
# Evaluation of diagnostic and prognostic features of breast cancer

**Ph. D. Thesis**

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LIST OF FULL 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

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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

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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

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## 1. 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. As such, it is one of the most investigated fields of cancer medicine with research in many aspects of the disease. We focused on the immunophenotyping of triple negative BCs (TNBCs) with breast markers, on the prognostic subclassification of TNBCs and on the comparison of different tumor regression grading systems in patients having locally advanced breast cancer (LABC) and receiving primary systemic treatment (PST).

The expression of estrogen receptor (ER), progesterone receptor (PR), sometimes even human epidermal growth factor receptor-2 (HER2), or "breast markers" like GATA3 (GATA binding protein 3), mammaglobin (MG), GCDFP15 (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. 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.

Prediction of prognosis remains essential to clinicians in their decision-making process, helps stratifying patients by risk and better allows preparing individual treatment plans.

The Nottingham Prognostic Index (NPI) takes tumor size, nodal stage and tumor histological grade into consideration on the basis of a multivariable prognostic assessment of BCs. On the basis of its equation and the values of the NPI, patients were originally divided into three prognostic categories, but later the prognostic groups were further subdivided to form the very good (VGPG), the good (GPG), the moderate I (MPGI), the moderate II (MPGII), the poor (PPG) and the very poor prognostic groups (VPPG). The NPI has proven to be a valid prognostic tool in BC risk stratification and treatment.

A more complex prognostic model, PREDICT was published by Wishart et al in 2010. Based on the factors that were found to hold independent prognostic value, a prognostic model was

established; it includes the presence of carcinoma in situ, age at diagnosis, menopausal state, ER, PR, HER2 and Ki-67 status, invasive tumor size, tumor grade, method of tumor detection, number of positive lymph nodes and whether nodal involvement is only micrometastatic. The online calculator estimates overall survival (OS) for 5, 10 and 15 years.

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. A pooled analysis of 2148 patients 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. Based on the results, an equation and an online tool (PrognosTILs) were developed for survival estimates of early stage TNBCs. With this application, the 5-year and 10-year OS and disease free survival (DFS) estimates can be calculated.

Treatment of LABC patients has been one of the great challenges of breast oncology for a long time. 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. This downstaging can allow some patients who would have had mastectomy as surgical treatment to be treated with breast conservation.

The characterization of regression differs from country to country due to lack of international consensus on definitions. The histology of post-NAT tumors represents a spectrum from pathological complete regression (pCR) to tumor growth and progression. The evaluation of regression remains a complicated and versatile task further compromised by the worldwide application of numerous grading systems. The firstly described National Surgical Adjuvant Breast and Bowel Project (NSABP) B18 was followed by Chevallier, Sataloff, Miller-Payne, Denkert-Sinn, Residual Cancer Burden (RCB), TR/NR (Tumor regression/ Nodal regression) and Residual disease in breast and nodes (RDBN) regression grading systems.

The quantification of residual tumor can be performed by using the RCB calculation. The algorithm developed by Symmans and coworkers takes notice of the two largest diameters of the residual tumor, tumor cellularity, the proportion of DCIS and the number of metastatic lymph nodes with the size of the largest nodal metastasis. The evaluation of RCB is supported

by the online RCB calculator. Although these grading systems are validated, neither is used universally.

## 2. AIMS

1. To assess the added value of SOX10 (SRY-related HMG-box 10) immunohistochemistry (IHC) to known GATA3, MG, GCDFP15 and NY-BR-1 statuses in highlighting mammary origin in a series of CK5 positive primary TNBCs;

2. To compare the validity of three multivariable analysis derived prognostic systems, the Nottingham Prognostic Index, 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, RDBN and RCB in BC patients receiving PST.

## 3. MATERIALS AND METHODS

### 3.1. IMMUNOHISTOLOGICAL EVALUATION OF TNBCS

A series of CK5 positive TNBCs characterized by GATA3, MG, GCDFP15 and NY-BR-1 IHC in a previous analysis was used for SOX10 IHC. Tissue microarrays (TMAs) constructed from archived paraffin-embedded blocks and incorporating 20 tumor tissue cores were cut at 3 to 4-micrometer-thick sections for SOX10 IHC using a monoclonal mouse antibody. 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.

### 3.2. EVALUATION OF NPI, PREDICT AND PROGNOSTILS IN TNBCS

Patients operated on for histologically verified TNBC 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 were collected from medical charts. For DFS and OS, 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 NPI was calculated as 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. The Nottingham Prognostic Groups were classified as very good prognostic group (VGPG): ≤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.

The predicted OS and DFS estimates of PrognosTILs were obtained from an online calculator (https://cesp-proxy2.vjf.inserm.fr/shiny/prognosTILs/). TILs were quantified according to the International TILs Working Group recommendations and rules.

The anticipated OS evaluations of PREDICT were also determined with the relevant online calculator (https://breast.predict.nhs.uk/predict_v2.0.html).

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. 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 significant parameters from the univariate models were entered in a multivariable Cox proportional hazard model to identify independent prognostic factors. The 3 predictive models were compared by means of receiver operating characteristic (ROC) curves.

### 3.3. EXAMINATION OF TUMOR REGRESSION GRADING SYSTEMS IN BC PATIENTS WHO RECEIVED NAT

NAT receiving, consecutive patients operated on for histologically verified invasive breast carcinoma at the Departments 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.

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-

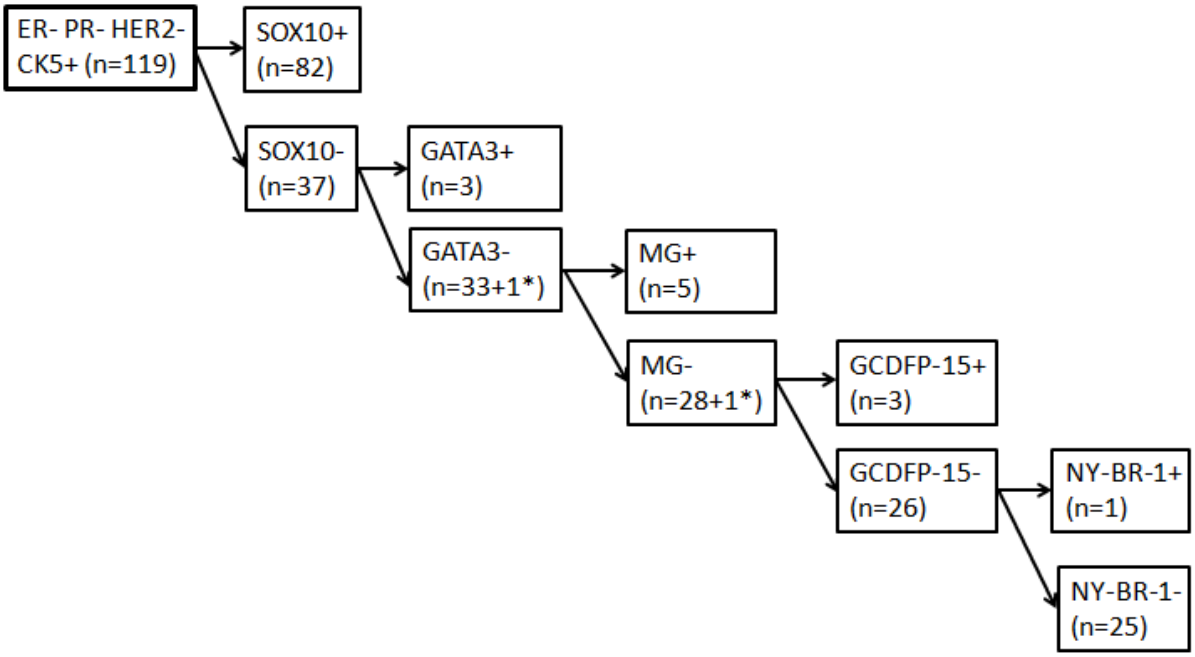
rank test was used for pairwise comparisons. All statistical tests were two-sided and $p<0.05$ values were considered statistically significant. The parameters found significant in the univariable models were entered in multivariable Cox proportional hazard model to identify factors of independent prognostic significance.

## 4. RESULTS

### 4.1. IMMUNOHISTOLOGICAL EVALUATION OF TNBCS

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, 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.

Of the 94 GATA3 negative cases, 61 cases were positive with SOX10. 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, as shown below:




* 1 not assessable for GATA3 and MG

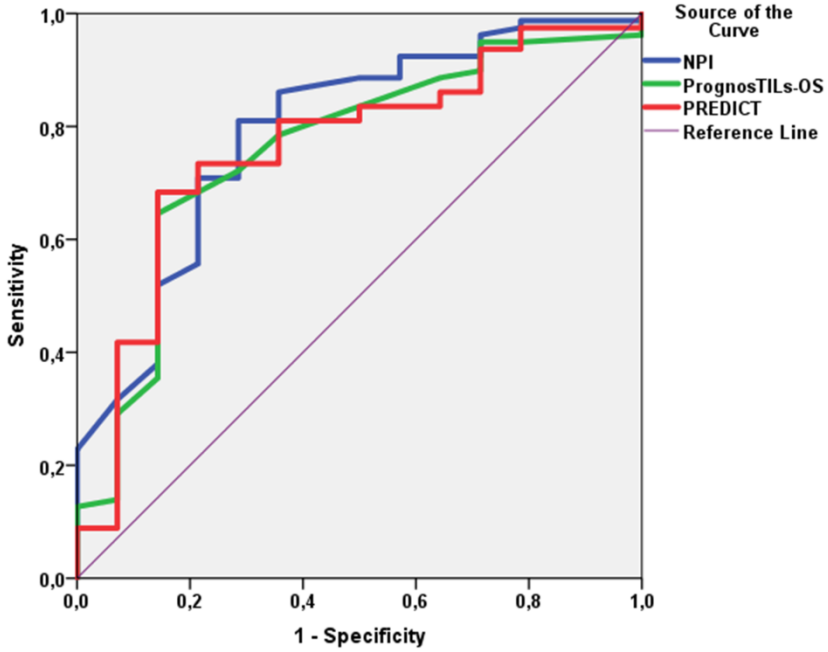
## 4. 2. EVALUATION OF NPI, PREDICT AND PROGNOSTILS IN TNBCS

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 OS and DFS were 66.8 months and 59.9 months, respectively (range for OS: 7-170 months; range for DFS: 2-170 months). There were 11 cases with local or regional recurrence, 23 cases with distant metastasis and 2 cases with both local and distant types of recurrence. The median time to recurrence was 41 months (range: 2-170 months). The median follow up was 56 months (range: 7-170 months).

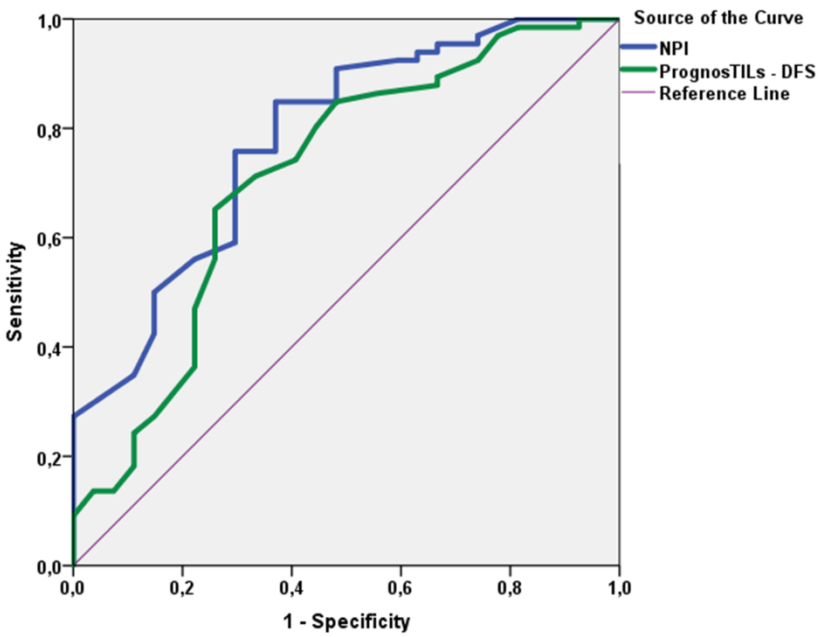
The predictions from PrognosTILs and PREDICT and the NPI scores were established in 93, 126 and 125 cases, respectively. For PrognosTILs, the comparison of predicted survival estimates and outcomes revealed that the predicted OS estimates of the patients dead of disease (DOD) were significantly lower 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 ($p<0.001$). For PREDICT, the statistical analysis strengthened, that the predicted OS estimates of patients DOD were significantly lower, than those of patients who were alive ($p=0.020$).

Significant differences were detected between OS and DFS estimations of different NPI derived prognostic groups. In the univariate Cox-regression, type of surgery (pDFS=0,017), pT (pDFS=0,009) and pN categories (pDFS<0,001), stage (pDFS<0,001), adjuvant therapy (pDFS=0,003) and NPI (pOS=0,022; pDFS<0,001) were found to be a significant prognostic variables. The multivariate Cox proportional hazard model revealed that among the variables found significant in univariate models, 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).

For the 5-year-OS estimates, the area under the ROC curve (AUC) of PrognosTILs, PREDICT and NPI were 0.759, 0.762 and 0.792, respectively:




For DFS, the AUC values of PrognosTILs and NPI were 0.713 and 0.781, respectively:

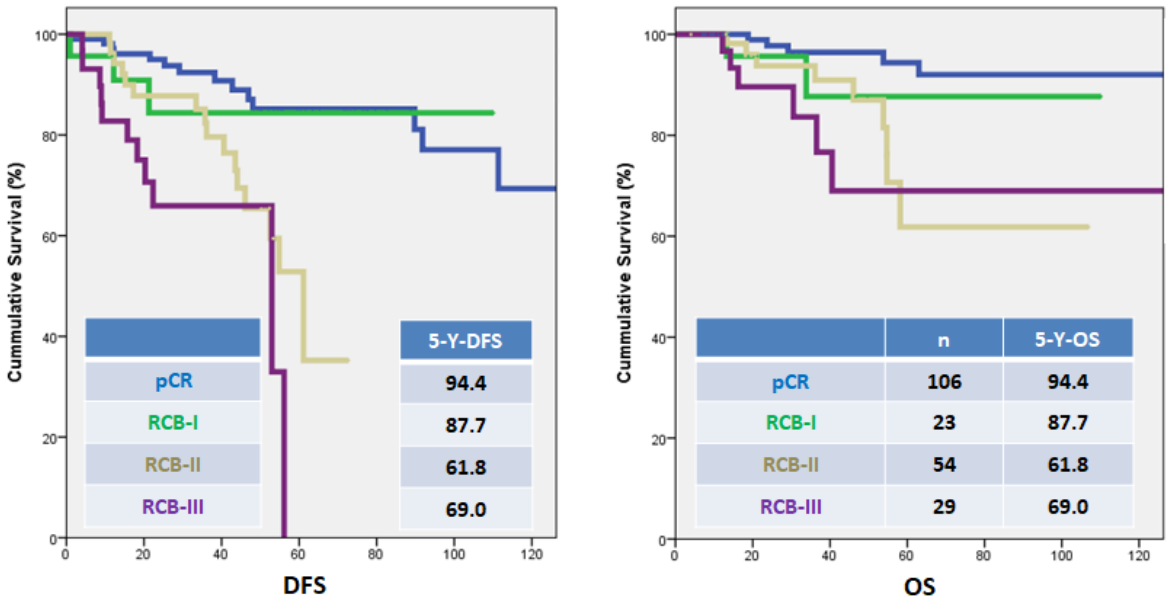



## 4.3. EXAMINATION OF TUMOR REGRESSION GRADING SYSTEMS IN BREAST CANCER PATIENTS WHO RECEIVED NAT

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. 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 cyclophospamide 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.

According to the original histopathology reports and previous databases, the numbers of patients evaluated with the different regression grading systems were 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. 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.

## 5. DISCUSSION

### 5.1. IMMUNOHISTOLOGICAL EVALUATION OF TNBCS

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. 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, GCDFP15 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. When looking at literature data, 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). In 41% of our cases, the SOX10 staining was focal, in these, the application of the TMA technique can be a limitation.

### 5.2. EVALUATION OF PROGNOSIS IN 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. PREDICT, to our knowledge has not yet been evaluated for TNBCs alone, whereas PrognosTILs is relatively recent for larger validation on comparison studies.

Concerning the NPI, we demonstrated that there are significant differences among OS and DFS estimates of certain prognostic groups. In univariate Cox analysis, type of surgery, pT, pN, stage, NPI and adjuvant therapy were found significant prognostic variables. The multivariate Cox-regression strengthened that NPI is an independent predictor of OS and DFS in TNBCs. The other two combined prognosticators could not be entered in this analysis, but their predicted survivals (DFS and OS, respectively) were significantly shorter in patients developing recurrences or dying of disease than those in patients without such events.

The direct comparison of the multivariable prognosticators was performed with ROC curve analysis. All three predictors of outcome reflect fair performance with AUCs 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. It can be inferred that any of the three compared combined prognostic approaches 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 VGPG 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. 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.

## 5.3. EXAMINATION OF TUMOR REGRESSION GRADING SYSTEMS IN BREAST CANCER PATIENTS WHO RECEIVED NAT

Due to the increasing use of NAT in patients having LABC, an increasing number of articles about its effectiveness have been published. The examination of residual tumor and grading regression is the pathologists' job. 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.

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. In keeping with previous 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. 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, which are 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 three 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 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.

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.

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