the grading of response to NAT in 746 patients, we have evaluated and compared the impact of different regression grading systems on DFS and OS. According to our results, the RCB was the best prognostic factor, therefore we would encourage its utilization in routine histopathological reports.

7. ACKNOWLEDGEMENT

I am very grateful to my supervisors Professor Gábor Cserni from the Department of Pathology, University of Szeged and the Department of Pathology, Bács-Kiskun County Teaching Hospital and Tamás Zombori from the Department of Pathology, University of Szeged, for their constant help, guidance and patience during my work.

I am grateful for all the coauthors I had the privilege to work with, including András Vörös, Arbel Golan, Tibor Nyári, Zsuzsanna Kahán and Renáta Kószó.

I would like to thank Professor Béla Iványi and László Tiszlavicz, Heads of Department during the time of my research for their support.

I also thank the previously unlisted members of the multidisciplinary breast team of the University of Szeged, including László Kaizer, Sándor Hamar, Orsolya Oláh-Németh, Professor György Lázár, Attila Paszt, Zsolt Simonka, Zoltán Horváth, Kalatin Ormándi, Alíz Nikolényi, Ágnes Dobi, Dóra Sántha, who helped me with my studies in general breast pathology.

Special thanks to all my coworkers at the Department of Pathology, University of Szeged. I am grateful for the doctors for helping learn my field and for all our technicians, without whose high quality work we would not be able to work or do research. Similarly, I am also indebted to all clinicians treating the patients analyzed and recording their data making the analyses possible. Naturally, the patients themselves merit recognition, for contributing to the analyses of their disease and the potential benefit of the results to other patients.

I would like to thank Bence Kővári who always challenges me in every field of pathology.

I am thankful for my TDK students during the time, including Fanni Hegedűs, Veronika Szelestei and Arbel Golan for their curiosity, interest and help in our studies.

Special thanks to Mihály Dezső for his help in high-quality pictures and figures.

I am very grateful for my family and friends, without whom, this work could not have been completed.

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9. APPENDIX

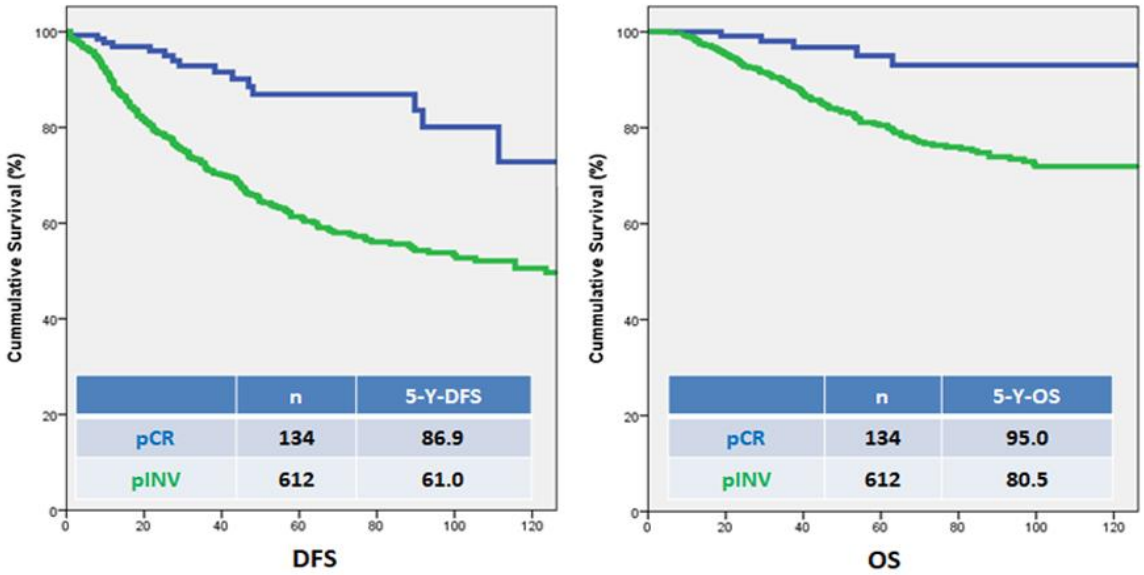
9.1. MAJOR NEW FINDINGS

Based on our data, SOX10 proved to be the most sensitive breast marker in CK5 expressing TNBCs, likely to correspond to basal-like TNBCs on the basis of the IHC based surrogate classification. With the additive value of GATA3, MG, GCDFP-15 and NY-BR-1, more than three quarters of the investigated 119 cases could be identified as BCs. With the joint use of SOX10, GATA3, MG and GCDFP-15, 78.2% (95% CI: 69.7-82.2) sensitivity was achieved. We propose SOX10 as first line approach to identify TNBCs, with the addition of GATA3, MG and GCDFP-15 for the negative cases; NY-BR-1 has little added value in this context.

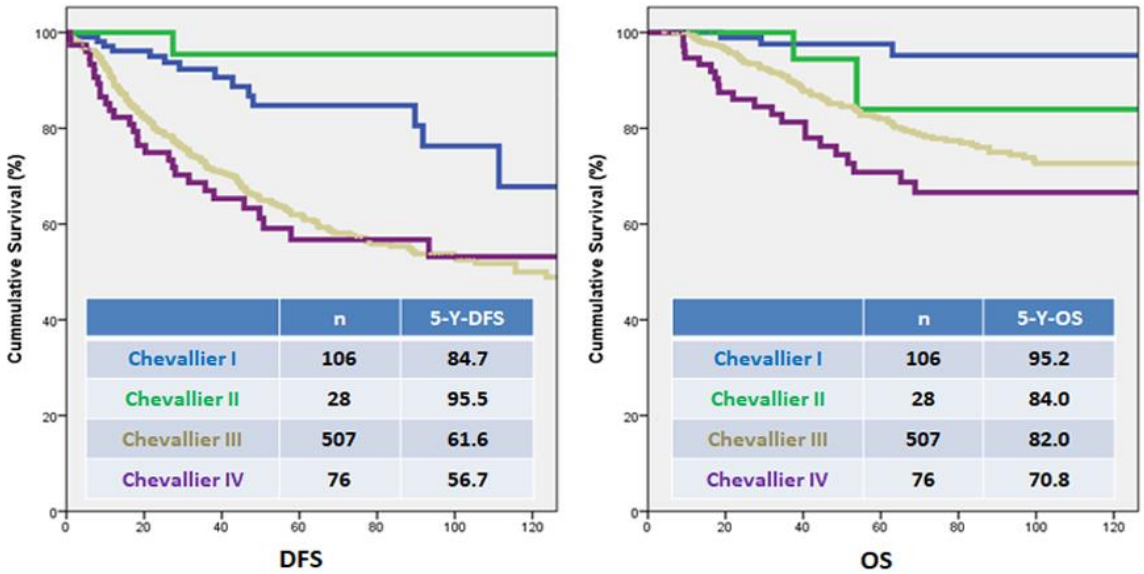
Our further findings reflect the diverse nature of TNBC and highlight the difficulties of predicting the outcome of this disease. Although the NPI seemed to give somewhat higher AUC values in the direct comparisons with PREDICT and PrognosTILs, none of the multivariable prognosticators is inferior to the others according to our data.

In our retrospective study involving the grading of response to NAT in 746 patients, we have evaluated and compared the impact of different regression grading systems on DFS and OS. According to our results, the RCB was the best prognostic factor, therefore we would encourage its utilization in routine histopathological practice.

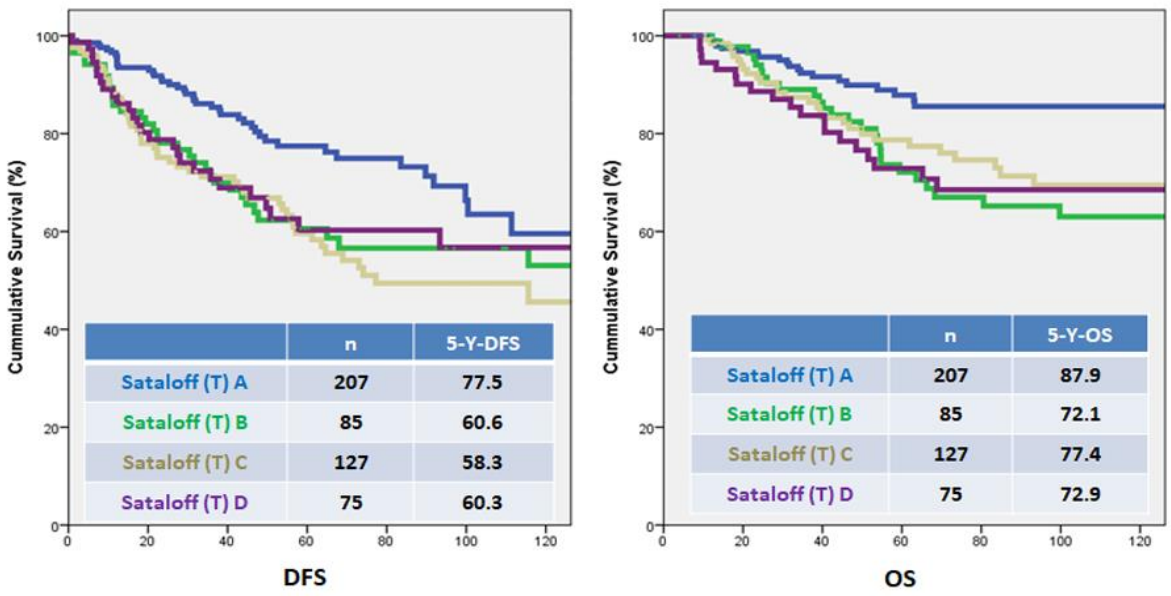
9.2. SUPPLEMENTARY FIGURES



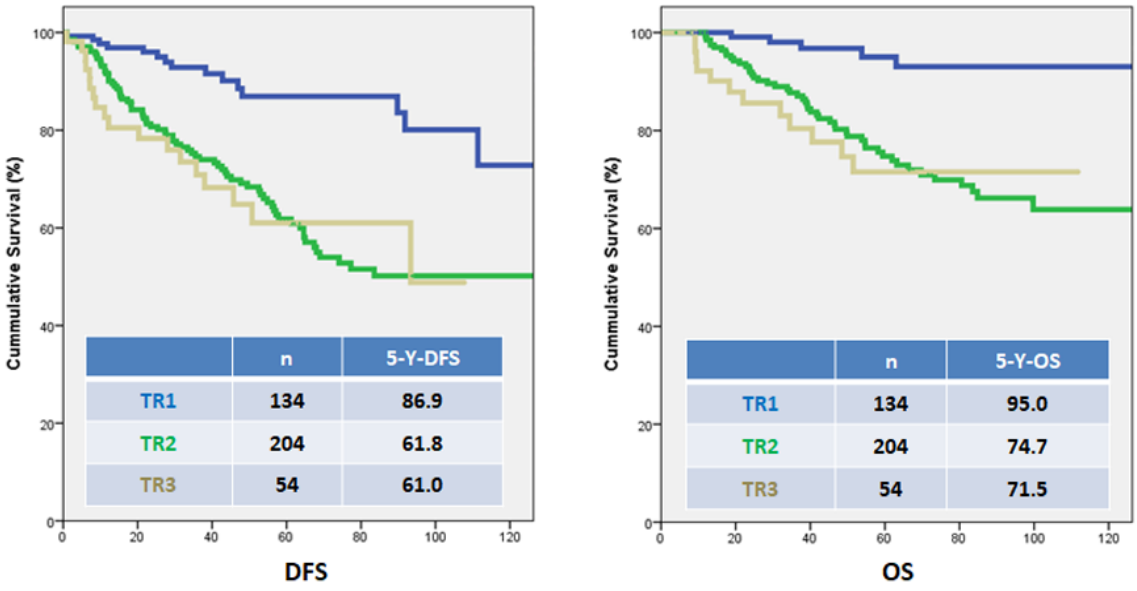
Supplementary Figure 1 Kaplan-Meier evaluation of the NSABP-B18 response scheme - Significant differences were defined between DFS and OS estimates of pCR vs. residual invasive tumors (pINV) [p<0.001].



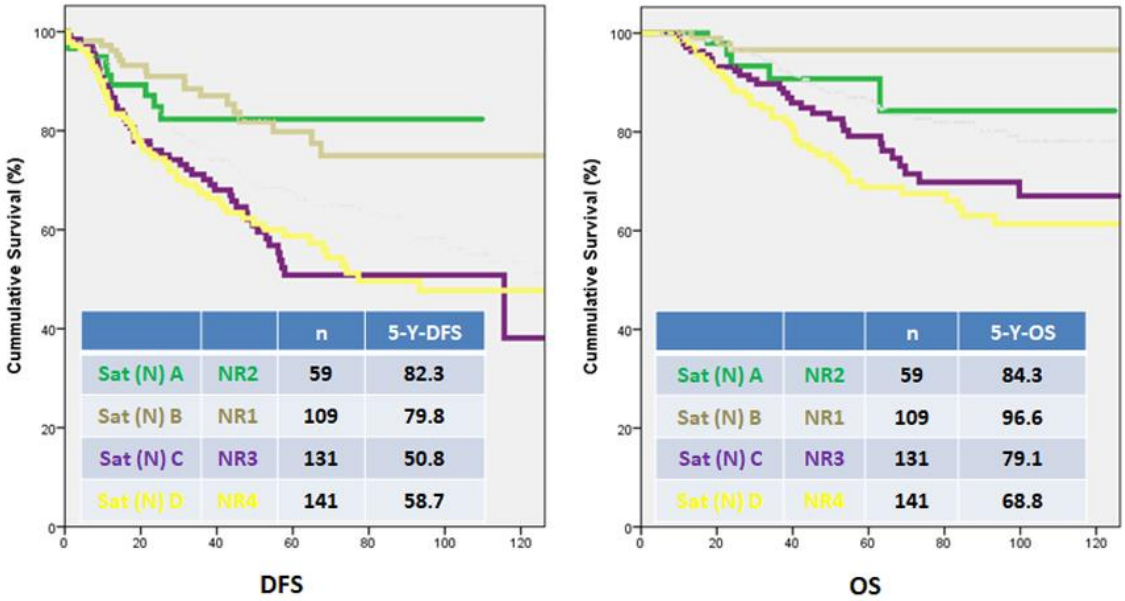
Supplementary Figure 2 Kaplan-Meier evaluation of the Chevallier grading system - Significant differences were observed between DFS estimates of group I vs. III (p<0.001); group I vs. IV (p<0.001); group II vs. III (p<0.001) and group II vs. IV (p<0.001). Significant distinction was detected between OS estimates of group I vs. III (p<0.001); group I vs. IV (p<0.001) and group II vs. IV (p=0.05).



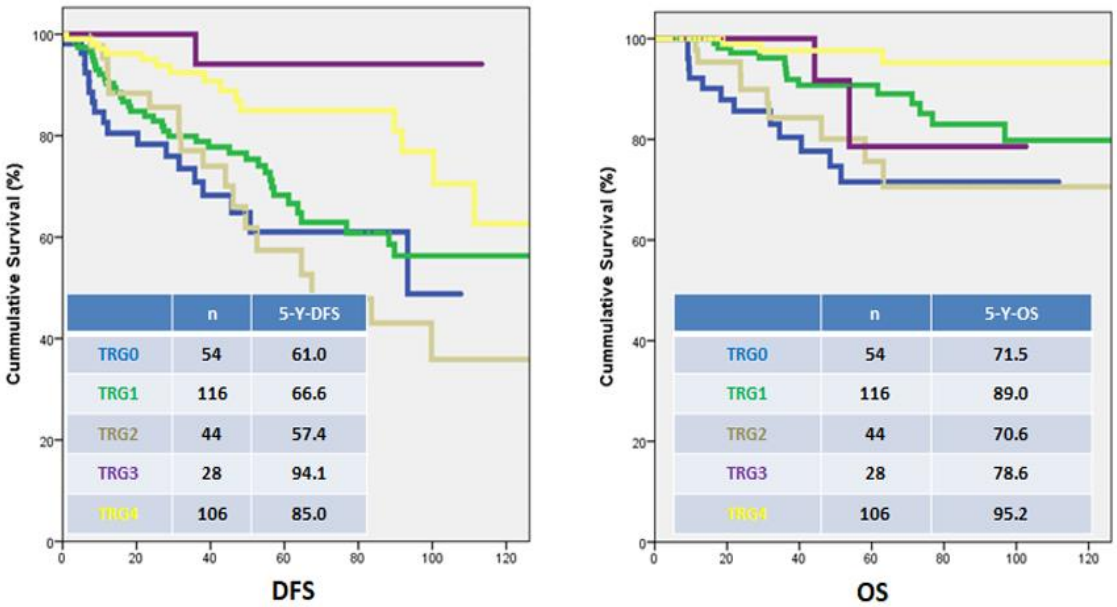
Supplementary Figure 3 Kaplan-Meier evaluation of the Sataloff (T) grading system - Significant differences were seen between DFS estimates of TA vs. TB (p=0.005), TA vs. TC (p<0.001) and TA vs. TD (p=0.009) along with significant distinction between OS estimates of TA vs. TB (p=0.005), TA vs. TC (p=0.016) and TA vs. TD (p=0.003).



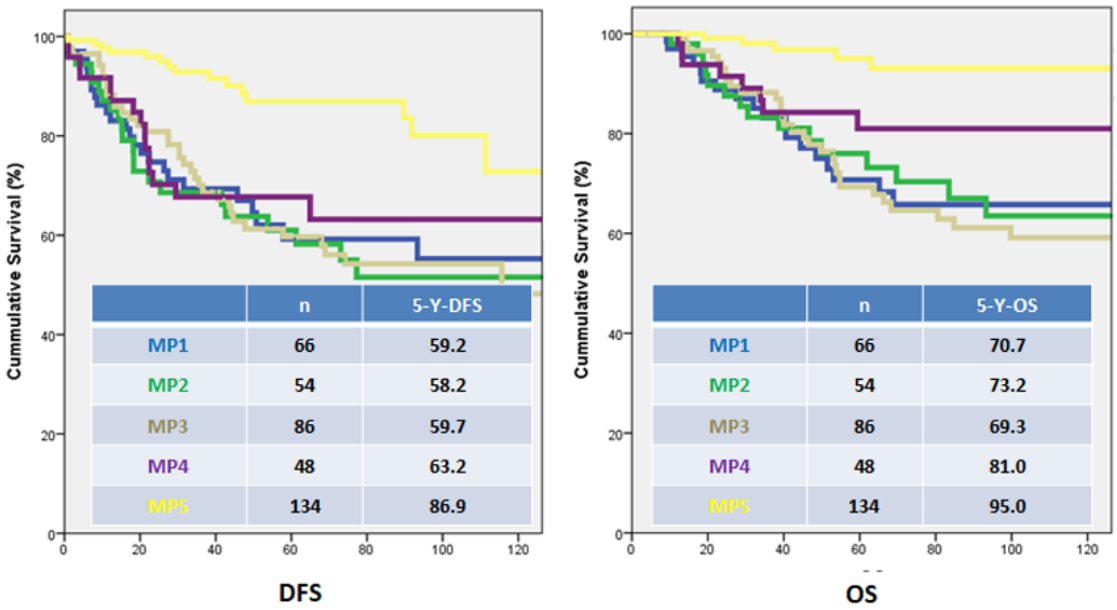
Supplementary Figure 4 Kaplan-Meier evaluation of the TR grading system - Significant differences were found between DFS and OS estimates of TR1 vs. TR2 (pDFS<0.001; pOS<0.001) and TR1 vs.TR3 (pDFS<0.001; pOS<0.001), respectively.



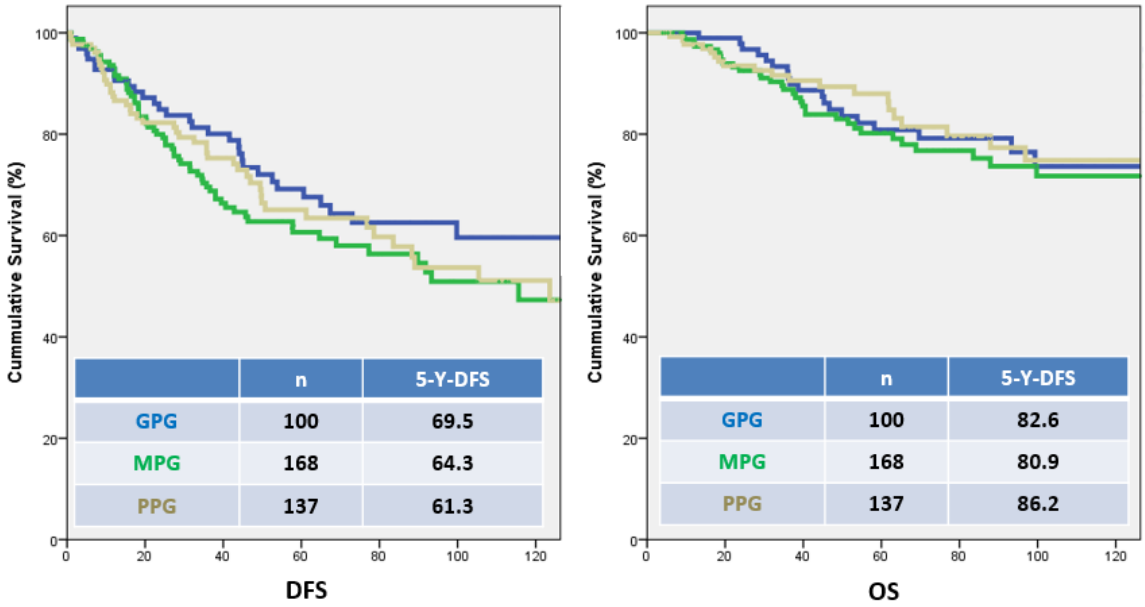
Supplementary Figure 5 Kaplan-Meier examination of Salatoff (N) and NR grading systems - Significant differences were found between DFS estimates of NR2 vs. NR3 (p=0.027), NR2 vs. NR4 (p=0.020), NR1 vs. NR3 (p<0.001), NR1 vs. NR4 (p<0.001) and between OS estimates of NR2 vs. NR4 (p=0.029), NR1 vs. NR3 (p<0.001) and NR1 vs. NR4 (p<0.001).



Supplementary Figure 6 Kaplan-Meier evaluation of the Denkert-Sinn grading system - Significant differences were defined between DFS estimates of TRG3 vs. TRG0 (p=0.006), TRG3 vs. TRG1 (p=0.020), TRG3 vs. TRG2 (p=0.006), TRG4 vs. TRG0 (p<0.001), TRG4 vs. TRG1 (p=0.012), TRG4 vs TRG2 (p<0.001) and between OS estimates of TRG4 vs. TRG0 (p<0.001), TRG4 vs. TRG1 (p=0.038), TRG4 vs. TRG2 (p<0.001).



Supplementary Figure 7 Kaplan-Meier evaluation of the Miller-Payne grading system - The DFS and OS estimates of MP5 group showed significant differences from other groups regarding DFS and OS.



Supplementary Figure 8 Kaplan-Meier evaluation of the RDBN grading system - There was no sign of significant difference among subgroups.

9.3. MAGYAR NYELVŰ ÖSSZEFOGLALÓ

Az emlőrák továbbra is a nők leggyakoribb daganata és a daganatspecifikus halálozási listán is az elsők között áll. Az elmúlt évtizedekben a szervezett szűrőprogram megjelenésével, a molekuláris szubtípusok és genetikai eltérések felismerésével, valamint a neoadjuváns terápia megjelenésével jelentős előrelépés volt megfigyelhető mind a diagnosztikus, mind a terápiás lehetőségek terén. Ugyanakkor ezen területeken még számos aktuális és megválaszolandó kérdés maradt. Kutatásainkban a tripla negatív emlőrákok immunhisztokémiai jellemzőinek vizsgálatát (1), a tripla negatív emlőrákok prognosztikai szisztémák szerinti összehasonlítását (2), valamint neoadjuváns terápiával kezelt emlőrákos betegek esetében a tumor regressziós grádus rendszerek összevetését tűztük ki célul.

(1)

Az emlőrákok megközelítőleg 15%-át ún. tripla negatív daganatok teszik ki, melyek jellegzetessége, hogy ösztrogén-, progeszteron- és HER2 receptorokat nem tartalmaznak kimutatható mennyiségben. A hormonreceptor negativitás mellett ezen daganatok a klasszikus, emlőrákokra specifikus markereket (GATA3, a MG, a GCDFP-15 és az NY-BR-1) sem expresszálják mindig, így áttétek esetén az emlő eredet megerősítése kihívást jelent. Vizsgálatunkban a korábban vizsgált markerek mellett SOX10 immunhisztokémiai reakciót végeztünk tissue microarray technikával, korábban igazolt, CK5 pozitív, tripla negatív emlődaganatos eseteken. Pozitívnak definiáltunk egy esetet, ha a tumorsejtek legkevesebb 10%-a jelölődött. Eredményeink alapján a vizsgált esetek 68.9%-ában (95% CI: 59.8-77.1) volt SOX10 pozitivitás, valamint 9.3%-ban olyan esetekben mutatkozott pozitivitás, melyekben a klasszikus emlőmarkerek mindegyike negatívnak bizonyult, ami egyértelműen a SOX10 additív szerepét igazolja. Az esetek 21%-ban nem észleltünk festődést egyetlen markerrel sem.

Mindezek alapján a SOX10 bizonyult a leggyakrabban pozitivitást mutató immunhisztokémiai markernek, így diagnosztikai algoritmusunk tripla negatív, CK5 pozitív daganatok esetében, szenzitivitás alapján a következőképpen módosult: SOX10 → GATA3 → MG → GCDFP-15 → NY-BR-1.

Javasoljuk hasonló esetekben a módosított immunhisztokémiai panel vizsgálatát.

(2)

A tripla negatív emlőrákok heterogén betegségcsoportot alkotnak. Elmondható róluk, hogy fokozott a lokális recidíva veszélye, a korai hematogén áttétképzés, valamint célzott terápia hiányában igen rossz kórjóslatúak. Habár a szakirodalomban számos prognosztikus faktort azonosítottak, valós túlélési adatok becsléséhez több tényezőt magába foglaló, összetett rendszerekre van szükség. Retrospektív kutatásunk célja a Nottingham Prognostic Index, a PREDICT és a PrognosTILs rendszerek értékelése, valamint a prognózist befolyásoló egyéb tényezők vizsgálata volt tripla negatív emlőrákos esetekben.

Egyváltozós Cox-regresszióval a műtét típusa, a pT, a pN, a stádium, az adjuváns terápia típusa, valamint a Nottingham Prognostic Index bizonyult szignifikáns változónak. Többváltozós Cox-regresszióval a Nottingham Prognostic Index mind a betegségmentes, mind a teljes túlélés tekintetében önálló, prognosztikus tényezőnek bizonyult. A három fentebb említett prognosztikai rendszert egymással ROC görbe analízissel hasonlítottuk össze. Noha valamennyi rendszer esetében a görbe alatti terület (AUC) értéke 0.7 és 0.8 közöttinek bizonyult, a Nottingham Prognostic Index értékei némileg magasabbak voltak. Eredményeink híven tükrözik a tripla negatív emlőrákok kórlefolyásának megjóslási nehézségeit.

A vizsgált Nottingham Prognostic Index, a PREDICT és a PrognosTILs prognosztikai rendszer prognosztikus szerepét megerősítettük és a szisztémák közül egyik sem volt rosszabb a másiknál, látszólag a Nottingham Prognostic Index emelkedett ki.

(3)

A neoadjuváns terápia kiváló kezelési lehetőség lokálisan előrehaladott emlőrákok esetében. A módszer előnye, hogy alkalmazásával downstaging, vagy akár komplett patológiai regresszió érhető el, ezáltal számos esetben emlőmegtartó műtét végezhető. Általánosságban elmondható, hogy a tripla negatív, valamint a HER2 pozitív emlődaganatok esetében érhető el leggyakrabban komplett patológiai regresszió. Habár a neoadjuváns kezelés hatására kialakuló tumorregresszió mértékének megítélésére számos rendszert fejlesztettek ki, nemzetközi konszenzus vagy használatukra vonatkozó ajánlás nem született mindezidáig. Vizsgálatunk célja az volt, hogy a jelenleg használatos regressziós grádus rendszerek, így a TR/NR, a Chevallier, a Sataloff, a Denkert-Sinn, a Miller-Payne, az NSABPB18, a Residual Disease in Breast and Nodes, valamint a Residual Cancer Burden prognosztikus értékét megvizsgáljuk a betegségmentes- és a teljes túlélés alapján.

Megállapítottuk, hogy a komplett patológiai regressziót (ypT0ypN0ycM0) mutató, vagy esetlegesen kizárólagosan reziduális in situ carcinomát (ypTisypN0ycM0) tartalmazó esetek betegségmentes és teljes túlélési becslései szignifikánsabban magasabbnak bizonyultak (pDFS< 0.001). Residual Cancer Burden használatával jelentős különbségeket állapítottunk meg a részleges regressziót mutató, és a regressziót egyáltalán nem mutató daganatok betegségmentes túlélésében. Többváltozós Cox-regresszióval a betegségmentes túlélés alapján a Residual Cancer Burden osztályozás (p=0.019), a teljes túlélés alapján az ystage (p=0.011) és a nyirokcsomó státusz (p=0.045) bizonyult önálló prognosztikus változónak.

Eredményeink alapján a Residual Cancer Burden alkalmazását javasoljuk neodjuváns kezelés hatására kialakuló tumorregresszió jellemzésére.

I. **Anita Sejben**, András Vörös, Arbel Golan, Tamás Zombori, Gábor Cserni. The added value of SOX10 immunohistochemistry to other breast markers in identifying cytokeratin 5 positive triple negative breast cancers as of mammary origin. Pathobiology. 2021 Feb 10; 1-6. doi: 10.1159/000512006

IF (2019/2020): 1.985

Pathobiology

Research Article

Pathobiology
DOI: 10.1159/000512006

Received: July 9, 2020
Accepted: October 5, 2020
Published online: February 10, 2021

The Added Value of SOX10 Immunohistochemistry to Other Breast Markers in Identifying Cytokeratin 5-Positive Triple Negative Breast Cancers as of Mammary Origin

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Keywords
Triple negative breast cancer · Immunohistochemistry · SOX10 · Tissue microarray

Abstract
Aims: Triple-negative breast cancer (TNBC) represents a specific group that lacks the expression of estrogen receptors, progesterone receptors, and human epidermal growth factor receptor-2 and might also lack the expression other breast markers like GATA3, mammaglobin (MG), GCDFP15 (growth cystic disease fluid protein 15), and NYBR1; when this occurs, proving the breast origin of a metastasis is a challenging task. In the present study, we assessed the added value of SOX10 immunohistochemistry to known GATA3, MG, GCDFP15, and NY-BR-1 statuses in a series of CK5-positive primary TNBCs. ***Methods:*** Tissue microarrays were made from the formalin-fixed and paraffin-embedded blocks of 120 TNBCs, and 3-4-mm-thick sections were immunostained for SOX10. The cut-off for a positive reaction was at least 10% of tumor cells staining. ***Results:*** In our cohort, SOX10 positivity was seen in 82/119 cases, 61, 74, 76, and 82 all of which were GATA3, MG, GCDFP15, and NY-BR-1 negative, respectively. Of the SOX10 negative cases, 12 stained with at least another breast marker. Nevertheless, 25/119 (21%) cases remained negative with all markers assessed. ***Discussion:*** SOX10 proved to be the most commonly positive breast marker in our CK5 expressing TNBCs, but the other markers also had some additive value to SOX10.

Introduction

About 15% of breast carcinomas belong to the so called triple negative category (TNBC), lacking the expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor-2 (HER2) [1]. Most (although not all) of these cancers are aggressive and may give rise to metastasis relatively early in the course of the disease [2, 3]. The expression of ER, PR, sometimes even HER2, or "breast markers" like GATA3, mammaglobin (MG), GCDFP15 (growth cystic disease fluid protein 15), and NY-BR-1 can point to the breast

Anita Sejben and András Vörös contributed equally to the present work and qualify as first authors.

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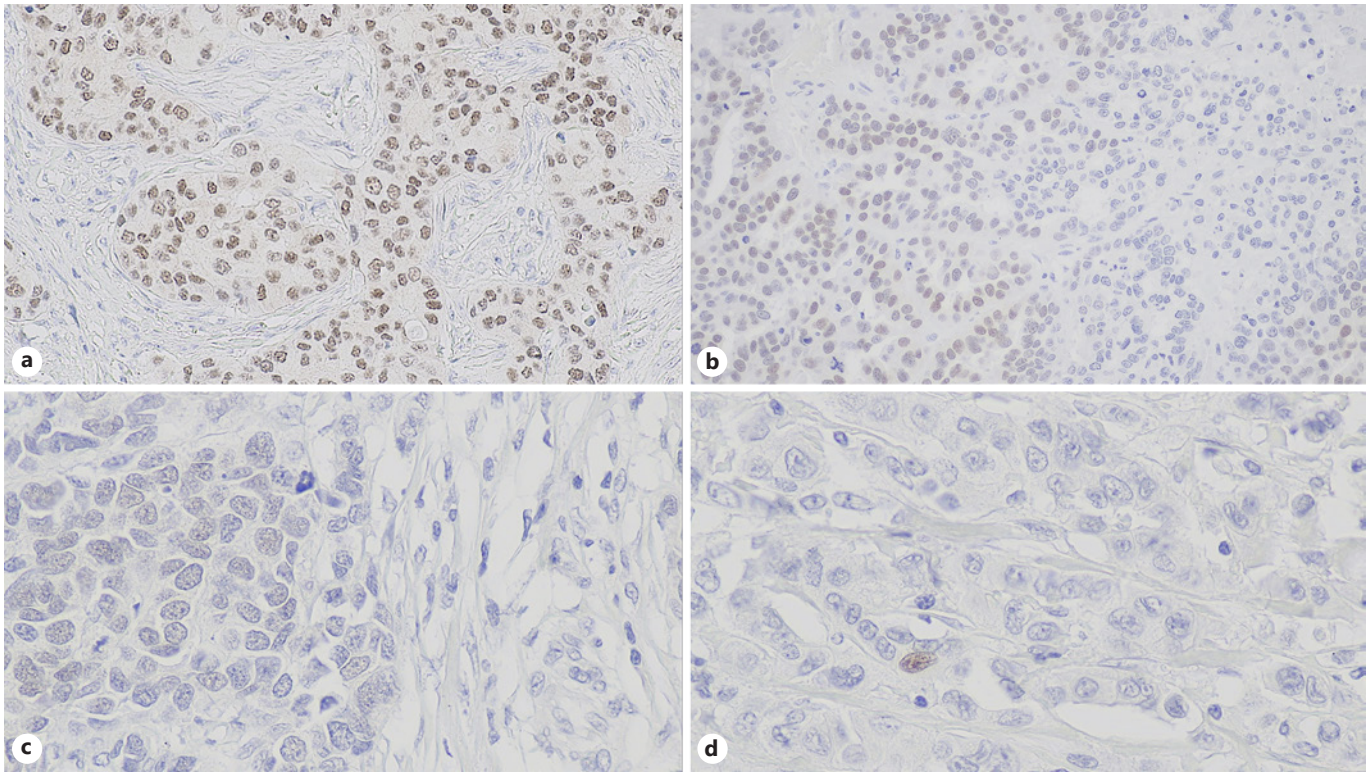

Fig. 1. Examples of SOX10 immunostaining. Diffuse strong nuclear staining (×20) (**a**); partial staining in >10% of the cells with moderate intensity (×20) (**b**); over 10% of nuclei staining weakly (×40) (**c**); less than 1% of nuclei staining (×40) (**d**).

origin of a metastasis, but TNBCs – by definition – lack the first 3 and might also lack the others [4, 5]. In a previous work, we identified GATA3 as the most gratifying breast marker, which could still be complemented by MG and GCDFP15, with practically no added value of NY-BR-1. Acknowledging that neither of these markers are absolutely specific, we also suggested that only about half of cytokeratin 5 (CK5) expressing TNBCs could be proven to be of mammary origin with their help; therefore, better or alternative markers would be useful in clinical practice [5].

SOX10 is a transcription factor involved in neural crest differentiation [6]. Accordingly, SOX10 positivity can be seen in melanoma, nerve sheath tumors [7–10], and is also expressed in myoepithelial cells in the breast [11]. SOX10 positivity has been described in salivary gland and cutaneous adnexal gland tumors, as well [12, 13]. As many TNBCs are basal-like on the basis of gene expression profile [14], show CK5 and/or epidermal growth factor receptor positivity, and classify as basal-like on the basis of an immunohistochemistry (IHC) based surrogate classification proposed by Nielsen and Perou [15], it is not surprising that these cancers may also stain for another myoepithelial marker, SOX10. Indeed, SOX10 has been reported to be positive in 40–70% of TNBCs [4, 16–18] and may be positive even in GATA3 negative cases [18]. In the present study, we assessed the added value of SOX10 IHC to GATA3, MG, GCDFP15, and NY-BR-1 IHC in a series of CK5-positive TNBCs.

Methods

A series of CK5-positive TNBCs previously characterized by GATA3, MG, GCDFP15, and NY-BR-1 IHC in a previous analysis was used for SOX10 IHC [5]. The tumors were assessed in tumor microarrays (TMAs) being represented by dual 2-mm-diameter tissue cores; the details of TMA building were reported earlier [5].

Briefly, the tumors were derived randomly from patients operated on and diagnosed with TNBC at the Bács-Kiskun County Teaching Hospital, Kecskemét between August 2005 and August 2015. The surgical specimens were fixed in 10% neutral buffered formalin for at least 24 h. The TMAs had been constructed from archived paraffin-embedded blocks using a TMA builder device (Histopathology Ltd, Pécs, Hungary), with each TMA incorporating 20 tumor tissue cores. The TMA blocks were stored at room

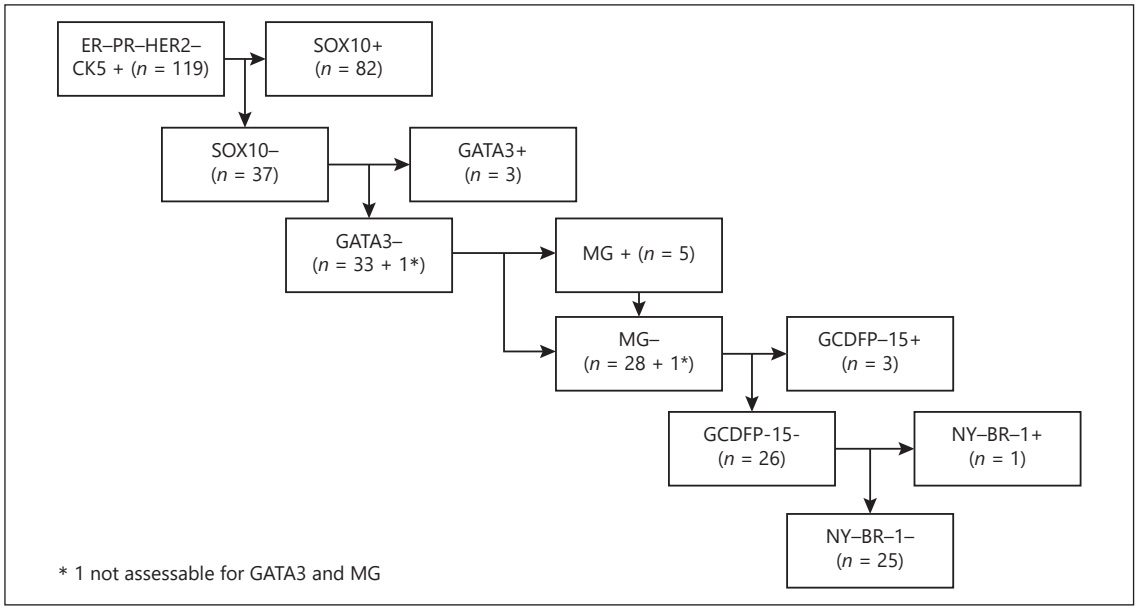

Fig. 2. Hierarchical labeling of the tumors with SOX10, GATA3, MG, GCDFP15, and NY-BR-1 as "breast specific" markers. MG, mammaglobin.

Table 1. Pairs of breast markers and their expressions in the tumors investigated

Any staining	SOX10+	SOX10–	GATA3+	GATA3–	MG+	MG–	GCDFP-15+	GCDFP-15–
GATA3+	20	3						
GATA3–	61	33						
MG+	7	6	2	11				
MG–	74	30	21	83				
GCDFP-15+	5	4	2	6	2	6		
GCDFP-15–	76	33	21	88	11	98		
NY-BR-1+	0	2	0	2	0	1	1	1
NY-BR-1–	82	35	23	93	13	103	7	108

MG, mammaglobin.

temperature, similarly to other paraffin blocks. 3–4-mm-thick sections were cut for SOX10 IHC using a monoclonal mouse antibody specific for an epitope mapping between amino acids 2–29 at the N terminus of SOX-10 of human origin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The antibody was used with 1:500 dilution for 30 min incubation period and pretreatment was performed at pH 9.

TMAs were scanned, and the proportion of positive cells was independently evaluated on the digital slides by the authors; the few discrepant cases were reassessed by consensus on the original slides. Rate, localization, and intensity were registered in all cases. The data for GATA3, MG, GCDFP15, NY-BR-1 were taken from a previous analysis [5].

The institutional ethical committee of the Bács-Kiskun County Teaching Hospital of the University of Szeged was consulted and approved this non-interventional retrospective study. The institutional data safety manager also gave approval for this study not requiring patients' identity related data. The study was finally approved by the Ethical Committee of the Albert Szent-Györgyi Medical Center of the University of Szeged.

Results

Of the 120 TNBCs represented in the TMA cores, 119 could be assessed for SOX10 staining. SOX10 staining was generally a nuclear staining occurring in <1–100% of tumor cells (Fig. 1); therefore, 2 different cutoffs for positive staining were evaluated. With a positivity threshold of >1 and ≥10%, 93 and 82 were defined as positive. Be-

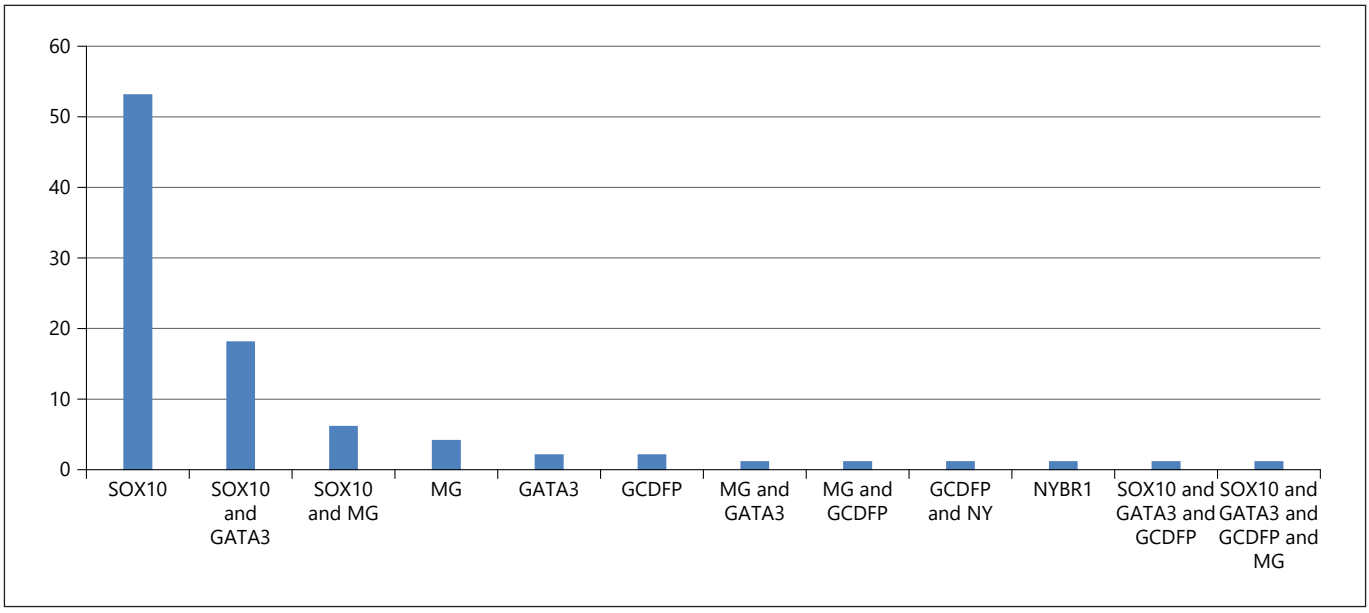

Fig. 3. Number of cases demonstrating a given pattern of breast marker expression in the series investigated. MG, mammaglobin.

cause the proportion of cases with 1–10% staining was relatively low, and the cases are less easy to pick up, the greater threshold was used for further analysis.

Of the 94 GATA3-negative cases, 61 cases were positive with SOX10. Similar results were observed with the other breast markers. Seventy four out of 104 MG negative cases, 76 out of 109 GCDFP15 negative cases, and 82 out of 117 NY-BR-1-negative cases stained positive with SOX10. Our series included 78 cases that were negative with all the previously tested markers, 53 of which were identified as positive with SOX10, still leaving 25 (21%) as breast marker negative. The sequential hierarchical staining for breast markers from the most commonly positive to the least commonly positive is shown in Figure 2. Mutual staining figures of pairs of breast markers are shown in Table 1. Figure 3 and online suppl. Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000512006) illustrate the proportion of single and multiple marker expression in the series with all staining combinations experienced.

Discussion

The expected lifetime risk of developing cancer in women is around 1 out of 3 [19], making the possibility of developing multiple primary cancers a real possibility and giving emphasis to the distinction between metastasis of a known primary tumor and an independent second primary cancer. TNBCs represent a minority of breast cancers, but as triple negative no special type carcinomas are often of poor prognosis, metastases may occur at a relatively higher rate. Owing to their phenotype overlapping with myoepithelial differentiation, TNBCs may show variable histologies, including spindle cells (sarcomatoid appearance), squamous metaplasia, and other rarer (e.g., sebaceous, chondroid or osseous) metaplasias, making their recognition as metastatic breast carcinomas at a metastatic site more difficult. This is why IHC markers supporting the breast origin of TNBCs is important.

In our previous study, by using GATA3, MG, GCDFP15, and NYBR1, we came out with an algorithmic value of these "breast markers" in CK5 expressing TNBCs believed to represent basal-like breast carcinomas, where GATA3 was the mostly expressed marker. Our results suggested that only about half of these cancers could be classified as of mammary origin on the basis of these 4 markers [5].

Cimino-Mathews et al. [16] published the first study investigating the utility of SOX10 IHC labeling in TNBC and metaplastic breast carcinoma cases. TMA blocks from 168 primary breast cancers were investigated; 40% showed positivity with SOX10. These were primarily basal-like unclassified TNBCs and metaplastic carcinomas.

There were 66% of TNBCs, but only 5% of luminal and HER2-positive cases were positive with SOX10 [16].

Nelson and coauthors reported about promising SOX10 results. TMA blocks were made from 26 patients' samples; 38% stained positively with SOX10, while no cases were positive with ER and HER2. A retrospective study was also performed in metastatic carcinomas of possible breast origin, and 57% of cases were labeled with SOX10. All SOX10-positive cases were confirmed to be negative with ER. Nelson and coauthors recommended the use of SOX10 in metastatic cases from unknown primary tumors to prove their melanoma or TNBC origin [20].

Since 2017, several research groups started to investigate SOX10. Al-Zahrani and coauthors compared its application with androgen receptor (AR). AR staining was positive in 95% of cases, mostly along with HER2 or ER and PR positivity, but no special breast cancer subtype could be identified on the basis or AR staining alone. SOX10 proved to be positive in one-third of the cases that were triple-negative [21].

Tozbikian examined the IHC profile of 57 TNBC cases. Among them, 82% showed positivity with GATA3, 58% with SOX10, and 25% with AR; 95% proved to be positive with either GATA3 or SOX10 and 46% showed dual positivity; and 80% of GATA3-negative cases were SOX10 positive. Their study concluded that while GATA3 was a more sensitive marker for TNBC cases, it is useful to add SOX10 to the IHC panel [18].

Harbhajanka et al. [22] investigated 48 TNBC cases in TMAs, and SOX10 showed positivity in 37.5% of the cases. A negative correlation with AR-positive molecular subtypes of TNBCs was observed, and a positive correlation was proved with WT1. However, no correlation was seen with the breast markers GATA3, MG, and GCDFP15, and basal-like subtype markers epidermal growth factor receptor and CK5/6 [22].

The most comprehensive study is the one by Laurent et al. [4] who reported their results about SOX10, GATA3, GCDFP15, AR, and MG in 207 metastatic TNBC cases and compared them with 152 primary lung adenocarcinomas. SOX10 showed the best sensitivity (62.3%) and specificity (100%) in comparison with GATA3 (30.4% sensitivity and 98.7% specificity), GCDFP15 (20.8% sensitivity and 98% specificity), MG (38.2% sensitivity and 81.6% specificity), and AR (30% sensitivity and 86% specificity); 6.3% of TNBC cases had no reaction with any of the above mentioned markers [4].

This year, Qazi et al. [23] suggested the combined use of SOX10 and GATA3 in cases of low ER expression and reduced GATA3 intensity. The study included 246 patients' samples as TMA blocks containing both ER-positive and ER-negative cases. Overall, it was 93%, and of TNBC cases, 63% showed positivity with GATA3; in parallel, 15% of all and 74% of TNBCs were positive with SOX10. Less than 1% had no reaction with either antibody and only 3% of ER-positive cases were SOX10 positive [23].

In our current study, by adding SOX10 IHC to our previous "breast marker" panel, there was an improvement in identifying CK5-expressing TNBCs as of mammary origin, and the algorithm could be changed substantially (Fig. 2). Using the 10% cutoff for SOX10 positivity, 68.9% (95% CI: 59.8–77.1) of the cases were found to be SOX10 positive and 9.3% of cases were positive only with the previously used markers, which proves the added value of GATA3, MG, GCDFP15, and NYBR1; 21% of the cases remained negative with all "breast markers," suggesting that negativity of all the examined markers does not securely exclude mammary origin. These results are in keeping with former reports [4, 23] and suggest that SOX10 is probably the best "breast marker" of TNBCs, followed by GATA3. Minor discrepancies in the proportion of cases staining and the value of MG versus GCDPF15 may stem from our cohort being restricted to CK5 expressing (and most likely basal-like) TNBCs, whereas others also included apocrine TNBCs which are expected to be positive with GCDFP15 (also an apocrine marker) more commonly. In 41% of the cases in our study, the staining observed was focal; in these cases, the application of the TMA technique can be a limitation.

Based on our data, SOX10 proved to be the most sensitive breast marker in CK5-expressing TNBCs, likely to correspond to basal-like TNBCs on the basis of the IHC-based surrogate classification. With the additive value of GATA3, MG, GCDFP15, and NY-BR-1, more than 3 quarters of the investigated 119 cases could be identified as breast cancers. With the joint use of SOX10, GATA3, MG, and GCDFP15, 78.2% (95% CI: 69.7–82.2) sensitivity was achieved. We propose SOX10 as the first-line approach to identify TNBCs, with the addition of GATA3, MG, and GCDFP15 for the negative cases; NY-BR-1 has little added value in this context.

Acknowledgement

This study was funded by the National Research, Development and Innovation Office grant GINOP-2.3.2-15-2016-00020.

Statement of Ethics

The institutional Ethical committee of the Bács-Kiskun County Teaching Hospital was consulted and approved this non-interventional retrospective study, which was also approved by the Regional Ethical Committee of the Albert Szent-Györgyi Medical Center of the University of Szeged.

Conflict of Interest Statement

No editorial or financial conflicts of interest exist for this submission.

Author Contributions

Concept and design – Anita Sejben, András Vörös, and Gábor Cserni. Evaluation of immunostained slides - all authors. Consensus on near cut-off staining cases – all authors. Search and evaluation of references – all authors. Drafting the manuscript – Anita Sejben, András Vörös, and Gábor Cserni. Approval of final manuscript – all authors.

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II. **Anita Sejben**, Tibor Nyári, Tamás Zombori, Gábor Cserni: Comparison of Nottingham Prognostic Index, PREDICT and PrognosTILs in triple negative breast cancer –a retrospective cohort study. Pathol Oncol Res. 2020;26:2443-2450. doi: 10.1007/s12253-020-00846-8

IF (2019/2020): 2.826

Pathology & Oncology Research (2020) 26:2443–2450
https://doi.org/10.1007/s12253-020-00846-8

ORIGINAL ARTICLE

Comparison of Nottingham Prognostic Index, PREDICT and PrognosTILs in Triple Negative Breast Cancer –a Retrospective Cohort Study

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Received: 28 May 2020 / Accepted: 10 June 2020 / Published online: 20 June 2020

Abstract

Triple-negative breast cancer (TNBC) represents a heterogenous subtype of breast cancer with generally poor prognosis. The prediction of its prognosis remains essential to clinicians in their therapeutical decision-making process. The aim of our study was to compare the validity of three multivariable analysis derived prognostic systems, the Nottingham Prognostic Index (NPI), PREDICT and PrognosTILs (a prognosticator including tumor infiltrating lymphocytes, TILs) in a series of TNBCs. Patients operated on with TNBC at the Department of Surgery, Bács-Kiskun County Teaching Hospital, Kecskemét between 2005 and 2016 were included. Clinical and pathological parameters and follow-up data were collected from medical charts. TILs were assessed retrospectively, following international recommendations. Estimated survivals of PrognosTILs, PREDICT and NPI were recorded and compared with real outcomes. Altogether 136 patients were included in this retrospective study. In univariate Cox analysis, type of surgery, pT, pN, stage, NPI and type of adjuvant therapy were the significant prognostic variables. The multivariate Cox-regression strengthened that NPI is an independent predictor of overall and disease-free survivals in TNBCs. The NPI, PREDICT and PrognosTILs could be compared directly only in a ROC curve analysis: the sensitivities and specificities of these predicting systems are rather similar with area under the curve values falling between 0.7 and 0.8, and NPI having the highest values. Our findings reflect the diverse prognosis of TNBC and highlight the difficulties of predicting its outcome. None of the three multivariable prognosticators is inferior to the others, the NPI can reliably be used for TNBCs.

Keywords Triple negative breast cancer · Nottingham Prognostic Index · Predict · Tumor infiltrating lymphocytes · PrognosTILs · Prognosis

Introduction

Triple-negative breast cancer (TNBC) represents a heterogeneous subtype of breast cancer (BC) defined by the lack of immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2), and by variable though distinct molecular profiles [1, 2]. Epidemiological data on TNBC revealed its higher prevalence among women of African ancestry, young BC patients and patients with Breast Cancer Gene-1 (BRCA-1) mutations [3]. The treatment of TNBC remains a challenge for clinicians due to its poor overall prognosis. Distant hematogenous metastasis formation and local recurrence are frequent and the treatment efficiency of TNBC is lower than in other types of BC [1, 4, 5]. By taking molecular profiles and BRCA deficiency into account, more personalized treatment methods are currently available [6]. Besides chemo- and radiotherapy, the role of immuno- and targeted therapy is increasing, both being currently under investigation with promising results [7–9].

Prediction of prognosis remains essential to clinicians in their decision-making process, helps stratifying patients by risk and better allows preparing individual treatment plans [10]. Various prognostic factors have already been presented in TNBC. Ovcaricek and coauthors described nodal status and age as independent prognostic factors for disease-free survival

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(DFS), whereas for overall survival (OS), only nodal status proved to be an independent factor [11]. Urru et al. have demonstrated that tumor stage at diagnosis and positive lymph node ratio are relevant predictors of survival and tumor recurrence, with the addition of Ki-67 status for recurrence prediction [12]. Asaga and coworkers have used a different approach, and analyzed clinical response to preoperative systemic chemotherapy [13].

The Nottingham Prognostic Index (NPI) was described by Haybittle and coauthors in 1982 and it was originally designed for primary operable BC. It takes tumor size, nodal stage and tumor histological grade into consideration [14]. On the basis of its equation and the values of the NPI, patients' could be divided into three prognostic categories according to the original article: Category I (good prognosis); Category II (moderate prognosis) and Category III (poor prognosis) [14, 15]. Later the prognostic groups were subdivided to form the very good, the good, the moderate I, the moderate II, the poor and the very poor prognostic groups [16]. Different cut-off values and diverse definitions of NPI-based groups (ranging from three to ten classes) have been used by some research groups [10]. The NPI has been proven to be a valid prognostic tool in BC treatment [17].

A more complex prognostic model, PREDICT was published by Wishart and coauthors in 2010. The algorithm was developed from 5694 patients' data from the Eastern Cancer Registration and Information Centre. The selected patients were operated on for invasive breast cancer. Based on the factors that were found to hold independent prognostic value, an algorithm was established that includes the presence of ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), age at diagnosis, menopausal state, ER, PR, HER2 and Ki-67 status, invasive tumor size, tumor grade, method of tumor detection and number of positive lymph nodes [18]. PREDICT is also endorsed by the American Joint Committee of Cancer [19]. The on line calculator estimates OS for 5, 10 and 15 years. Although the tool generally received good ratings for validity, Maishman and coauthors' results showed that PREDICT was a great tool only in long-term survival estimates, and overestimated short-time survivals, especially in ER-positive tumors [17, 20, 21].

Tumor infiltrating lymphocytes (TILs) reflect prognosis in TNBC, since their higher proportion correlates with better outcome in this subset of breast tumors, and indicates the prominent role the immune system plays in TNBC. While TNBCs lack targeted therapy, the interest for immune modulators has increased [22, 23]. Loi and coworkers conducted a pooled analysis of 2148 patients and identified the following factors that independently influence the prognosis of primary TNBCs: percentage of stromal TILs, age, tumor size, number of positive lymph nodes, histological grade and treatment. Invasive disease-free survival (i-DFS), distant disease-free survival (d-DFS) and OS results were examined in 3 and 5 year intervals [24]. Based on the results, an equation was developed for survival estimates. For easier utilization, an online tool named PrognosTILs was developed for early stage TNBCs [25]. With this application, the 5-year and 10-year OS and DFS estimates can be calculated.

The aim of our study was to compare the validity of NPI, PREDICT and PrognosTILs in a series of TNBC cases.

Materials and Methods

Patients operated on for histologically verified triple negative, invasive breast carcinoma at the Department of Surgery, Bács-Kiskun County Teaching Hospital, Kecskemét between 2005 and 2016 were included in our consecutive and retrospective study. Follow up data (OS and DFS) were collected from medical charts. For these outcomes, patients were followed from the date of surgical treatment until the time of recurrence or tumor-related death; those alive without recurrence and those dying from other causes were censored at the time of the last follow-up and death, respectively.

The following clinical and pathological variables were obtained for analysis: age, gender, localization, type of surgical and adjuvant treatments, histological type and grade of cancer, vascular invasion, tumor size, pT and pN categories, and stage. The NPI was calculated with the following equation: NPI = tumor size (cm) × 0.2 + nodal score (1 for pN0, 2 for pN1, 3 for pN2 or pN3) + number value from the histological grade [14]. The Nottingham Prognostic Groups were classified as excellent (EPG): ≤2.4; good (GPG): 2.41–3.4; moderate-1 (MPG1): 3.41–4.4; moderate-2 (MPG2): 4.41–5.4; poor (PGP): 5.41–6.4 and very poor (VPPG): ≥6.41 [16].

The predicted OS and DFS estimates of PrognosTILs were obtained from an online calculator [23, 25]. The estimations were based on the following parameters: age, number of positive lymph nodes, tumor size, histological grade, type of chemotherapy and proportion of stromal TILs. For the determination of the latter, the International TILs Working Group (later acting as International Immunooncology Biomarker Working Group - IIOBWG) recommendations and rules were used [25, 26]. To help in the estimation of stromal TILs, the online calibration system described by the IIOBWG and found at was also used [27, 28]. After getting accustomed with the scoring system with a hundred cases evaluated in a study by the European Working Group for Breast Screening Pathology, the calibration (etalon) pictures for different rates of stromal TILs were screensaved and printed, and these printed pictures were compared with the microscopic images displayed on a monitor for at least three areas. The mean of these estimates was rounded to the closest 10% value also allowing for 5% and 1%, with the help of the calibration picture published in the first article of the IIOBWG for the latter value [29].

Table 1 Clinical and pathological characteristics of patients evaluated and the results of univariate Cox-regression [pT, pN categories defined by AJCC [27: Amin-AJCC], CMF: cyclophosphamide, methotrexate and 5-fluorouracil; second generation systemic treatment refers to anthracycline based regimens without taxanes; third generation refers to taxane containing regimens]

			pOS	pDFS
Age (years)	n	%	$p = 0.102$	$p = 0.207$
30–39	12	9.5		
40–49	15	11.9		
50–59	37	29.3		
60–69	35	27.8		
70–79	21	16.7		
80–91	6	4.8		
Laterality			$p = 0.645$	$p = 0.958$
Right	58	46.0		
Left	68	54.0		
Type of surgery			$p = 0.354$	$p = 0.017$
Mastectomy	24	19.0		
Breast conserving surgery	102	81.0		
Histology diagnosis			$p = 0.626$	$p = 0.566$
Carcinoma of no special type (NST)	112	88.8		
Medullary carcinoma	7	5.6		
Other	7	5.6		
Grade			$p = 0.967$	$p = 0.88$
2	5	4.0		
3	121	96.0		
pT			$p = 0.222$	$p = 0.009$
pT1	67	53.1		
pT2	55	43.7		
pT3	1	0.8		
pT4	3	2.4		
pN			$p = 0.006$	$p < 0.001$
pN0	75	59.6		
pN1mi	8	6.3		
pN1	31	24.6		
pN2	9	7.1		
pN3	2	1.6		
pNx	1	0.8		
Vascular invasion			$p = 0.573$	$p = 0.400$
Absent	100	79.4		
Present	26	20.6		
Stage			$p = 0.05$	$p < 0.001$
I	47	37.3		
II	51	40.5		
III	27	21.4		
no data	1	0.8		
Adjuvant therapy			$p = 0.151$	$p = 0.003$
Chemotherapy	10	7.9		
Radiotherapy	15	11.9		
Both	85	67.5		
Neither	16	12.7		
Generation of chemotherapy			$p = 0.092$	$p = 0.303$
Second generation	16	12.7		
Third generation	73	57.9		
Other (CMF)	6	4.8		
No data	31	24.6		

The anticipated OS evaluations of PREDICT were determined with the online calculator, that required the following data: age, menopausal state, ER status, HER-2 status, Ki67 status, size of invasive tumor, grade of tumor, type of detection, number of positive lymph nodes and presence of micrometastasis in the lymph nodes [18, 19].

The Wilcoxon rank sum test was applied to analyze the correlation between recurrence or tumor-specific death and DFS or OS prediction rate of PrognosTILs and OS prediction rate of PREDICT. The OS and DFS data could not be correlated directly with the survival predictions of PrognosTILs and PREDICT, therefore the patients were classified in the following four categories: patients alive, patients who died of disease (DOD), patients alive with and without recurrence. The calculated OS and DFS survival predictions of PrognosTILs, the OS survival estimates of PREDICT and NPI scores were correlated with the 4 categories by receiver operating characteristic (ROC) curve analysis aiming to compare them and to find cut-off points. Patients DOD and patients alive categories were utilized in ROC curve analysis focusing on 5-year-OS prediction of PrognosTILs, PREDICT and NPI scores, while patients with recurrence and patients without recurrence categories were used in a ROC curve of 5-year-DFS estimates of PREDICT and NPI scores. The cut-off points identified by ROC curve analysis could show which OS and DFS rates of PrognosTILs, OS estimates of PREDICT and NPI scores are related to more frequent recurrence and tumor-specific death, respectively.

NPI was analyzed with the Kaplan-Meier method and the subgroups were compared with the log rank test. Cox-regression was utilized as univariate analysis. The parameters found significant in the univariate models were entered in a multivariable Cox proportional hazard model to identify factors of independent prognostic significance. PrognosTILs and PREDICT survival estimates could not be included in the multivariate analysis due to statistical reasons. Statistical models were fitted using SPSS Statistics V.23.0 software (IBM, SSPS 22.0, Armonk, NY USA). All statistical tests were two-sided and $p < 0.05$ values were considered statistically significant.

This retrospective study was approved by the institutional ethical committee of the Albert Szent-Györgyi Clinical Centre of the University of Szeged and the ethical committee of Bács-Kiskun County Teaching Hospital also gave a consent for the study.

Table 2 The 5-year overall survival (OS) and disease-free survival (DFS) predictions of PrognosTILs according to outcome. Significant differences were detected between OS predictions of patients who died of disease and patients alive, and DFS predictions of patients with and without recurrence

PrognosTILs predictions			average		median		range		Wilcoxon-test
	n	%	OS	DFS	OS	DFS	OS	DFS	pOS = 0.015
Patients deceased due to tumor	14	15.0	80.1	80.6	80	76	74–92%	69–92%	
Patients alive	79	85.0	85	82	85	83	49–95%	44–95%	
Patients with recurrence	27	29.0	80.3	77.3	80	77	49–93%	44–93%	pDFS < 0.001
Patients alive with recurrence	13	14.0	80.6	77.7	83	80	49–93%	44–93%	
Patients alive without recurrence	66	71.0	85.8	84	86	83	71–95%	67–95%	
All (where PrognosTILs was evaluated)	93	100.0	84.2	81.7	84	82	49–95%	44–95%	

Results

Altogether, 136 patients who underwent surgical resection were included in our study. Ten patients (7.4%) were censored due to non-tumor related death. Tumor-specific death was found in 23 cases (16.9%), while 103 patients (75.7%) were alive at the last follow up, including 20 patients with recurrence (14.7%). The mean and median OS and DFS were 66.8 months and 57.5 months, 59.9 months and 41 months, respectively (range for OS: 7–170 months; range for DFS: 2–170 months). Recurrence was observed in 43 cases, including 11 cases (25.6%) with local or regional recurrence, 23 cases (53.5%) with distant metastasis and two cases with both local and distant types of recurrence. The median time to recurrence was 41 months (range: 2–170 months) Novel malignancies were found in 3 cases (7.0%; ovary [$n = 1$] and lung cancer [$n = 2$]). The median follow up was 56 months (range: 7–170 months).

The basic clinical and pathological characteristics are displayed in Table 1 [30]. The mean and median age of the patients were 59.6 and 59 years, respectively (range: 32–91). In univariate Cox-regression, the type of surgery, the pT and pN categories, the stage of the disease and the type of adjuvant therapy were found to be significant variables.

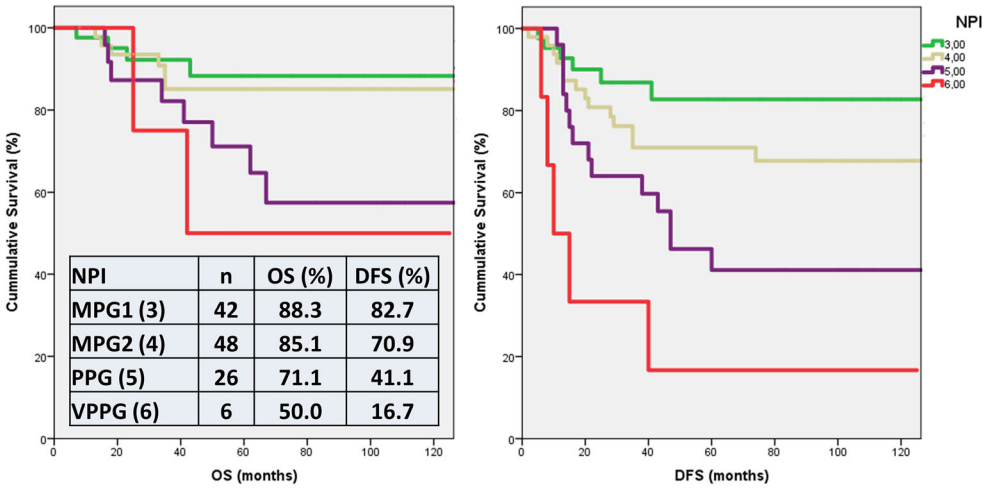
The predictions from PrognosTILs and PREDICT and the NPI scores were established in 93, 126 and 125 cases, respectively. Concerning the 5-year-OS and -DFS predictions of PrognosTILs, the mean, the median and the range of estimates are presented in Table 2. The comparison of predicted survival estimates and outcomes revealed that the predicted OS estimates of the patient DOD were significantly lower than those of patients who were alive ($p = 0.015$); similarly, the predicted DFS estimates of patients with recurrence were significantly lower, than those of patients without recurrence ($p < 0.001$). Table 3 highlights the mean, the median and the range of the 5-year-OS estimates of PREDICT. The statistical analysis strengthened, that the predicted OS estimates of patient DOD were significantly lower, than those of patients who were alive ($p = 0.020$).

The NPI-based GPG included only 3 cases, therefore this group was excluded from further evaluation. Figure 1 demonstrates the results of Kaplan-Meier analysis of the NPI subgroups. Significant differences were detected between OS and DFS estimations of different prognostic groups, namely the OS estimates of MPG1 vs. PPG ($p = 0.017$), MPG1 vs. VPPG ($p = 0.049$), MPG2 vs. PPG ($p = 0.026$); and the DFS estimates of PPG vs. MPG1 ($p = 0.002$), PPG vs. MPG2 ($p = 0.035$), PPG vs. VPPG ($p = 0.013$), VPPG vs. MPG1 ($p < 0.001$) and VPPG vs. MPG2. ($p = 0.001$). In the univariate Cox-regression, NPI was found to be a significant prognostic variable (pOS = 0.022; HR:1.71, 95%CI:1.08–2.72; pDFS<0.001; HR:2.02, 95%CI:1.43–2.86).

Figure 2 displays the results of ROC curve analysis focusing on 5-year-OS estimates of PrognosTILs, PREDICT and NPI scores. The area under the curve (AUC) of PrognosTILs, PREDICT and NPI were 0.759, 0.762 and 0.792, respectively. Figure 3 demonstrates the ROC curve analysis of 5-year-DFS estimates of PrognosTILs and NPI scores. The AUC values of PrognosTILs and NPI were 0.713 and 0.781, respectively. The findings of ROC curve analyses drew attention to the similarities of these predictive systems concerning sensitivity

Table 3 The basic characteristics of 5-year overall survival (OS) predictions of PREDICT according to outcome. The survival estimates of patients dying of tumor progression were lower than those of patients who were alive at last follow up

PREDICT estimates	n	%	mean	median	range	Wilcoxon-test pOS = 0.020
Patients deceased due to tumor	23	18.3	62.9	65.5	9.2–85.1%	
Patients alive	103	81.7	71.8	78.1	7.1–86.5%	
All (where PREDICT was evaluated)	126	100	70.1	75.3	7.1–86.5%	



NPI	n	OS (%)	DFS (%)
MPG1 (3)	42	88.3	82.7
MPG2 (4)	48	85.1	70.9
PPG (5)	26	71.1	41.1
VPPG (6)	6	50.0	16.7

Fig. 1 Kaplan-Meier analysis of NPI. According to the log rank test significant differences were observed between the overall survival (OS) estimates of MPG1 vs. PPG ($p = 0.017$), MPG1 vs. VPPG ($p = 0.049$) andMPG2 vs. PPG ($p = 0.026$); and the disease-free survival (DFS) estimates of PPG vs. MPG1 ($p = 0.002$), PPG vs. MPG2 ($p = 0.035$), PPG vs. VPPG ($p = 0.013$), VPPG vs. MPG1 ($p < 0.001$) and VPPG vs. MPG2 ($p = 0.001$) [MPG1: Moderate Prognostic Group 1, MPG2: Moderate Prognostic Group 2, PPG: Poor Prognostic Group, Very Poor Prognostic Group]

and specificity and to the fact that they are not ideal for defining cut-off values.

The multivariate Cox proportional hazard model revealed that among the variables found significant in univariate models (type of surgery, pT, pN, stage, adjuvant therapy and NPI), only NPI was an independent prognostic marker for triple negative breast cancer (pOS = 0.006; HR:1.66, 95%CI:1.16–2.37; pDFS<0.001; HR:1.92, 95%CI:1.46–2.53).

Discussion

TNBCs are generally considered as the worst IHC based molecular subtype of breast cancer, owing to their poor prognosis and the limited therapeutic success associated with them. Despite the overall bad prognosis of TNBC, there are some tumors that by definition fall into this category, but belong to a better prognostic group. These include rare tumors like tall cell carcinoma with reversed polarity, secretory carcinoma, non-high grade, i.e. classical adenoid cystic carcinoma [31–33]. Even without these low grade special type carcinomas, the prognosis of TNBC is heterogeneous and depends on a number of prognostic factors.

The presence of distant metastasis, nodal status, tumor size and histological grade are established prognostic factors of breast carcinomas, and have their role in predicting the outcome of TNBCs as well. More recently the proportion of stromal TILs has also been recognized as an independent

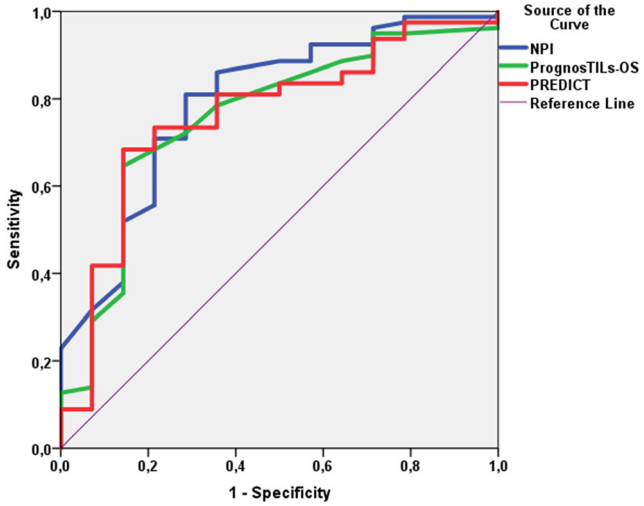


Fig. 2 ROC curve analysis of 5-year overall survival predictions of TIL, PREDICT and NPI scores (area under the curve values for TIL, PREDICT and NPI were 0.759, 0.762 and 0.792, respectively)

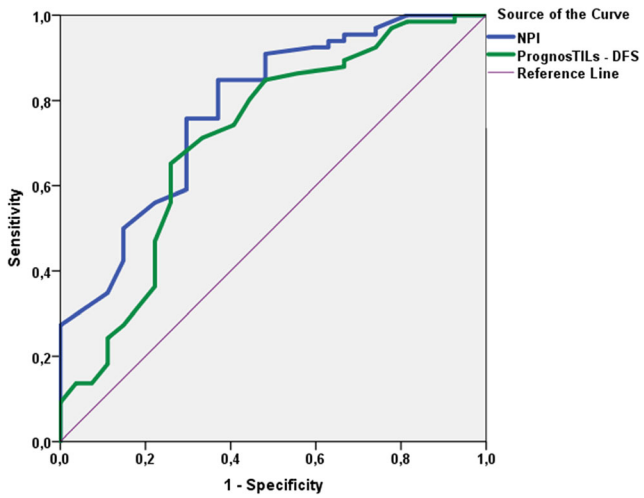


Fig. 3 ROC curve analysis of 5-year disease-free survival predictions of PrognosTILs and NPI scores (area under the curve values for TIL and NPI were 0.713 and 0.781, respectively)

prognosticator of TNBCs [24], and the prognostic value of TILs was also found in a more recent meta-analysis [34]. When prognostic factors show divergent features, i.e. clinicians are faced with a combination of factors toward good and bad prognosis, predictive models based on multivariable analysis of multiple prognostic factors are much more valuable than isolated factors. The NPI is one such factor and was derived from the multivariable analysis of 387 patients with different molecular subtypes of breast cancer and was later validated in a series of 320 independent consecutive cases [35]. Several external studies have demonstrated its ability to give a prognostic classification of breast carcinomas [36–38]. Although the improvements in treatment have significantly altered the outcomes of breast cancer, and this improvement is also reflected in the NPI prognostic group-specific survivals, the prognostic separation of breast cancers on the basis of the NPI was still found to be valid [39]. The PREDICT tool was derived from a much greater population and was also independently validated in a number of reports [17, 40]. PrognosTILs is a novel multivariable prognosticator model and calculator derived from the pooled analysis of 2148 individual patients' data from 9 studies on TNBCs proving the prognostic value of stromal TILs in the adjuvant setting [24]. This distinguishes it from NPI and PREDICT which were built on data from ER-positive and ER-negative tumors together, and theoretically could mean that it is better fitted to predict the prognosis of TNBCs.

The significance of the NPI in TNBC was first examined by Albergaria and coauthors in 2001 with reassuring results. NPI results correlated well with real survival data due to the facts that TNBCs are frequently high grade and large tumors [41]. PREDICT, to our knowledge has not yet been evaluated for TNBCs alone, whereas PrognosTILs is relatively recent for larger validation on comparison studies.

In univariate Cox analysis, type of surgery, pT, pN, stage, NPI and adjuvant therapy were found significant prognostic variables. We also found that lower 5-year OS and DFS predictions of PognosTILs are related with more frequent tumor specific death and recurrence (pOS = 0.015, pDFS<0.001), while the lower 5-year OS predictions of PREDICT are associated with higher rate of tumor specific death ($p = 0.02$). Concerning the NPI, we demonstrated that there are significant differences among OS and DFS estimates of certain prognostic groups (Fig. 1). PrognosTILs and PREDICT derived estimates of survival, as scale variables could not enter the Kaplan-Meier analysis. The direct comparison of the multivariable prognosticators was performed with ROC curve analysis. Regarding the OS follow up data, PrognosTILs, PREDICT and NPI, while regarding the DFS follow up data, PrognosTILs and NPI were compared. All three predictors of outcome reflect fair performance with areas under the ROC curves falling between 0.7 and 0.8. The sensitivity and specificity of these predicting systems are rather similar, although there seems to be a tendency for NPI values to better predict outcome on the basis of the somewhat greater AUC values. In keeping with the results of Albergaria et al., the multivariate Cox-regression strengthened that NPI is an independent predictor of OS and DFS in TNBCs (pOS = 0.006; HR:1.66, 95%CI:1.16–2.37; pDFS<0.001; HR:1.92, 95%CI:1.46–2.53) [41]. Considering that the ROC curve analysis yielded similar results for the three multivariable prognosticators studied, it can be inferred that any of these is suitable to predict the outcome of TNBCs, and none of these is inferior to the others.

The results also show that TNBCs are prognostically heterogeneous. No case was classified as of very good prognosis on the basis of the NPI, and only 3 cases fell into the good prognostic group. This is due to the fact that only 5 tumors were of histologic grade 2, whereas the remaining were high grade, and with this combination, their NPI value was immediately >4.

The lack of all prognostic markers for all cases and the fact that this was a single institution study of retrospective nature with limited number of cases are possible limitations of this work. A further limitation may be that values predicted by PrognosTILs and PREDICT, due to statistical reasons, could not be entered into the multivariate Cox-regression analysis, and could not be compared to NPI in this setting; but this drawback was compensated by the ROC curve analysis of the three prognosticators. Our study has strengths, as well. To our knowledge, this study is the first to evaluate the value of PREDICT in TNBCs, and these multivariable prognostic tools have never been compared in a single study. Another advantage of the study design was the uniform evaluation of TILs with rigorous adherence to internationally agreed guidelines.

In conclusion, our findings reflect the diverse nature of TNBC and highlight the difficulties of predicting the outcome of this disease. Although the NPI seemed to give somewhat higher AUC values in the direct comparisons with PREDICT and PrognosTILs, none of the multivariable prognosticators is inferior to the others according to our data.

Authors' Contributions All authors contributed to the study conception and revision. Data collection was performed by Anita Sejben, Tamás Zombori and Gábor Cserni. Statistical analysis was performed by Tibor Nyári and Tamás Zombori. The first draft of the manuscript was written by Anita Sejben, Tamás Zombori and Gábor Cserni and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Information Open access funding provided by University of Szeged.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Consent to Participate Not applicable.

Consent for Publication Not applicable.

This retrospective study was approved by the institutional ethical committee of the Albert Szent-Györgyi Clinical Centre of the University of Szeged.

Conflict of Interest The authors declare that they have no conflict of interest.

Code Availability There are no restrictions on the availability of materials, data and code.

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III. **Anita Sejben**, Renáta Kószó, Zsuzsanna Kahán, Gábor Cserni, Tamás Zombori: Examination of tumor regression grading systems in breast cancer patients who received neoadjuvant therapy. Pathol Oncol Res. 2020;26:2747-2754. doi: 10.1007/s12253-020-00867-3

IF (2019/2020): 2.826

Pathology & Oncology Research (2020) 26:2747–2754
https://doi.org/10.1007/s12253-020-00867-3

ORIGINAL ARTICLE

Examination of Tumor Regression Grading Systems in Breast Cancer Patients Who Received Neoadjuvant Therapy

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Received: 16 April 2020 / Accepted: 29 June 2020 / Published online: 20 July 2020

Abstract

Neoadjuvant therapy is a common form of treatment in locally advanced breast cancer (LABC) patients. Besides some guidelines for grading regression, a standardized general scheme is not yet available. The aim of our study was to compare the prognostic impact of different regression grading systems, namely the TR/NR, Chevallier, Sataloff, Denkert-Sinn, Miller-Payne, NSABP-B18, Residual Disease in Breast and Nodes and Residual Cancer Burden (RCB) on disease-free (DFS) and overall survival (OS). Data of 746 breast cancer patients treated in neoadjuvant setting between 1999 and 2019 have been included. The different regression grades and follow-up data were collected from medical charts. Statistical analysis included the Kaplan-Meier method, log-rank test and multivariate Cox regression. The average patient age was 55 years. The DFS and OS estimates of patients with complete pathological regression and residual in situ carcinoma have been significantly more favorable than those having partial regression or no signs of regression (pDFS<0.001, pOS < 0.001). Significant differences were found between DFS estimates of classes with partial regression and without regression defined by RCB. Concerning DFS estimates, the RCB classification ($p = 0.019$), while regarding OS data the y-stage ($p = 0.011$) and the nodal status (ypN; $p = 0.045$) were significant prognosticators by multivariate Cox regression. Regression grading systems help the evaluation of regression in LABC patients treated with neoadjuvant therapy. Of the several grading systems compared, the RCB classification makes the best distinction between the outcomes of the different classes, therefore we recommend the inclusion of RCB into the histopathological findings.

Keywords Breast cancer · Neoadjuvant therapy · Regression pattern · Grading systems · Residual cancer burden

Introduction

Treatment of locally advanced breast cancer (LABC) patients has been one of the great challenges of breast oncology for a long time. Patients with such advanced disease benefit from treatment devised by a multidisciplinary team of specialists: oncologists, surgeons, pathologists and radiologists [1].

Neoadjuvant therapy (NAT) has changed the management of LABC, since it can achieve reduction or even complete regression of the primary tumor and its metastases [2, 3]. This downstaging can allow some patients who would have had mastectomy as surgical treatment to be treated with breast conservation [4]. While receiving NAT, patients have to be under constant oncological and radiological follow-up [5]. The effectiveness of NAT completed with surgical and if needed postoperative endocrine treatment seems to be equivalent with adjuvant therapy on the basis of disease-free (DFS) and overall survivals (OS) [6, 7]. Pathological complete regression occurs more frequently in triple negative or HER-2 positive cancers than in ER positive ones [8, 9].

The work-up of surgical specimen after NAT requires the undivided attention of the pathologist. The identification of the primary tumor bed can be challenging because of its resemblance to fibrotic breast tissue. Insertion of metal clips into the tumor and/or specimen mammography can simplify the identification process. Specimen sampling requires adequate radio-pathological correlation [10, 11]. The evaluation of

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12253-020-00867-3) contains supplementary material, which is available to authorized users.

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tumor regression after NAT has to be established with full consideration given to radiology, gross morphology and microscopy.

The characterization of regression differs from country to country due to lack of international consensus on definitions. Pathological complete regression (pCR) implies no residual tumor in the surgical specimen, but the meaning is interpreted variously. In some European countries, pCR generally means the absence of in situ or invasive tumor tissue in the specimen. A significant difference in DFS between ypT0ypN0M0 and ypTisypN0M0 was demonstrated by the German and Austrian Breast Groups [12]. The United States Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research and the American Joint Committee on Cancer define pCR as the absence of residual invasive cancer in the surgical specimen [13, 14].

The histology of post-NAT tumors represents a spectrum from pCR to tumor growth and progression (Fig. 1) [15]. Regression can be reflected by the changes in tumor size, the cellularity of the tumor bed, the presence of lymph node metastases and of ductal carcinoma in situ (DCIS). Since all of these factors may affect prognosis, it is essential that all are represented in the histopathological findings [16]. One of the most essential prognostic factors in breast cancer after NAT continues to be the size of the invasive cancer. In case of unifocal tumors the largest tumor dimension will produce the ypT category, while in cases of multifocal ones the largest diameter of cancer cell containing tissue will be the defining factor.

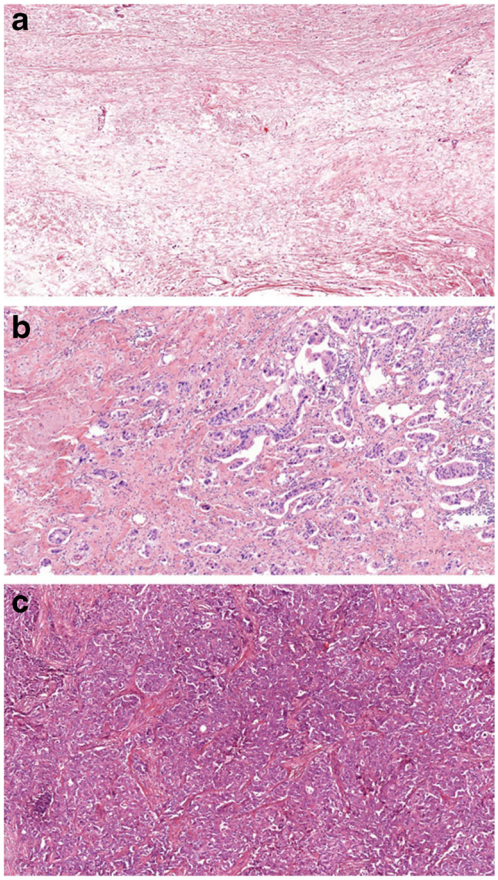

Fig. 1 Spectrum of tumor regression: Complete pathological regression (**a**), partial regression (**b**) and lack of regression (**c**) (HE, A: 4x, B and C: 10x)

The evaluation of regression remains a complicated and versatile task especially due to worldwide application of numerous grading systems. The firstly described National Surgical Adjuvant Breast and Bowel Project (NSABP) B18 classifies all NAT cases into two groups. The first group contains pCR cases (including ypT0 and ypTis) whereas the second group refers to all residual invasive tumor cases [17]. Further regression grading systems, namely Chevallier, Sataloff, Miller-Payne, Denkert-Sinn, Residual Cancer Burden (RCB), TR/NR (suggested system in the European guidelines for measuring tumor regression and nodal regression) and Residual disease in breast and nodes (RDBN) define the presence or absence of complete pathological regression with one or more categories for tumors with some regression [18–24]. The TR/NR, Sataloff and RCB systems take residual tumor burden into account, the Chevallier grade considers the presence of some regression, while the Denkert-Sinn grade includes tumor size, and the Miller-Payne system integrates change of cellularity between the biopsy and the resection specimen. The Sataloff, TR/NR and RCB grading systems include lymph node status as well [22, 19, 11]. The RDBN score can be calculated by the following equation RDBN = 0,2xtumor size (mm) + Nottingham histologic grade (1–3) + lymph node involvement (0–3). According to the RDBN score a good (⩽3.4), a moderate (3.4 < and ⩽5.4), and poor (>5.4) prognostic group were identified [24]. The quantification of residual tumor can be performed by using the RCB calculation. The algorithm was developed by Symmans and coworkers and takes notice of the two largest diameters of the residual tumor, the presence and proportion of DCIS and the number of metastatic lymph nodes with the size of the largest nodal metastasis [22]. The evaluation of RCB is supported by the online available RCB calculator (http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3).

Table 1 represents tumor regression grading systems evaluated in our study and defines the differences among them. Although these grading systems are validated, none of them are accepted internationally. The Hungarian protocol in regression grading was recommended by the 3rd Hungarian Consensus Conference on Breast Cancer in 2016 and is practically identical with the recommendation of the European Working Group for Breast Screening Pathology (EWGBSP) [11, 23]. In Germany, the Denkert-Sinn grade is utilized, while in the USA and many other countries the RCB becomes increasingly adopted.

Table 1 Tumor regression grading systems for breast cancer specimen after NAT (DCIS: ductal carcinoma in situ, RCB: Residual Cancer Burden, pCR: pathological complete regression, NAT: neoadjuvant therapy)

TR/NR [23]	Chevallier [18]	Sataloff [19]	RCB [22]	Denkert-Sinn [21]	Miller-Payne [20]
TR1a: No residual carcinoma.	G1: Disappearance of all tumor either on macroscopic or microscopic assessment.	TA: Total or nearly total therapeutic effect (i.e. isolated tumor cells).	pCR: ypT0 and ypTis: 0	TRG0: No signs of regression.	G1: No change or some alteration to individual malignant cells but no reduction in overall cellularity.
TR1b: No residual invasive tumor but DCIS present.	G2: Presence of in situ carcinoma only in the breast, without invasive tumor and tumor cells in the lymph nodes.	TB: Therapeutic effect subjectively superior to 50%.	(RCB index score) RCB-I: 0,1–1,35 (RCB index score)	TRG1: Tumor sclerosis with focal inflammation and/or minimal cytopathic changes (>5 mm).	G2: A minor loss of tumor cells but overall cellularity still high; up to 30% loss.
TR2a: Minimal residual disease/near total effect (e.g. < 10% of tumor remaining).	G3: Presence of invasive carcinoma with stromal alteration, such as sclerosis or fibrosis.	TC: Therapeutic effect less than 50%, but evident effect.	RCB-II: 1,36-3,27	TRG2: Great amount of tumor sclerosis. May be multifocal, presence of minimally invasive tumor (Not more than 5 mm, usually with intraductal spread).	G3: Between an estimated 30% and 90% reduction in tumor cells.
TR2b: Evidence of response to therapy but with 10–50% of tumor remaining.	G4: No or few modifications of the tumoral appearance.	TD: No therapeutic effect.	(RCB index score) RCB-III: >3,28	TRG3: No signs of residual invasive tumor.	G4: A marked disappearance of tumor cells such that only small clusters or widely dispersed individual cells remain; more than 90% loss of tumor cells.
TR2c: > 50% of tumor cellularity remains evident, when compared with the previous core biopsy sample, although some features of response to therapy present.			(RCB index score)	TRG4: No signs of invasive or in situ tumor.	G5: No malignant cells identifiable in sections from the site of the tumor; only vascular fibroelastotic stroma remains often containing macrophages. However, ductal carcinoma in situ (DCIS) may be present.
TR3: No evidence of response to therapy					
NR1: No evidence of metastatic disease and no evidence of changes in the lymph nodes.		NA: Therapeutic effect, but no metastasis.			
NR2: Metastatic tumor not detected but evidence of response/down-staging, e.g. fibrosis.		NB: No metastasis, no therapeutic effect.			
NR3: Metastatic disease present but also evidence of response, such as nodal fibrosis.		NC: Therapeutic effect, but metastasis.			
NR4: Metastatic disease present with no evidence of response to therapy.		ND: Metastasis, no therapeutic effect.			

The aim of our study was to evaluate the prognostic impact (on disease-free and overall survival) of the different tumor regression grading systems in breast cancer patients treated with NAT. We also aimed to identify which of the grading systems could best reflect prognosis.

Materials and Methods

NAT receiving, consecutive patients operated on for histologically verified invasive breast carcinoma at the Department of Surgery, University of Szeged or Bács-Kiskun County Teaching Hospital, Kecskemét between 1999 and 2019 were included in our retrospective study. Follow up data were collected from medical charts.

The following clinical and pathological variables were obtained for analysis: age, gender, localization, type of neoadjuvant and surgical treatments, DFS and OS; histological type and grade of cancer in previous core biopsy and surgical specimen, completeness of the resection, vascular invasion, size - possibly in 2 dimensions, ypT, ypN, ystage, tumor cell density, tumor cellularity in biopsy and resection specimens, presence and proportion of DCIS, presence of metastasis and/or regression in lymph nodes, size of metastatic deposits and receptor status (estrogen receptor - ER, progesterone receptor – PR, and human epidermal growth factor receptor-2 – HER2). Tumor cell density was defined as the proportion of viable tumor cells in the complete tumor bed, not including necrosis or DCIS.

Regression grades (NSABP-B18, TR/NR, Chevallier, Sataloff, Denkert-Sinn, Miller-Payne, and RCB) and morphological variables were correlated with DFS and OS data using Kaplan-Meier estimates. Patients were followed from the date of initiation of NAT until the time of recurrence or tumor-related death. Patients alive without recurrence and patients dying from other causes were censored at the time of the last follow-up and death, respectively. The log-rank test was used for pairwise comparisons. All statistical tests were two-sided and $p < 0.05$ values were considered statistically significant. The parameters found significant in the univariable models were entered in multivariable Cox proportional hazard model to identify factors of independent prognostic significance. Statistical models were fitted using SPSS Statistics V.22.0 software (IBM, SSPS 22.0, Armonk, NY USA).

This retrospective study was approved by the regional ethical committee of the Albert Szent-Györgyi Clinical Centre of the University of Szeged.

Results

Data of 746 patients who underwent NAT and surgical resection were collected. The median patient age was 55 years (range: 26–91) and 2 of them were males. Table 2 summarizes the oncological and surgical treatments of all patients in the examined population. The majority of patients received primary chemotherapies, whereas 16.4% got primary endocrine therapy. Regarding primary systemic chemotherapy, the majority of patients were given third generation (taxane containing) regimens. 11.2% of the patients had been given second generation (anthracycline based) chemotherapeutics. Patients who received a combination of platinum compounds with cyclophospamide fell into the "others" category. Anti-Her2 treatment was essentially given in combination with chemotherapy. Concerning primary endocrine therapy, the most frequent agents used were aromatase inhibitors and the average hormonal therapy treatment period was 1 year. The majority of patients underwent mastectomy. Re-excisions were rarely performed and were done because of positive or close resection margins. Regional lymph nodes were examined in almost all cases, most commonly by means of axillary lymph node dissection.

As Table 3 demonstrates, with histological examination, 87.8% of patients had invasive carcinoma „No Special Type" in surgical specimens. Invasive tubular, mucinous, medullary and metaplastic breast cancers were categorized into the others category. The presence of residual DCIS was described in 212 cases. One fifth of the patients achieved pCR. The most frequent pathological tumor category was ypT2

Table 2 Types of NAT and surgical treatment in the examined population (LHRH: Luteinizing hormone-releasing hormone, HER2: Human epidermal growth factor receptor 2, SNB: Sentinel node biopsy, ALND: Axillary lymph node dissection)

Neoadjuvant therapy		
Primary hormonal therapy ($n = 123 = 100\%$)	n	%
Tamoxifen	4	3.25
Aromatase inhibitor	102	82.93
Tamoxifen and LHRH-analogue	3	2.44
Aromatase inhibitor and LHRH-analogue	14	11.38
Primary chemo- and target therapy ($n = 623 = 100\%$)	n	%
Second generation chemotherapy	70	11.24
Third generation chemotherapy	550	88.28
Others	3	0.48
Anti-HER2 (in combination therapy)	91	14.60
Number of cycles should go under Primary chemo-and target therapy)	5.60	6.00
Surgical treatment ($n = 746 = 100\%$)	n	%
Breast conserving excision	249	33.38
Mastectomy	497	66.62
Re-excision	17	2.28
SNB	72	9,65
ALND	593	79,49
SNB + ALND	60	8.04

Table 3 Morphological features of breast cancer in the examined population (NST: Invasive carcinoma „No Special Type", ILC: Invasive lobular carcinoma, DCIS: Ductal carcinoma in situ, R: Resection, V: (Lympho) vascular invasion, Pn: Perineural invasion, HR: Hormone (estrogen and/or progesterone) receptor, HER2: Human epidermal growth factor receptor 2; ypT and ypN categories are defined by AJCC. Not all evaluated features were available for all cases, hence the differences in the sums of some rows

Histological subtype (core)	*n*	%
NST	655	87.80
ILC	55	7.37
others	36	4.83
grade	*n*	%
1	35	4.69
2	246	32.98
3	420	56.30
No data	45	6.03
DCIS (present)	212	28.41
R (R1/R0)	130/616	17.42
V (V1/V0)	151/560	21.23
Pn (Pn1/Pn0)	10/324	2.99
Hormonal state	*n*	%
HR +, HER-2 -	439	58.85
HER-2 +, HR +/-	126	16.89
Triple negative	181	24.26
ypT	*n*	%
ypT0	106	14.21
ypTis	28	3.75
ypT1a	48	6.43
ypT1b	25	3.35
ypT1c	110	14.75
ypT2	151	20.24
ypT3	55	7.37
ypT4	29	3.90
No data	194	26.00
ypN	*n*	%
ypN0	290	38.87
ypN1	227	30.43
ypN2	127	17.02
ypN3	61	8.18
No data	41	5.50
ystage	*n*	%
0	9	1.21
I	75	10.05
II	209	28.02
III	207	27.75
IV	6	0.80
No data	240	32.17

(20.2%), while 38.9% of the patients fell in with ypN0 category. Most cases expressed ER and PR, while HER-2 positivity was observed in 126 cases (17%). Median patient follow up was 53.8 months (range: 4–238 months; average: 65.1 months). Relapse occured in 34.85% of cases during the follow-up period and tumor specific death was observed in 122 (16,3%) cases.

According to the original histopathology reports, the numbers of patients evaluated with the different regression grading systems are as follows: NSABP-18 grade: 746, Chevallier-grade: 717, Sataloff (T) grade: 494, Miller-Payne grade: 386, TR grade: 392, Denkert-Sinn grade: 348, RDBN grade: 405 and RCB: 212. Figure 2 *and* Supplementary Fig. 1-8 show the disease-free survival and overall survival estimates of the different grading systems, respectively. The DFS and OS estimates of complete pathological regression (ypT0) and residual in situ carcinoma (ypTis) together were significantly different from the survivals of tumors without regression and moderate regression categories in all grading systems ($p < 0.001$). There was no significant DFS and OS difference observed between the ypT0 and ypTis categories. Survival values associated with different partial or no response categories showed no significant differences between each other, with the exceptions of DFS for the RCB-I vs III and II vs III categories.

As all regression grading systems showed a significant effect on survival in the univariable models, they were all entered in the multivariable Cox-regression analysis. According to our results the RCB ($p = 0.019$) proved to be an independent prognostic marker for DFS, whereas the ystage ($p = 0.011$) and lymph node status ($p = 0.045$) showed similar results for OS.

Discussion

Due to the increasing use of NAT in patients having locally advanced breast cancer, more and more articles about its effectiveness have been published [8]. Although imaging techniques serve as great options to monitor regression after NAT, histopathological review remains the gold standard in the evaluation procedure [25]. Although several national guidelines aiming at the standardization of specimen cut up and reporting have been introduced, for example in Australia, Belgium, Germany, the UK, Netherlands, the USA and Hungary, there is no international agreement in the interpretation of tumor regression, in the definition of pCR, and in the measurement of tumor size in cases where fibrosis develops as a result of NAT or multifocality is present [11, 26–31].

Several regression grading systems have been introduced which are based on prognostic markers such as tumor size (in one or more dimensions), change in cellularity, presence of DCIS, presence of regression or metastasis in lymph nodes and the size of lymph node metastasis [17–23]. The definition of pCR and the complete lack of regression -as the extreme ends of the regression spectrum- are common features of these systems which also define one or more subgroups for partial regression

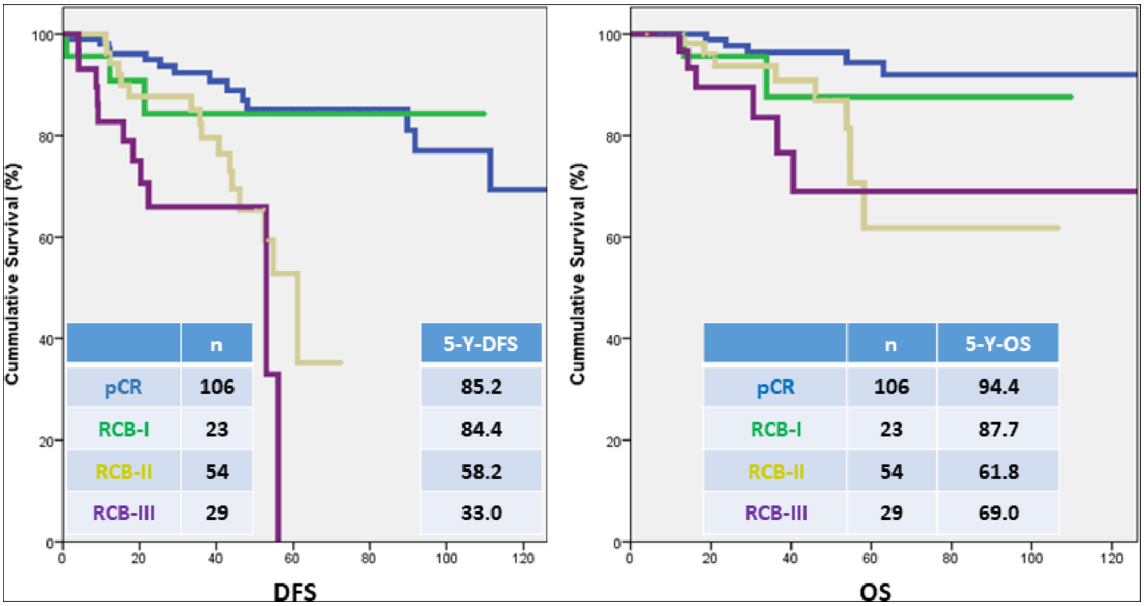

Fig. 2 Kaplan-Meier evaluation of the RCB grading system for DFS and OS. Significant differences were found between DFS estimates of pCR vs. RCB-II ($p < 0.001$), pCR vs. RCB-III ($p < 0.001$), RCB-I vs. RCB-III ($p = 0.035$), RCB-II vs. RCB-III ($p = 0.05$). Regarding OS, significant differences were observed between estimates of pCR vs. RCB-II ($p = 0.005$) and pCR vs. RCB-III ($p < 0.001$), respectively (RCB: Residual Cancer Burden, DFS: disease-free survival, OS: overall survival, pCR: pathological complete regression)

categories. Despite of the relative abundance of regression grading systems, there is a lack of international consensus on their application. All grading systems attempt to quantify the degree of regression or the amount of residual tumor, and there is agreement that a quantitative characterization of tumor regression is necessary for the evaluation of the effectiveness of NAT, and may have further role in therapeutic decisions (e.g. alternative treatments if no regression is present).

Although the presence of residual DCIS has been reported to convey a worse prognosis than complete absence of in situ and invasive carcinoma, there was no significant difference between OS and DFS estimates of ypT0ypN0 and ypTisypN0. Our results are therefore supporting the more permissive definition of pCR (including ypTis) defined by the United States' FDA and endorsed by the AJCC [13, 14] and the European Guidelines [23]. Our findings regarding the prognostic impact of pCR are in keeping with those of others, since patients with pCR had a favorable prognosis (both in DFS and OS) compared to patients having partial regression. Concerning the subcategories of partial regression, we observed significant differences only between DFS estimates of certain RCB classes, namely between RCB-I vs. RCB-III and RCB-II vs. RCB-III classes. No other regression classification system showed subgroups of partial response with significant differences between each other.

The RCB system was developed by Symmans and coworkers in 2007. In their study, the prognostic role of morphological variables was evaluated by Cox-regression, and from variables found statistically significant, a complex equation was produced to determine the RCB index score. The RCB index score was correlated with survival data and cut-off scores were assigned to identify the RCB classes. In concordance with the original results by Symmans et al., there were no significant differences in DFS and OS estimates between RCB-0 (pCR) and RCB-I (nearly pCR) classes. Furthermore, the multivariable Cox regression models for DFS suggest that the RCB system is the only significant prognosticator among regression grades ($p = 0.019$) [22].

In a subsequent publication, Symmans and co-authors have demonstrated that the RCB is a prognostic marker independent from the type of primary chemotherapeutic regime and significant differences have been described between RCB classes among hormone receptor positive (ER+ and/or PR+, HER2-), HER-2 positive (hormone receptor positive or negative) and triple negative (ER-, PR, HER2-) breast cancer cases [32]. Our results support these conclusions, and moreover, by adding primary endocrine therapy to our calculations, RCB remained an independent prognostic marker.

Considering literature data and our results, RCB is highly recommended to be included in routine histopathological reports of breast cancers treated with NAT. Although most elements of RCB are routinely part of histopathological reports, the characterization of some others, namely the second largest dimension of tumor size, the cellularity and the proportion of DCIS, require experience in practice. The standardization of reporting these markers are supported by the concise guidance at the RCB calculator website [32].

Corben and co-authors emphasized the role of the presence and size of lymph node metastasis. Those grading systems that include lymph node status (RCB, Sataloff, TR-NR, RDBN)

show better correlation with long term survival than those including only invasive tumor size and cellularity [5]. In keeping with Corben's results, we found the ypN category as a significant prognostic marker according to OS estimates. The presence of nodal metastasis was associated with poor prognosis regardless of the presence or absence of nodal regression. Corben and coworkers suggested the RDBN grade to be the most optimal regression grading system among the 5 investigated [5]. However, we found no significant differences in DFS or OS between the RDBN groups with Cox regression. This contrast may be due to different factors, like the differences in patients and in cohort sizes (62 vs 746) and the inclusion of primary endocrine therapy in the present analysis.

Concerning the limitations of our study, it has to be mentioned that not all grading systems were assessed in all cases. Several patients had gone through lymphadenectomy prior to NAT and this could influence the prognostic value of a given grading system. Furthermore, the institution where the core needle biopsy was taken differed from the place of surgery in many cases, therefore the comparison of these samples was not always possible. On the other hand, the strengths of our evaluation include a large cohort of patients having primary endocrine treatment or chemotherapy with relatively long follow-up data. Our multicenter study was based on two Hungarian departments with identical cut-up and reporting protocol, following the recommendations of the 3rd Hungarian Consensus Conference on Breast Cancer. Although not all grading systems were evaluated in all cases, even the smallest group included more than 200 patients, and this proved sufficient for statistical analysis.

In our retrospective study involving the grading of response to NAT in 746 patients, we have evaluated and compared the impact of different regression grading systems on DFS and OS. According to our results, the RCB was the best prognostic factor, therefore we would encourage its utilization in routine histopathological reports.

Acknowledgements We gratefully acknowledge the assistance of Mihály Dezső in designing figures.

Authors' Contributions All authors contributed to the study conception and revision. Data collection was performed by Anita Sejben, Renáta Kószó and Zsuzsanna Kahán. Statistical analysis was performed by Anita Sejben and Tamás Zombori. The first draft of the manuscript was written by Anita Sejben and Tamás Zombori and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Information Open access funding provided by University of Szeged.

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code Availability There are no restrictions on the availability of materials, data and code.

Compliance with Ethical Standards

This retrospective study was approved by the institutional ethical committee of the Albert Szent-Györgyi Clinical Centre of the University of Szeged.

Conflict of Interest The authors declare that they have no conflict of interest.

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