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Dynamic evaluation of the immune infiltrate and immune function genes as predictive markers for neoadjuvant chemotherapy in hormone receptor positive, HER2 negative breast cancer

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ABSTRACT

Gene expression (GE) signatures and Tumor Infiltrating Lymphocytes (TIL) enumeration are predictive for response to neoadjuvant chemotherapy in HR- and in HER2+ breast cancer, but data are conflicting in HR+/HER2- disease. This study aimed to explore their predictive value in this subset, measured both at baseline and after short exposure to chemotherapy. Specifically, the PROMIX phase 2 trial enrolled patients with locally advanced HER2- BC to receive six cycles of epirubicin and docetaxel, plus bevacizumab during cycles 3–6. Patients underwent tumor biopsies at baseline and after cycle 2 for GE profiling and enumeration of TIL, FOXP3+ T-cells and CD163+ macrophages. An immune related gene module and the quantification of the immune infiltrate were analyzed for association with pathologic complete response (pCR), decrease in tumor size and disease-free survival (DFS). Of the 150 patients enrolled in PROMIX, 113 were HR+/HER2-. Baseline GE and immune cell enumeration data were available from 71 patients, while data after 2 cycles of chemotherapy were available from 41. At baseline, only GE was statistically significantly associated with higher pCR rates (OR 2.29, 95% CI 1.05 – 5.38, $p = 0.037$) and decrease in tumor size ($r = 0.25$, $p = 0.047$). In contrast, longitudinal data indicate that both GE ($r = 0.54$, $p < 0.001$) and TIL abundance ($p = 0.009$) are stronger predictors for the reduction of tumor size, while low FOXP3+ was statistically significantly associated with an improved DFS ($p = 0.027$). In conclusion, GE analysis, TIL and FOXP3+ enumeration after short-term exposure to chemotherapy carry important predictive information in HR+/HER2- breast cancer at the neoadjuvant setting.

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Introduction

The increasing adoption and use of neoadjuvant chemotherapy (NACT) in early and locally advanced breast cancer (BC) in recent years¹ can be attributed to three factors: the demonstration that it confers similar long-term outcomes to postoperative therapy due to the early eradication of micrometastatic disease;^{2,3} the increase in breast conserving surgical procedures; and the fact that it offers an appropriate platform for *in vivo* testing of chemotherapy activity and discovery of prognostic and predictive biomarkers, due to the ease of response assessment and tissue extraction. Among the putative biomarkers that are under development is the quantitative and qualitative assessment of the immune infiltrate.

The best characterized marker that describes the tumor – host interactions that occur in BC is the enumeration of tumor infiltrating lymphocytes (TILs). Higher TIL counts have been associated with an increased probability for pathologic complete response (pCR) in all disease subtypes, an important outcome following NACT.^{4–6} However, a meta-analysis of 13 studies and 3251 patients suggests that

this association is not apparent in hormone receptor (HR) positive, human epidermal growth factor receptor 2 (HER2) BC.⁷ Confusingly, an analysis of 3771 samples from patients enrolled in six randomized trials demonstrated that higher TIL counts were associated with decreased overall survival (OS) in HR positive BC, although TILs were associated with a higher probability for pCR in the same patients⁶ Moreover, few studies have reported on TIL kinetics under NACT, with the vast majority of those comparing pre-chemotherapy samples with surgical specimens, after the completion of the entire schedule of NACT. Again, the published results are inconsistent, with studies suggesting both improved^{8,9} and worse patient outcomes^{10,11} associated with high TIL counts after NACT. In addition, the prognostic value of Forkhead Box P3 (FOXP3+) T-lymphocytes in BC has been controversial; however, a recent meta-analysis suggests that high FOXP3+ cells are associated with poor recurrence-free survival.¹² A similar association between tumor associated macrophages and poor prognosis in BC has also been suggested.¹³

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 Supplemental data for this article can be accessed on the [publisher's website](#).

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On the other hand, gene expression (GE) signatures have emerged as potent prognostic and predictive biomarkers.^{14,15} Among the biologic processes that can be assessed using GE analysis is immune activation in the tumor microenvironment. Indeed, immune-related gene signatures have been previously developed and validated in BC and shown to be associated with the probability for pCR after NACT in all BC subtypes.^{5,16} Nevertheless, little is known regarding the modulation of gene expression caused by the effects of cytotoxic chemotherapy and whether these short-term changes harbor any predictive or prognostic value. In one published study, 24–96 hours after the administration of chemotherapy a downregulation of immune-related and proliferation genes was noted, while increased residual interferon gamma signaling was associated with poor outcomes.¹⁷

The effect of short periods of NACT on the immune infiltrate and immune-related GE and their comparison as biomarkers are not well characterized. Following previously published results in the same cohort that established immune function as a predictor for pCR in HR positive, HER2 negative patients¹⁸ using a gene module previously published by Desmedt et al¹⁹ we herein explore the predictive power for chemosensitivity of baseline and longitudinal changes after 2 cycles of NACT in the composition of the immune infiltrate and of immune gene expression. The present study is a direct continuation of a previous correlative analysis published from our group regarding HR positive metastatic BC²⁰ where we used another immune gene signature published by Denkert et al.²¹

Results

Patient characteristics and outcomes

The clinical and demographic characteristics, treatment and short and long-term outcomes of the entire study population of the PROMIX trial have been previously presented.¹⁸ In total, 150 patients were enrolled of which 113 were HR positive and 37 ER negative. Both GE and immune cell (IC) data at baseline were available from 71 of the HR positive patients (cohort A) and from 41 HR positive patients after 2 cycles of

NACT (cohort B) (Figure. 1). Table 1 presents the patient characteristics.

pCR events were rare in this HR positive, HER2 negative population with locally advanced tumors: there were $n = 5$ (7.0%) and $n = 2$ (4.8%) patients with pCR after six cycles of NACT in cohort A and B, respectively. After a median follow-up of 5 years, there were 19 and 11 DFS events among patients enrolled in cohort A and B, respectively. The corresponding five-year disease free survival (DFS) rates were 76% (95% Confidence Interval [CI] 67% – 87%) in cohort A and 73% (95% CI 61 – 88%) in cohort B.

Association of baseline immune gene expression and immune cell enumeration with pathologic complete response after neoadjuvant chemotherapy

An Immune Module Score (IMS) was tested for an association with the probability for attaining a pCR at the end of NACT. There was a statistically significant association between the probability for pCR and baseline IMS (Odds ratio [OR] 2.29, 95% CI 1.05 – 5.38, $p = 0.037$) (Figure. 2). In contrast, no statistically significant associations were noted between the probability for pCR and baseline abundance of TILs (OR 1.81, 95% CI 0.08 – 14.34, $p = 0.61$) or the abundances of immunosuppressive cells (OR 1.81, 95% CI 0.08 – 14.34, $p = 0.61$ for CD163+ macrophages and OR 5.25, 95% CI 0.23 – 54.04, $p = 0.19$ for FOXP3+ T regulatory lymphocytes) (Figure. 2).

Baseline immune function and decrease in tumor size after neoadjuvant chemotherapy

As detailed in the discussion section, pCR is a dichotomous endpoint that rarely occurs after NACT in HR positive, HER2 negative breast cancer. Thus, in order to better characterize the relationship of immune function and chemosensitivity at the neoadjuvant setting, we evaluated the association of baseline GE analysis and IC cell enumeration with the percentage of tumor decrease at the final specimen compared to the initial measurement. There was a statistically significant association between greater tumor shrinkage and a higher baseline

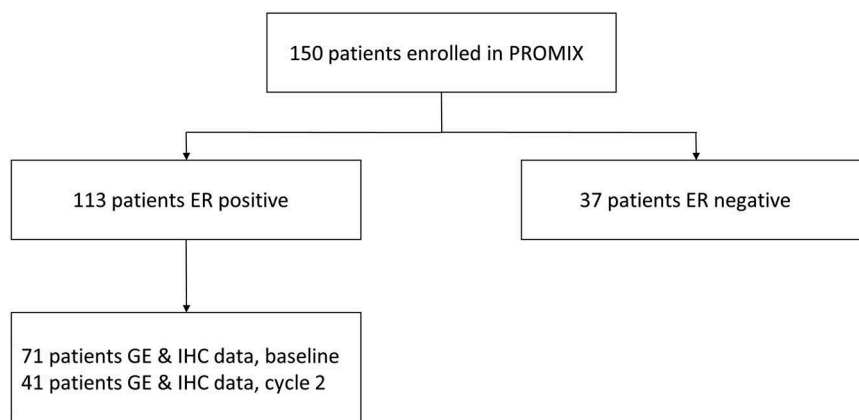


Figure 1. CONSORT flow diagram showing the 150 patients that participated in the PROMIX study and the 71 and 41 estrogen receptor positive patients that were included in cohort A and B respectively of the correlative analysis. Abbreviations: ER: estrogen receptor; GE: gene expression; IHC: immunohistochemistry.

Table 1. Patients' clinical and demographic characteristics.

	Cohort A N = 71 ¹	Cohort B N = 41
Age		
Median (range)	49 (27 – 69)	50 (33 – 69)
Nodal status		
Positive	41 (58%)	23 (56%)
Negative	17 (24%)	11 (27%)
Unknown	13 (18%)	7 (17%)
Intrinsic subtype²		
Luminal A	15 (21%)	4 (10%)
Luminal B	21 (30%)	14 (34%)
HER2 Enriched	11 (15%)	8 (19%)
Basal Like	5 (6%)	2 (5%)
Normal Like	20 (28%)	6 (14%)
Unknown	0 (0%)	7 (17%)
Ki-67		
<15%	20 (28%)	9 (22%)
≥15%	46 (65%)	30 (73%)
Unknown	5 (7%)	2 (5%)

¹In one patient, the receptor status was imputed from gene expression data

²The molecular subtype was determined using the Absolute assignment of breast cancer intrinsic molecular subtype (AIMS) methodology, as described by Paquet et al 2014

IMS ($r = 0.25$, $p = 0.047$). In contrast, there were no statistically significant associations between the degree of tumor shrinkage and baseline immune cell abundances (Figure. 3).

Longitudinal changes in immune biomarkers after short-term exposure to chemotherapy and their association with chemosensitivity

Among patients with available GE and IC data after 2 cycles of NACT (cohort B), there was a stronger association between greater tumor shrinkage and a higher IMS after short-term exposure to chemotherapy ($r = 0.54$, $p < 0.001$) (Figure. 4).

For patients with longitudinal GE data at baseline and cycle 2 ($n = 49$), IMS at cycle 2 but not IMS at baseline was statistically significant in a multivariate analysis (supplemental table S1), indicating that short-term exposure values may carry more information about chemosensitivity than baseline values. In line with this, TILs abundance after 2 cycles of NACT (cohort B) was also statistically significantly associated with tumor shrinkage ($p = 0.009$, Figure. 4). A similar trend

was seen for FOXP3+ cells after 2 cycles ($p = 0.057$) (Figure. 4).

There were only 2 pCR events in patient cohort B, precluding any statistically significant associations with this endpoint.

Immune function as a predictor of long term outcomes

Baseline immune function as assessed by GE or IC was not found to predict DFS (supplemental Figure. S1). In contrast, a FOXP3+ abundance of <10% at cycle 2 was statistically significantly associated with an improved DFS ($p = 0.027$), while there was also a trend for association between lower IMS at cycle 2 and prolonged DFS ($p = 0.066$) (supplemental Fig. S2).

Discussion

A previous analysis in the same patient cohort indicated that among other gene signatures, immune related gene expression – using a different gene signature compared to the present study – was correlated with pCR in HR positive patients.¹⁸ However, previously published studies have not definitively established the predictive value of TILs at the neoadjuvant setting of HR positive BC, nor have they evaluated the dynamic evolution of the host immune response during treatment. Thus, we aimed to explore different aspects of host immunity and evaluate the predictive power of the longitudinal changes in the immune response under the effects of chemotherapy. This correlative study from a prospective trial contributes to the existing literature and demonstrates that, at baseline, only GE was predictive for short term outcomes after NACT. These results are consistent with previous work from our group that demonstrated that immune related gene expression (using the same signature as in the present study) in RNA extracted from metastatic biopsies, but not TILs, was predictive for chemosensitivity in metastatic HR positive, HER2 negative BC.²⁰ Moreover, in the present study the baseline abundance of FOXP3+ T-lymphocytes and CD 163+ macrophages was shown to be neither predictive nor prognostic.

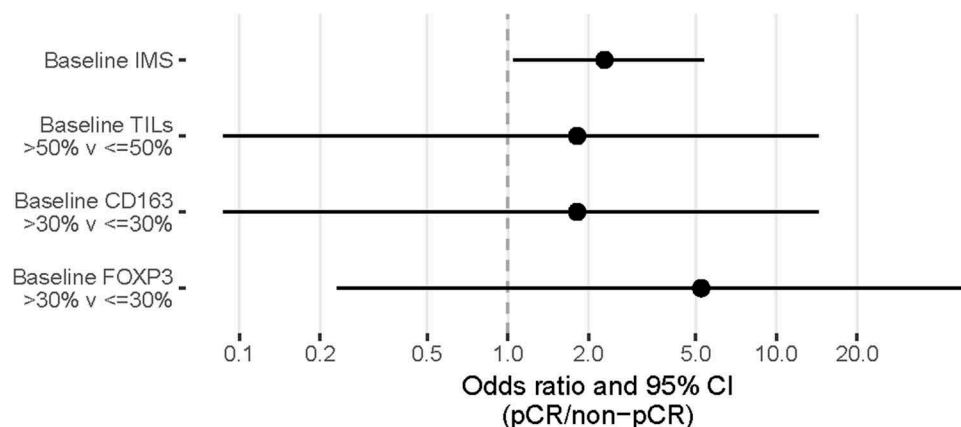


Figure 2. Baseline immune function and pathologic complete response after neoadjuvant chemotherapy (univariate logistic regression, cohort A). Abbreviations: CD163: cluster of differentiation 163; CI: confidence interval; FOXP3: forkhead box P3; IMS: immune module score; pCR: pathologic complete response; TILs: tumor infiltrating lymphocytes.

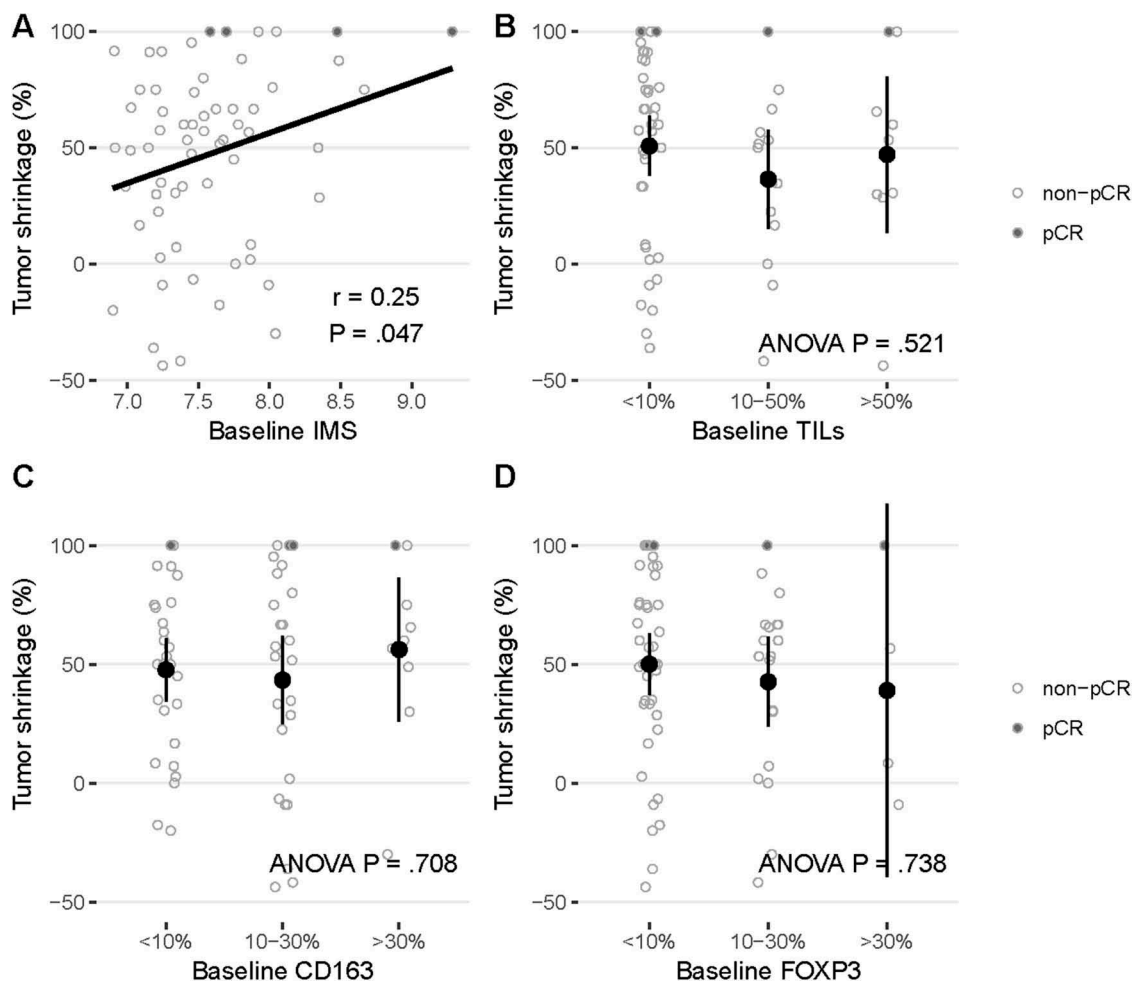


Figure 3. Correlation of baseline immune function with the percentage decrease in tumor size after neoadjuvant chemotherapy (cohort A). Abbreviations: ANOVA: analysis of variance; CD163: cluster of differentiation 163; FOXP3: forkhead box P3; IMS: immune module score; pCR: pathologic complete response; TILs: tumor infiltrating lymphocytes.

Importantly, the evaluation of longitudinal data on the immune gene signature after short-term exposure to chemotherapy was shown to provide additional predictive information. In addition, both GE analysis and TILs enumeration at cycle 2 were found to be associated with chemosensitivity, while low FOXP3+ T-lymphocytes were prognostic for long term outcomes. This observation is consistent with the hypothesis that priming the anti-tumor immune response with the release of neoantigens caused by the effects of short-term exposure to antineoplastic therapy may be a critical step towards the induction of immunogenic cancer cell death.²¹ Interestingly, the priming of anti-tumor immunity is not limited to cytotoxic therapy but has been demonstrated with targeted therapy as well.²² Supporting the putative role of TIL as a longitudinal biomarker is the recently published correlative study from the neoadjuvant PAMELA trial, where TIL at two weeks after treatment initiation with dual HER2 blockade, but not at baseline, were predictors for pCR at the multivariable analysis.²³ This finding is in agreement with previous reports regarding the usefulness of the early assessment of biologic markers²⁴ and in contrast with the failure of the adaptation of NACT according to imaging findings during treatment to improve outcomes.²⁵

The aforementioned observations support a change of paradigm in NACT for HR positive, HER2 negative early BC, from a cancer cell centric paradigm to the assessment of the tumor microenvironment and from a static assessment at baseline and at surgery to the evaluation of longitudinal biomarkers during NACT in order to capture the response dynamics. This hypothesis generating study, with its dual novelty of comparing different aspects of immune function in a less studied BC subtype and doing so longitudinally after short-term exposure to NACT, serves as a proof of principle for the feasibility of serial biopsies at this setting and the potential predictive value that the evaluation of immune function holds. In addition, another possible application could be the selection of patients enrolled in studies evaluating agents that modulate the immune system, such as immune checkpoint inhibitors, by treating patients that either initially harbor an immunologically “hot” microenvironment or that demonstrate a shift in the microenvironment after short-term exposure to cytotoxic therapy.

On the other hand, our study suffers from some limitations that need to be acknowledged. This was a retrospective analysis of material collected from prospectively enrolled patients in a non-randomized trial. Moreover, three factors could have

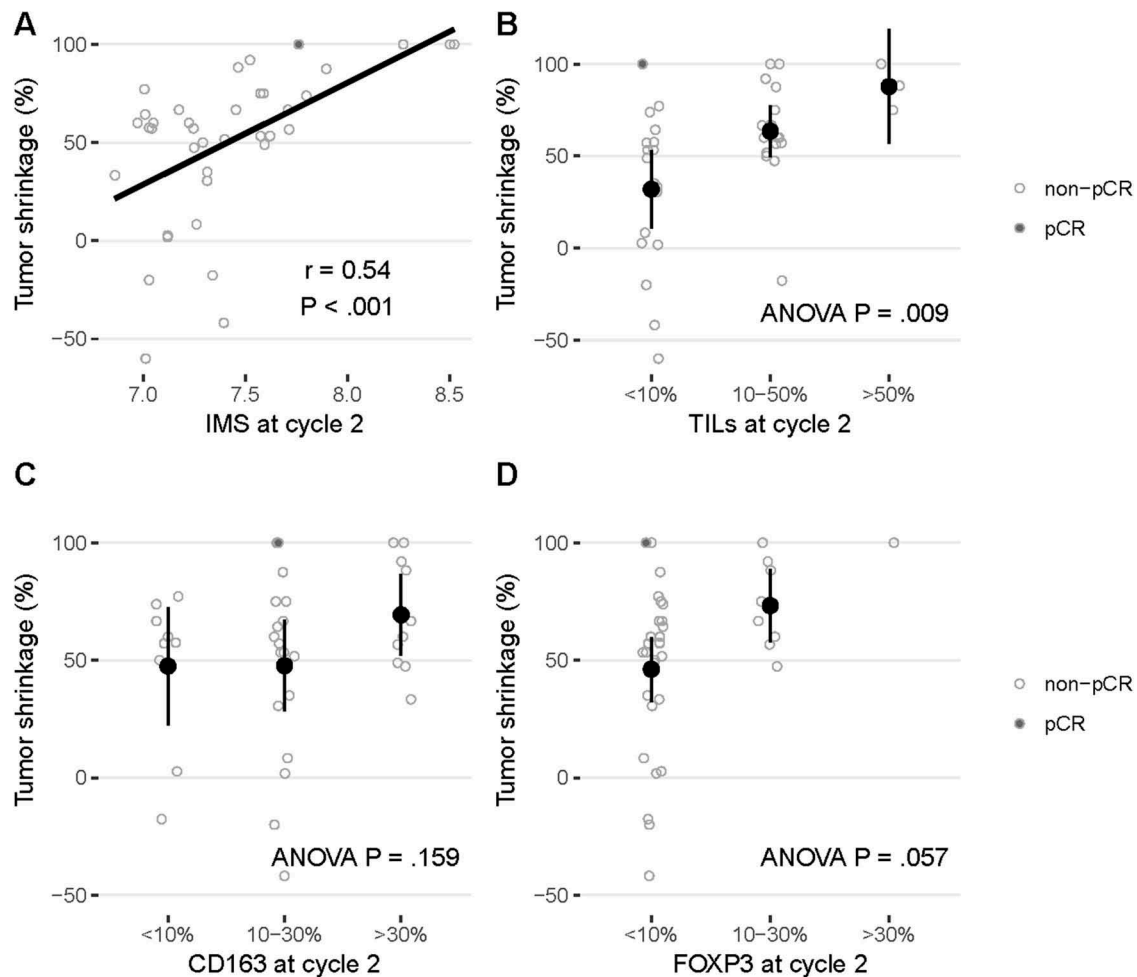


Figure 4. Correlation of immune function after short-term exposure to chemotherapy (2 cycles) with the percentage decrease in tumor size after neoadjuvant chemotherapy (cohort B). Abbreviations: ANOVA: analysis of variance; CD163: cluster of differentiation 163; FOXP3: forkhead box P3; IMS: immune module score; pCR: pathologic complete response; TILs: tumor infiltrating lymphocytes.

masked potential associations due to lack of statistical power: the relatively small number of patients, the inherent rarity of pCR in HR positive BC and the lack of GE and IC data because of the insufficient quality of tumor tissue under the effects of cytotoxic therapy. The latter could also introduce bias, since better responding patients (thus, with lower quality tumor material) could have been excluded from cohort B. As a result, these exploratory analyses should be interpreted with caution, pending their prospective validation in larger studies. In addition, the prolonged natural history of the disease and the paucity of relapses or survival events during the follow-up period precluded the use of relapse free and overall survival as endpoints. Finally, we should acknowledge the possibility that other immunohistochemistry (IHC) based markers or combinations thereof could offer superior predictive information, since immune cells exist in a continuum and the dichotomous categorization does not take into account the plasticity that T lymphocytes and macrophages exhibit, while their relative abundances, such as the CD8+/FOXP3+ ratio, could also offer further predictive and prognostic information.²⁶

In conclusion, the expression of immune related genes but not the assessment of the immune infiltrate was predictive for response to NACT in patients with locally advanced HR

positive, HER2 negative BC at baseline. Moreover, the evaluation of immune function after short-term exposure to chemotherapy carries important predictive value regarding the response to NACT. These results, if validated independently, could offer the basis for patient selection and early assessment in novel adaptive neoadjuvant strategies.

Patients and methods

Clinical trial and biopsies

The clinical trial and enrolled patients have been previously described in detail.¹⁸ PROMIX was an academic, multicenter, non-randomized, phase II trial (ClinicalTrials.gov identifier NCT00957125) that was conducted in five centers across Sweden. Eligible patients had tumors >20 mm, HER2 negative BC and received two initial cycles of NACT with docetaxel and epirubicin, administered every three weeks. Those achieving a clinical complete response, as assessed by radiology and physical examination every 2 cycles of treatment, continued with four further cycles of the same regimen, while those who did not receive additionally bevacizumab (15 mg/kg every three weeks) until cycle 6. Adjuvant therapy

was administered according to the Swedish national guidelines and local clinical practice. The primary endpoints of the study were the early objective response rate as assessed by conventional radiology (mammography and ultrasound) and the pCR rate.

Enrolled patients underwent a core biopsy at baseline prior to treatment initiation and after the second cycle of NACT. In addition, tissue was collected from the surgical specimen after the completion of NACT. Hormone receptor status was considered as positive if $\geq 10\%$ of cancer cells stained positive for ER or progesterone receptor at the baseline core biopsy.

The clinical study including the correlative analyses was approved by the Ethics Committee at Karolinska Institutet (2007/1529-31/2), which had jurisdiction for all participating centers and by the Swedish Medical Product Agency. All patients received oral and written information and consented to participate.

Gene expression profiling and data analysis

RNA was extracted from core biopsies obtained at baseline and cycle 2 as well as from the surgical specimen and profiled on Illumina Human HT-12 v4.0 Expression BeadChip (Illumina Inc., San Diego, CA), as described previously¹⁸ and available at the Gene Expression Omnibus (GEO) database under accession number GSE87455.

An immune-related gene signature (IMS, derived from⁵), was explored for its predictive value at the neoadjuvant setting of HR positive, HER2 negative BC. Gene module score was derived as the weighted average of the expression values of the constituent signature genes, where the weight for each gene is +1 or -1 depending on the direction with the phenotype in the original publication (Supplemental Data). Gene expression data was first collapsed to gene level using a non-specific filter keeping only the probe-sets with highest interquartile range in the case of multiple mappings to the same Entrez Gene ID. Only original probe-sets or genes that could be mapped to Entrez Gene IDs were used.

Hematoxylin-eosin and immunohistochemistry staining and scoring

From all specimens, serial sections of 4 μm thickness were prepared for Hematoxylin-eosin (H&E) staining and IHC. For the IHC, the sections were de-paraffinized followed by antigen retrieval in EnVision FLEX Target Retrieval Solution, high pH (Dako, Glostrup, Denmark) using PT-link at 97 $^{\circ}\text{C}$ for 20 minutes. Thereafter, slides were incubated for 30 minutes at room temperature with either the monoclonal anti-human-FOXP3 antibody (clone 236A/E7, 1:50, eBioscience, San Diego, CA) or the monoclonal mouse anti-human-CD163 antibody (clone 10D6, 1:200, Novocastra, Leica Microsystems, Newcastle, United Kingdom). The IHC EnVision visualization system was used with the standard method of horseradish peroxidase and 3, 3'-diaminobenzidine, incubating the sections with a dextran polymer conjugated with secondary antibodies for 20 minutes and substrate working solution FLEX DAB sub-chromophore for five minutes in Autostainer Link 48 according to the manufacturer

(Dako). Counterstaining was performed using Mayer's hematoxylin and slides were dehydrated, cleared and mounted using Tissue-Tek coverslipping film (Sakura Finetek, Torrance, CA). Tonsil tissue was used as a positive control for both FOXP3 and CD163 antibodies.

TIL score was evaluated on H&E stained biopsies and tumor sections and was defined as the estimated proportion of area with TIL infiltration within the tumor and adjacent stroma. The TIL score was classified as low (<10%), intermediate (10-50%) and high (>50%) as described previously.^{27, 28} The estimation of the number of positive cells with immune reactivity for FOXP3 and CD163 within the tumor and adjacent stroma area was scored as follows low (<10%), intermediate (10-30%) and high (>30%).²⁹ The TIL, FOXP3 and CD163 score was assessed by two different observers blinded to the clinical data. Discordant cases were reviewed a third time, followed by a conclusive judgement. All scoring of IC was performed at x100 magnification with a resolution of 6.24 pixels/ μm . Images at x200 magnification were captured using a Leica DMD108 light microscope with an integrated camera.

Statistical analysis

The association between IMS and IC scores and pCR after neoadjuvant therapy was assessed using logistic regression models with the IMS standardized and as continuous variable and with IC scores dichotomised. The association between IMS and IC scores percentage decrease in tumor size after neoadjuvant therapy was analysed with Pearson's correlation coefficient and one-way analysis of variance (ANOVA), respectively. DFS outcomes in groups were estimated using Kaplan-Meier curves and compared with the log-rank test. An arbitrary level of 5% statistical significance (two-tailed) was used. All data analysis was done in R/Bioconductor (version 3.4.0).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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