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Immune phenotype of tumor microenvironment predicts response to bevacizumab in neoadjuvant treatment of ER-positive breast cancer

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Abstract

Antiangiogenic drugs are potentially a useful supplement to neoadjuvant chemotherapy for a subgroup of patients with human epidermal growth factor receptor 2 (HER2) negative breast cancer, but reliable biomarkers for improved response are lacking. Here, we report on a randomized phase II clinical trial to study the added effect of bevacizumab in neoadjuvant chemotherapy with FEC100 (5-fluorouracil, epirubicin and cyclophosphamide) and taxanes (n = 132 patients). Gene expression from the tumors was obtained before neoadjuvant treatment, and treatment response was evaluated by residual cancer burden (RCB) at time of surgery. Bevacizumab increased the proportion of complete responders (RCB class 0) from 5% to 20% among patients with estrogen receptor (ER) positive tumors (P = .02). Treatment with bevacizumab was associated with improved 8-year disease-free survival (P = .03) among the good responders (RCB class 0 or I). Patients treated with paclitaxel (n = 45) responded better than those treated with docetaxel (n = 21; P = .03). Improved treatment response was associated with higher proliferation rate and an immune phenotype characterized by high presence of classically activated M1 macrophages, activated NK cells and memory activated CD4 T cells. Treatment with bevacizumab increased the number of adverse events, including hemorrhage,

Abbreviations: BCT, breast conserving therapy; DFS, disease-free survival; ECOG, Eastern Cooperative Oncology Group; FEC100, epirubicine 100 mg/m^2, 5-fluorouracil 600 mg/m^2 and cyclophosphamide; GCSF, granulocyte-colony stimulating factor; HER2, human epidermal growth factor receptor 2; MDSCs, myeloid derived suppressor cells; NBCG, Norwegian breast cancer group; pCR, pathologic complete response; RCB, residual cancer burden; Tregs, regulatory T cells; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

The NeoAva study has been presented in part at: The 36th annual San Antonio Breast Cancer Symposium, 10-14 December 2013; The 37th annual San Antonio Breast Cancer Symposium, 9-13 December 2014; The 38th annual San Antonio Breast Cancer Symposium; The American Society of Clinical Oncology (ASCO) 2015 Annual Meeting, 29 May-June 2, 2015; The 41st annual San Antonio Breast Cancer Symposium, 4-8 December 2018.

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hypertension, infection and febrile neutropenia, but despite this, the ECOG status was not affected.

KEYWORDS
bevacizumab, immune cells, neoadjuvant chemotherapy, RCB, VEGF

1 | INTRODUCTION

Angiogenesis is essential for tumor growth and progression. Vascular endothelial growth factor (VEGF) is the only angiogenic factor present throughout the entire tumor life cycle,[1,2] and anti-VEGF treatment with the monoclonal antibody bevacizumab has been thoroughly investigated in neoadjuvant treatment of HER2-negative breast cancer.[3-9] However, the results from studies combining bevacizumab with chemotherapy are not conclusive, and molecular markers predictive of treatment response are desirable.

The primary objective of our study was to explore potential biomarkers for prediction of treatment response when bevacizumab is added to standard neoadjuvant chemotherapy regimens. Evidence suggests that VEGF has an immune suppressive role[10] as indicated in another publication from this trial measuring the serum levels of cytokines in the patients.[11] A favorable response to bevacizumab in combination with chemotherapy has been linked to elevated immune activity in patients with ER-positive tumors.[12] We hypothesized that bevacizumab may act as an enhancer of the immunomodulatory effect of chemotherapy and that benefit of bevacizumab treatment may be dependent on the immune cell composition in the tumor microenvironment at the start of treatment.[13] Our initial results that a higher response rate is observed for bevacizumab-treated patients with strong correlations between serum cytokine levels and tumor-infiltrating CD8T cells,[11] rendered further investigation as reported in this study.

Neoadjuvant chemotherapy followed by pathologic complete response (pCR) is associated with long-term survival.[14-19] While pCR is well acknowledged and commonly used as a primary end point in neoadjuvant trials, residual disease after neoadjuvant treatment includes a broad range of actual responses from near pCR to an intact remaining tumor. Here, we use residual cancer burden (RCB) as the primary end point; RCB offers a more detailed quantification of response and is derived from evaluation of the tumor size, cellularity and axillary nodal burden. Multiple studies have shown that RCB is an independent prognostic factor in several subtypes of breast cancer.[20-22] We present extended biological analyses in addition to the full clinical results from our study, including RCB classification and 8-year clinical follow-up data.

What's new?

The molecular effects of antiangiogenic therapy are largely unknown. In this neoadjuvant clinical trial studying the anti-VEGF monoclonal antibody bevacizumab, the authors demonstrated increased response in tumors with immune gene upregulation, using a 770-gene immune panel. Specific immune cell types were identified as determinants of drug response. Moreover, the results suggested improved recurrence-free survival in patients achieving a favorable response after neoadjuvant antiangiogenic therapy in combination with chemotherapy.

2 | MATERIALS AND METHODS

2.1 | Study design

A randomized controlled phase II trial was performed, enrolling women aged 18 and above, with operable, HER2 negative breast cancer with size 2.5 cm and larger, without metastatic disease. The patients included in the study were recruited at two sites in Norway (Oslo University Hospital [OUS], Oslo and St. Olav's Hospital, Trondheim), between November 2008 and July 2012. Written informed consents were obtained from all patients prior to inclusion. The study protocol was approved by the institutional protocol review board, the regional ethics committee, the Norwegian Medicines Agency and carried out in accordance with the Declaration of Helsinki, International Conference on Harmony/Good Clinical practice. The NeoAva study is registered in the http://www.ClinicalTrials.gov/ database with the identifier NCT00773695.

Key inclusion criteria included no previous treatment for breast cancer, ECOG performance status ≤2, adequate hematological and biochemical parameters, and no sign of metastatic disease. Additional prerequisites were normal organ function in general and normal left ventricular ejection fraction. Acetylsalicylic acid (160 mg or lower) was the only anti-aggregant drug allowed during treatment. A block randomization procedure was used, performed by the centralized research support facility at OUS.

Stratification was based on tumor size ($2.5 \leq T \leq 5$ cm or $T > 5$ cm) and hormone receptor status (estrogen receptor (ER) positive, progesterone receptor (PgR) positive or both), and the patients were randomized 1:1 to receive bevacizumab (combination-therapy arm) or not (chemotherapy arm). Of the 150 patients enrolled in the study, 138 were assigned to treatment with chemotherapy with four cycles of FEC 100 every 3 weeks followed by docetaxel/paclitaxel with or without the addition of bevacizumab. The endocrine therapy arm included 12 patients, independently randomized and is not reported here. Of the patients randomized to the combination treatment arm, two were ineligible to enter study protocol due to metastatic disease. Three of the patients discontinued treatment due to cardiac arrhythmia, neurological disorder and death of unknown cause, respectively. In the chemotherapy arm, one patient was ineligible to enter the study protocol due to elevated bilirubin. Adverse events and toxicity were reported for all patients. The efficacy analyses reported here were performed on data from all the 132 patients who underwent randomization and for whom RCB evaluation in the breast and toxicity reports were available. The study treatment period was 24 weeks followed by surgery (mastectomy/BCT and axillary clearance), radiotherapy and endocrine therapy according to the national treatment guidelines (nbcg.no).

2.2 | Treatment

Patients in both treatment arms received four cycles of FEC100 (5-fluorouracil 600 mg/m^2, epirubicin 100 mg/m^2 and cyclophosphamide 600 mg/m^2) given every 3 weeks, followed by docetaxel 100 mg/m^2 every 3 weeks or 12 weekly infusions of paclitaxel 80 mg/m^2. Patients randomly assigned to the bevacizumab treatment arm received a dose of 15 mg/kg every third week when given docetaxel/FEC or 10 mg/kg every other week in those receiving paclitaxel.

2.3 | Tumor evaluation, sampling and assessment of response

Before each chemotherapy administration hematological parameters were evaluated, whereas biochemical parameters, ECOG performance status and clinical tumor measurement were evaluated every third week. Tumor response before surgery was assessed using mammography and ultrasound and/or MRI of the mammary gland and axilla. The local pathologist, unaware of treatment assignments, performed histopathological examination of the breast using study-specific guidelines.

Biopsies used in this article were collected before the neoadjuvant treatment. Biopsies were frozen in liquid nitrogen and stored at −70°C. Altogether, 131 specimens were available for further expression analyses.

2.4 | Residual cancer burden

The extent of tumor remaining in the breast after neoadjuvant therapy was assessed by the RCB measure, using an algorithm developed at the University of Texas, M.D. Anderson Cancer Center.[21] The gross sectioning was performed as part of the routine pathology analysis starting by cutting the breast ablation specimen into approximately 1 cm thick slices from the posterior side toward the skin side, in the sagittal plane. For the RCB assessment, the largest two dimensions of the residual tumor bed were retrieved from the routine pathology report, based on the observations registered during the gross sectioning together with the subsequent microscopical analysis of the areas selected for paraffin block embedding. RCB assessment was subsequently performed by the study pathologists according to the M.D. Anderson algorithm instructions by examination of the histological sections for overall cancer cellularity as percentage of area, and percentage of cancer being in situ. These values, together with the number of tumor-positive lymph nodes and the diameter of the largest lymph node metastasis were entered into the MD Anderson RCB calculator, and the continuous-valued RCB index was found. Four response categories were defined based on the RCB index according to the criteria given by MD Anderson: RCB class 0 (pCR; stage yp T0, ypN0), RCB class I (minimal residual disease; $0 < \text{RCB} < 1.36$), RCB class II (moderate residual disease; $1.36 \leq \text{RCB} < 3.28$), and RCB class III (extensive residual disease; RCB ≥3.28).[20,21]

2.5 | Gene expression profiling and immune score

Gene expression profiling was performed using 40 ng total RNA and one color SurePrint G3 Human GE 8x60 k Microarrays (Agilent Technologies, Santa Clara, California) following the manufacturer's protocol. Gene expression profiles were successfully obtained from 131 fresh frozen thru-cut biopsies sampled before treatment start. Microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4439. Gene expression values were log2 transformed and probes corresponding to the same gene identifier were collapsed by calculating the average expression value. Samples were quantile normalized to obtain identical distribution of gene expression values across samples, and missing values were imputed using the LLSimpute algorithm in the Bioconductor package pcaMethods and with $k = 20$. Signal values were adjusted for differences in batch effect, site, RIN values and background, while preserving differences between time-points using GLM in SAS. The data was then mean centered on both rows and columns. Gene expression subtypes were assigned to tumors using PAM50.[23] The tumor expression profiles were collectively centered to align with the PAM50 training data set prior to application of the PAM50 algorithm. For this purpose, we first calculated separate expression centroids c_{neg} and c_{pos} for the ER-negative samples and the ER-positive samples, respectively, and then found an overall centroid $c = a\, c_{neg} + (1 - a)\, c_{pos}$ where $a > 0$ denotes the proportion of ER-negative tumors in the PAM50 training set. Finally, we subtracted

the centroid **c** from each expression profile in our data set. A proliferation score was calculated for each tumor by computing the mean of the log2 transformed expression values of 11 proliferation-related PAM50 genes (CCNB1, UBE2C, BIRC5, KNTC2, CDC20, PTTG1, RRM2, MKI67, TYMS, CEP55 and CDCa1).[24] An immune profile of the tumor microenvironment was obtained using the nCounter Nanostring Immune profiling panel measuring the expression of 770 genes and designed to capture features of the immune response in different cancer types.[25] Absolute fractions of 22 types of infiltrating immune cells were estimated from the gene expression profiles using CIBERSORT[26] with the signature matrix LM22 and 1000 permutations.

2.6 | Statistical and bioinformatical analysis

All patients who received at least one cycle of anthracycline-containing chemotherapy were included in the safety analysis. Statistical analysis was performed in R version 3.3.1[27] and SPSS version 24. Associations between different groups of patients were assessed by the T-test, Wilcoxon rank-sum test (Mann-Whitney test), Kruskal-Wallis test or Fisher's Exact test. Disease-free survival (DFS) was defined as time from randomization to disease recurrence. The cut-off date for the follow-up data was September 25, 2018. To test for differences in survival in subgroups the Logrank test was used, and the Kaplan-Meier estimator was used to estimate and visualize survival functions. In all tests, a two-sided alternative was used, with significance level 0.05. Hierarchical clustering was performed with Euclidean distance for samples and Pearson correlation distance for genes, and using complete linkage. Heatmaps and hierarchical clustering were produced using an in-house computational pipeline available as open source in R (https://rdrr.io/github/cbsteen/clustermap/). The number of clusters was calculated using Partitioning Algorithm using Recursive Thresholding (PART)[28] in the clusterGenomics package on Comprehensive R Archive Network (CRAN).

3 | RESULTS

3.1 | Patient and tumor characteristics

Between November 2008 and July 2012, 150 patients with large (>2.5 cm) HER2 negative mammary carcinomas were enrolled. Patients received either chemotherapy (n = 138) or endocrine therapy (n = 12; not reported in this study) by the physician's choice. In the chemotherapy arm, three patients were ineligible to enter protocol and three were discontinued due to toxicity (Section 2), resulting in 132 patients. Patients treated with chemotherapy were subject to a randomization, resulting in 66 patients being treated only with chemotherapy and 66 patients with a combination of bevacizumab and chemotherapy; these patients were included in the primary efficacy analysis. Patient and tumor characteristics were well balanced across the treatment arms (Table 1). No significant skewness in distribution of important clinical and molecular parameters such as tumor size, grade, lymph node status and hormone receptor status were observed.

Bevacizumab was administered intravenously at a dose of 15 mg/kg (n = 21) every third week or 10 mg/kg (n = 45) every second week in addition to docetaxel or paclitaxel, respectively. Tolerability was the main factor determining the type of taxane chemotherapy. Bevacizumab 10 mg/kg was administered to 68% of the patients (median 6 doses, mean 5.2 doses).

3.2 | Overall response to treatment

RCB class 0 or I, further denoted "good response", was achieved after neoadjuvant treatment for 38 of 132 patients (29%), with 23 patients obtaining RCB class 0 (pCR). For patients with residual disease, 15 (11% of the total patient population) achieved RCB class I, 73 (55%) RCB class II and 21 (16%) RCB class III. For patients with T2 tumors at diagnosis, 15 of 40 (37%) achieved a good response, compared to 20 of the 82 patients with T3 tumors (25%; Figure S1).

3.3 | Bevacizumab reduces RCB

The RCB class distribution (Table 2) did not differ significantly between the two treatment arms in the whole cohort or in the ER-negative subgroup (P = .113, n = 132 and P = .246, n = 21, respectively). In the ER-positive subgroup, the RCB class distribution differed significantly (P = .035, n = 111) and the proportion of patients achieving RCB class 0 increased from 5% to 20% with the addition of bevacizumab (P = .022, n = 111; see also Silwal-Pandit et al[12]). Patients in RCB class I and II were equally distributed between the treatment arms, while 62% of RCB class III patients belonged to the chemotherapy arm.

Patients receiving paclitaxel/bevacizumab 10 mg/kg (q2w; n = 45, 68%; P = .038, n = 66) had significantly better outcome than patients receiving docetaxel/bevacizumab 15 mg/kg (q3w; n = 21, 32%; Table S1). The proportion of patients achieving RCB class 0 was 5% with docetaxel/bevacizumab and 32% with paclitaxel/bevacizumab (P = .025, n = 66), and the proportion of patients achieving a good response was 10% vs 45% (P = .012, n = 66).

3.4 | Bevacizumab improves DFS in patients with good response

Mean follow-up time was 8.6 years in the combination treatment arm and 7.8 years in the chemotherapy arm (overall estimate: 8.3 years). Overall DFS after 8 years was 81.8% (24 events), while treatment specific DFS after 8 years was 77.3% (15 events) in the chemotherapy arm and 86.4% (9 events) in the combination therapy arm. DFS was not significantly different in the two treatment arms (P = .17, n = 132; Figure 1A) or in the ER-positive or ER-negative subpopulations. Among the good responders (RCB class 0 or I), DFS was significantly

better for patients receiving combination therapy than for patients receiving only chemotherapy (Logrank test: P = .025, n = 38; Figure 1B). No significant difference was present in the group of poor responders (RCB class II or III; Logrank test: P = .78, n = 94). In the group of patients achieving RCB class 0, there were three disease recurrences, of which two were observed in the chemotherapy arm. Recurrent disease was observed in 21 of the 109 patients with RCB class I-III; 13 (22%) of these in the chemotherapy arm, 8 (16%) in the combination treatment arm.

TABLE 1 Clinicopathological characteristics of patients

Variable	Number of patients		
	Chemotherapy arm	Combination therapy arm	*P*-value
Clinical tumor stage			
T2	21 (32%)	19 (29%)	.86
T3	39 (59%)	42 (64%)	
T4	6 (9%)	5 (8%)	
Nodal status			
cN0	29 (44%)	29 (44%)	.96
cN1-3[a]	37 (56%)	37 (56%)	
Histopathology			
Invasive ductal carcinoma	54 (82%)	52 (79%)	.68
Invasive lobular carcinoma	10 (15%)	13 (20%)	
Other	2 (3%)	1 (2%)	
Pathological tumor grade			
1	3 (5%)	8 (12%)	.28
2	42 (64%)	43 (65%)	
3	15 (23%)	12 (18%)	
Not available	6 (9%)	3 (5%)	
Estrogen receptor status			
ER-negative	9 (14%)	12 (18%)	.66
ER-positive	57 (86%)	54 (82%)	

Values of P are from Fisher's Exact test for 2×2 tables and Pearson's Chi-squared test otherwise.
[a]In 15% and 17% of the cases, respectively, metastatic lymph nodes were diagnosed by radiology only.

3.5 | Response to bevacizumab is associated with high tumor proliferation

To investigate the effect of tumor proliferation on treatment response, a proliferation score based on the expression of 11 genes[28] was calculated and compared in good and poor responders and individually for each treatment (Figure 2). In the combination therapy arm, a good response was borderline associated with higher proliferation score (t test: P = .049). A similar but nonsignificant trend was observed in the chemotherapy arm.

3.6 | Response to bevacizumab is associated with high immune activity

It has previously been reported that upregulation of a set of genes reflecting immune activity is associated with better bevacizumab response in ER-positive tumors.[12] To investigate this further, we considered the expression of the 770 genes in the Nanostring immune panel (Section 2) in the ER-positive samples.

Using hierarchical clustering and determining the number of clusters with the PART algorithm[28] we identified three distinct groups of tumors, hereby denoted cluster 1 (n = 13), cluster 2 (n = 21) and cluster 3 (n = 77; Figure 3A). Cluster assignments were significantly associated with RCB (P = .0009) and PAM50 subtype (P = .00008). Combined, cluster 1 and 2 were enriched for tumors with RCB class 0 (pCR) and for basal-like, normal-like and luminal B tumors. These clusters also showed the highest overall expression of the immune panel genes. Considering each treatment arm separately revealed no association between good response and immune cluster with chemotherapy (P = .45; Figure 3B) and a strong association between good response and immune cluster with combination therapy (P = .0067; Figure 3C).

3.7 | Response to bevacizumab is associated with a distinct immunophenotype

Absolute fractions of 22 distinct types of immune cells were estimated from bulk tumor transcription profiles using CIBERSORT.[29] (Complete list of fraction for all 22 cell types given in Table S2, FDR adjusted P values [Kruskal-Wallis Test]). In the chemotherapy arm, there was only minor differences in immune cell composition

TABLE 2 RCB class distribution

RCB	ER-positive subgroup		ER-negative subgroup	
	Chemotherapy	Combination therapy	Chemotherapy	Combination therapy
0	3 (5%)	11 (20%)*	5 (56%)	4 (33%)**
I	7 (12%)	6 (11%)	1 (11%)	1 (8.5%)
II	34 (60%)	30 (56%)	3 (33%)	6 (50%)
III	13 (23%)	7 (13%)	0 (0%)	1 (8.5%)

Note: Number of patients within each Residual Cancer Burden (RCB) class, ER subgroup (ER-positive [n = 111] vs ER-negative [n = 21]) and treatment arm (chemotherapy vs combination therapy). Wilcoxon test: *P = .035; **P = .246.

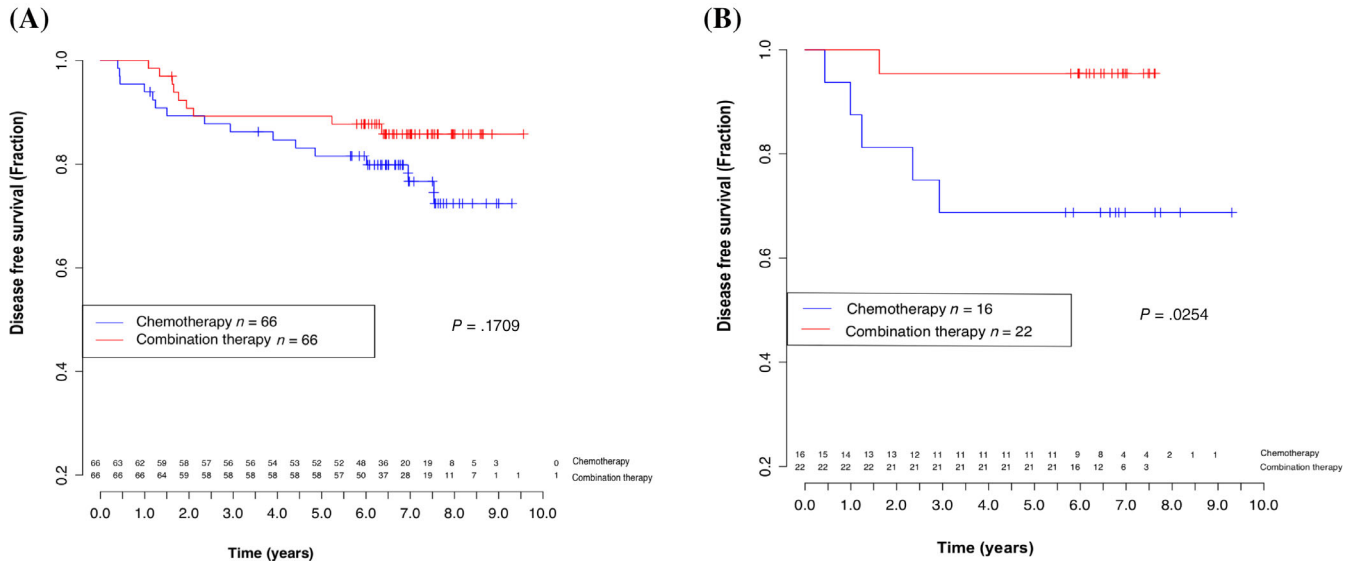

FIGURE 1 Disease-free survival (DFS). Kaplan-Meier estimate of DFS in the chemotherapy arm vs the combination therapy arm. Results are shown for, A, the whole study population (P = .171, n = 132) and B, patients in residual cancer burden (RCB) class 0 and I (P = .025, n = 38)

in tumors from patients achieving RCB class 0 (pCR) and RCB class I-III (Figure 3D). Most notably the fraction of follicular helper T cells before treatment was associated with treatment response (P < .001 after Bonferroni correction). In the combination therapy arm, there were pronounced differences in immune cell composition in tumors from patients achieving RCB class 0 and RCB class I-III (Figure 3E). Patients achieving RCB class 0 had significantly higher fractions of cytotoxic T cells, memory activated helper T cells, follicular helper T cells and classically activated (M1) macrophages and significantly lower fractions of regulatory T cells (Bonferroni adjusted P < .001).

In the ER-positive subgroup, the highest fractions of CD8+ T cells, memory activated CD4+ T cells, activated natural killer (NK) cells and M1 macrophages (all associated with higher levels of immune response) were found in cluster 1 and the lowest fractions in cluster 3 (Bonferroni adjusted P < .00001). The highest fraction of regulatory T cells (associated with dominant negative regulation of other immune cells) was found in cluster C and the lowest fraction in cluster A (Bonferroni adjusted P < .00001; Figure 3F).

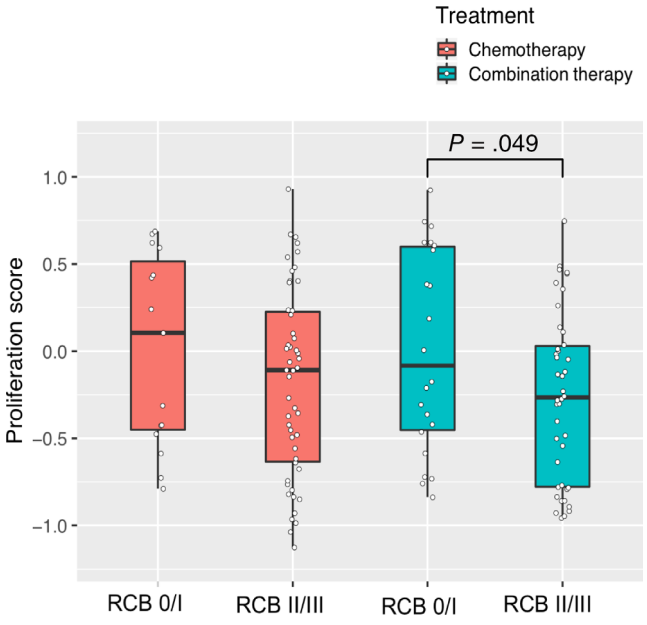

FIGURE 2 Proliferation score. The distribution of proliferation scores, stratified on response (Residual Cancer Burden [RCB] class 0 or I vs RCB class II or III) and type of treatment (chemotherapy vs combination therapy). Proliferation scores are based on the expression of 11 proliferation-related genes and were calculated as described in Nielsen et al.[25] For patients in the combination therapy arm, good response (RCB class 0 or I) was associated with high proliferation score (P = .049). A similar trend was observed in the chemotherapy arm, but this was not statistically significant

3.8 | Adherence to treatment and adverse events

Neoadjuvant chemotherapy was planned for 24 weeks according to the protocol, and all treatment-related details were available for the patients. There was no difference in the dosing between the treatment groups, which were completed (all doses given) in 49 of the 66 patients (74%) in each of the treatment arms.

In the combination arm, chemotherapy was delayed for 24 (36%) patients during FEC treatment, and for 20 patients (30%) in the chemotherapy arm. The majority of these were delayed due to administrative reasons. The FEC dose was reduced in three (4.5%) patients in each treatment arm, and was discontinued for only one patient. Taxane related side effects resulted in a reduction of treatment (to less than 10 weeks of taxanes) in five patients treated in the chemotherapy arm and four patients in the combination arm. Taxane treatment was delayed for three (4.5%) and dose reduced for six (9%) patients in the combination treatment arm. A delayed taxane dose

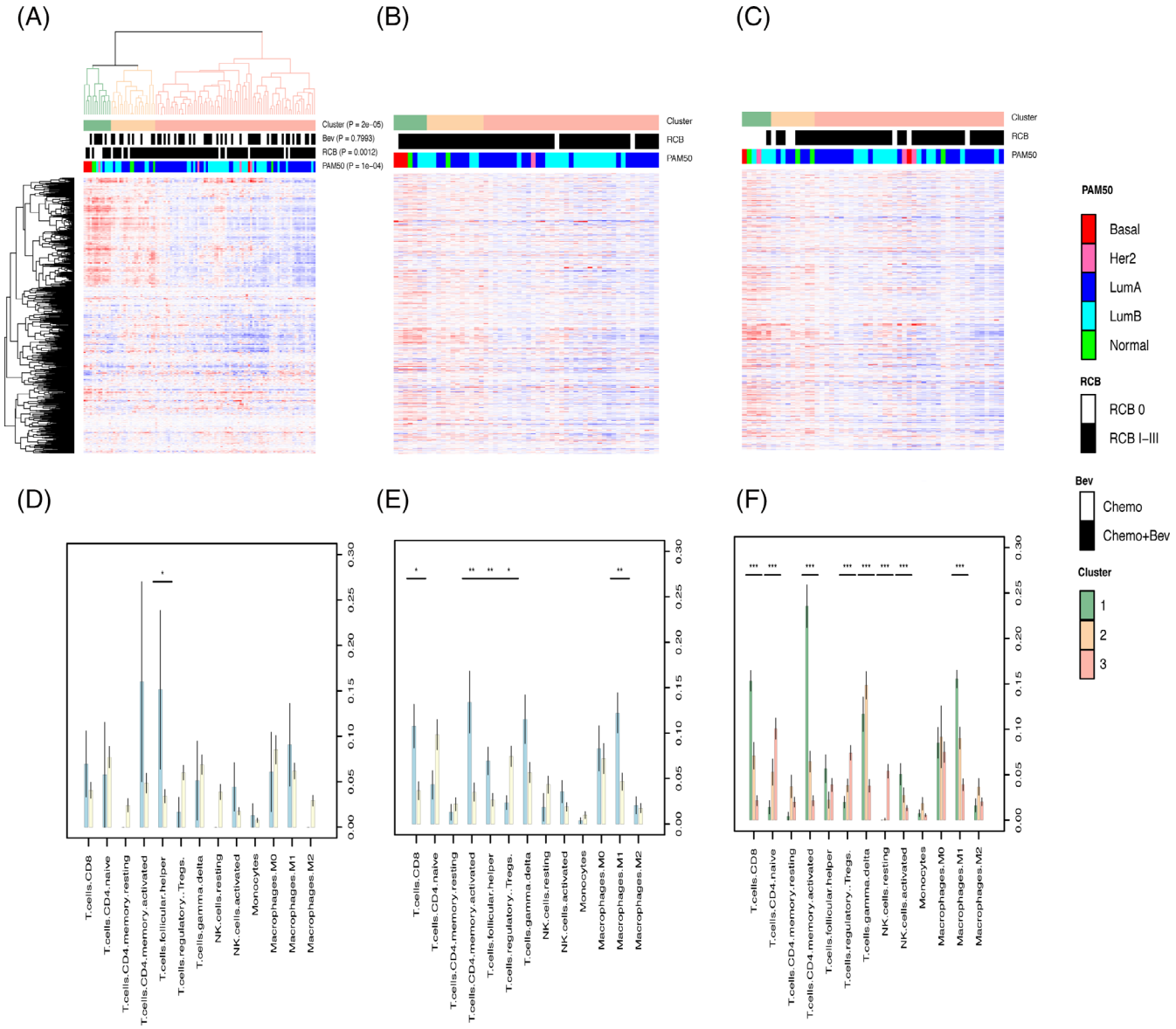

FIGURE 3 Tumor immunophenotype. A, Clustered heatmap of expression values for all ER-positive samples (represented by columns) and the 770 genes (represented by rows) in the Nanostring immune panel. B, Heatmap with same ordering of columns and rows as in, A, but showing only samples in the chemotherapy arm. C, Heatmap with same ordering of columns and rows as in A but showing only samples in the combination therapy arm. Color bars on top of the heatmaps show cluster assignment by the PART algorithm (cluster 1-3), treatment arm (chemotherapy or combination therapy), residual cancer burden (Residual Cancer Burden [RCB] 0 vs RCB I-III) and PAM50 expression subtype (basal-like, Her2-like, luminal A, luminal B or normal-like). *P* values are from Fisher's exact test of the association between cluster assignment and Bev/RCB/PAM50 group. D, Estimated fractions of different immune cell types for samples in the chemotherapy arm, stratified on treatment response with RCB 0 samples shown in blue and RCB I-III samples shown in yellow. E, Estimated fractions of different immune cell types for samples in the combination therapy arm, stratified on treatment response with same color coding as in D. F, Estimated fractions of different immune cell types for all ER-positive samples, and separately for the three clusters (green: cluster 1, yellow: cluster 2, red: cluster 3). All fractions in D-F were estimated using CIBERSORT in Absolute mode. Fractions are only shown for the 13 most relevant immune cell types; a complete list of fractions for all 22 cell types estimated by CIBERSORT is given in Table S2. Lengths of bars indicate mean values, while the vertical lines through the bars indicate ± 1 SD. An ANOVA test was performed to assess the association between treatment response (RCB 0 vs RCB I-III) and immune cell fraction. Code: ***$P < .00001$; **$P < .0001$; *$P < .001$ (Bonferroni adjusted *P* values with $n = 22$ tests)

was administered to four (6%) patients in the chemotherapy arm, without dose reduction.

Of the 66 patients receiving bevacizumab, 23 (35%) had a delayed treatment, however, 21 (32%) of these were due to administrative reasons. One or more bevacizumab doses were omitted in nine (13%) patients due to bevacizumab related side effects, or due to upcoming surgery date. Among patients receiving combination therapy, 14 (21%) reported side effects related to bevacizumab. The overall main reasons for receiving fewer cycles were treatment toxicities during taxane treatment with and without the addition of bevacizumab, and patient withdrawal.

TABLE 3 Adverse events

Adverse event	Chemotherapy arm	Combination therapy arm	*P*-value
Febrile neutropenia[a]	25	53	<.001
Proteinuria	37	43	NS
Hypertension	14	37	<.001
Bleeding/ Hemorrhage	13	48	<.001
Infection[b]	6	16	.033
Neutropenia	3	5	NS
Fever	2	3	NS
Stomatitis	1	2	NS
Arrhythmia supraventricular	1	2	NS
Hypersensitivity reaction	2	0	NS
Syncope	2	0	NS
Death	0	1	NS
Other[c]	21	18	NS

Note: Number of patients with adverse events, stratified on treatment arm. Values of P were found using Fisher's Exact test. Serious adverse events: Only those marked with superscript letters (a and b) were significantly different between the treatment groups ([a]Fisher's exact test $P < .001$ and [b]$P = .055$). For a complete listing of SAE (including superscript letter (c) other), refer Table S3.
Abbreviation: NS, not significant.

Febrile neutropenia, proteinuria and hypertension were the most frequent adverse events observed in both treatment arms (Table 3). In the combination-therapy arm, significantly higher frequencies of hemorrhage and hypertension were observed ($P < .001$). Serious adverse events (SAEs), mainly febrile neutropenia and infection were also significantly more frequent in this arm ($P < .001$ and $P < .05$, respectively; Table S3). One death occurred after 12 weeks in the combination-therapy arm, but despite autopsy, a specific cause could not be established.

Performance status (ECOG) was evaluated by the physician at the study visits. We compared the reported ECOG status before, during and after treatment between the two treatment arms. No statistically significant difference was observed, despite the significantly higher frequency of reported SAE in the combination treatment arm.

4 | DISCUSSION

Clinical studies in patients undergoing neoadjuvant therapy create a unique possibility for integrating molecular profiling with clinical endpoints. In this randomized study, we extended the translational results and report the clinical 8-year outcome in patients treated with chemotherapy and antiangiogenic therapy compared to chemotherapy alone. We recently reported that in patients treated with bevacizumab in addition to standard chemotherapy, increased expression of immune-related genes was associated with good response when comparing patients that achieved a pCR with those that did not.[12] These studies were followed by the characterization of the cytokine profiles in these patients and their correlation to tumor-infiltrating immune cells.[11] Here, we used a standardized immune panel (nCounter Nanostring) and the CIBERSORT bioinformatics tool to confirm the relationship between response and immune gene expression and furthermore, to elucidate the cell types behind this response.[25,30] This demonstrates a link in ER-positive tumors between improved response to bevacizumab and active immune response at baseline using the standardized immune gene panel.[12] A phenotype characteristic of a strong immune response to tumor growth was significantly enriched in tumors from patients achieving RCB class 0. This was corroborated by the strong association between this immune phenotype and cluster 1. This phenotype was defined by high presence of cytotoxic T cells, memory activated helper T cells and M1 macrophages and low presence of regulatory T cells. Regulatory T cells are a highly immune-suppressive subset of CD4+ T cells and a high level of these cells is associated with a diminished survival in breast cancer.[31] Low levels of regulatory T cells in a tumor can be associated with tumor response to chemotherapy in combination with bevacizumab, and is suggested as a predictive marker on the efficacy of antiangiogenic combination treatment.[32] In addition, a higher level of tumoricidal M1 macrophages were detected in patients on antiangiogenic treatment achieving RCB class 0.[33]

These results suggest an additional immunomodulatory effect for bevacizumab in cancer treatment and might aid to select patients more likely to benefit from the addition of bevacizumab to conventional chemotherapy. It supports the theory of immune activity as a predictor of response to bevacizumab,[10] and that cellular immune response in tumors has the potential to enhance clinical predictions, determine prognosis, in addition to identify potential candidates for immunotherapy.[12] In addition to its well-defined role in angiogenesis, VEGF is considered to play a role in cancer immune evasion.[10] Immune-related pathways have been shown to be predictive of higher pathological complete response (pCR) rates in different subsets of breast cancer.[34]

The primary objective of our study was to explore possible biomarkers that may predict the treatment response of bevacizumab. With the number of patients included, and the relatively few events in the study, the bevacizumab combination therapy did not result in a statistical significant increase in DFS. This is similar to many of the previously reported neoadjuvant trials, with the exception of one.[3,6,9,15]

pCR is commonly used as a primary endpoint in neoadjuvant trials and a potential surrogate marker for survival. Residual disease after neoadjuvant treatment includes a spectrum of responses from near pCR to an intact remaining tumor[20] and is not a perfect surrogate marker since many patients with residual disease after neoadjuvant chemotherapy may still have a good prognosis. Assessment of pCR as a prognostic marker and surrogate endpoint has until recently been most relevant for high proliferative tumors like triple-negative (TNBC) and HER 2 positive breast cancer, and less relevant for the ER-

positive/HER2 negative subgroup. The latter group constitutes the majority of patients in our study and are less likely to achieve a complete response after neoadjuvant therapy.[19] RCB has been demonstrated to be prognostic for long-term survival after neoadjuvant treatment in all subtypes of breast cancer. Patients with minimal residual disease (RCB class I) have an improved outcome compared to patients with more remaining tumor after treatment. In our study, we determined the RCB of the tumors using the criteria described.[20,21,35] The addition of bevacizumab resulted in improved response, most pronounced in the ER-positive subset where more patients achieved a good response (RCB class 0 and I) and a decrease in the number of tumors with limited treatment response (RCB class III). In line with this, treatment with combination therapy resulted in a significantly improved DFS in the good responders compared to patients treated with chemotherapy only, independent of ER status. These results are in contrast to the ARTEMIS trial[9] were the addition of bevacizumab seemed to result in a slight survival disadvantage. However, the studies are different both in treatment regimens, sequencing of the treatment, the duration of bevacizumab treatment, size and patient population, and the results should therefore be interpreted and compared to caution. Notably, in the NSABP-B40 trial demonstrating an effect on the survival, the patients received bevacizumab for 18 weeks preoperatively, and for 30 weeks after surgery.

Patients in the combination therapy arm received bevacizumab first in combination with antracycline then with docetaxel or paclitaxel for an additional 12 weeks. Due to lack of tolerability, some patients given docetaxel were shifted to paclitaxel, and received bevacizumab in a dose of 10 mg/kg every other week. Patients with the more frequent combined biweekly administration seemed to achieve an improved response. An inhibitory action on the proliferation of endothelial progenitor cells have been described for taxanes, with an antiangiogenic effect at lower doses than those needed to inhibit the proliferation of cancer cells. The resulting hypoxia induces the autocrine production of proangiogenic agents, mainly belonging to the HIF-1/VEGF pathway. Bevacizumab may inhibit this reaction, thus potentiating the antiangiogenic action of chemotherapy.[2,36,37] This could represent a biological rationale for the combination of paclitaxel with bevacizumab. A more frequent administration of paclitaxel has been described to enhance the proapoptotic and antiangiogenic properties, increasing the antineoplastic effect.[38] A specific synergism of paclitaxel and bevacizumab in combination has also been suggested in other studies.[39-41] The increased response observed in the patients treated with biweekly bevacizumab in our study support the theory about synergism with paclitaxel and might also be explained by the increased delivery with combined therapy. However, this comparison was not preplanned, and the results describing a difference in effect based on type of drug in addition to scheduling, should be interpreted with caution, also due to the low number of patients in this subset.

VEGF is the main proangiogenic factor and a potent mediator of tumor angiogenesis. VEGF mediates numerous changes within the tumor vasculature, including endothelial cell proliferation, migration, invasion, vascular permeability and vasodilation.[42] Blocking VEGF by the monoclonal antibody bevacizumab affects vascular function and has been proposed to cause inhibition of new-vessel formation and normalization of the remaining vasculature, resulting in an improved and more uniform delivery of drugs.[43] This may certainly enhance the clinical response observed in tumors treated with the combination of bevacizumab with conventional chemotherapy.

The mobilization of Gr-1$^+$ CD11b$^+$ myeloid-derived suppressor cells (MDSCs), have been described as one mechanism for resistance to anti-VEGF therapies, and may be influenced by the administration of G-CSF, to induce BV8/prokineticin 2 expression in MDSCs, and contribute to the mobilization and treatment resistance conferred by these cells.[9,44-46] In this clinical study, G-CSF were administered to patients with febrile neutropenia, during antracycline treatment. Of the 26 patients receiving the addition of G-CSF during bevacizumab combination treatment, 38% (n = 10) achieved a good response and did not differ from the patients treated without G-CSF (P = .59).

The addition of bevacizumab in neoadjuvant trials has resulted in an increased number of SAEs.[3,6] A significant increase in the proportion of patients with neutropenia, hypertension and bleeding was observed in the combination treatment arm in our study. The mechanism behind bevacizumab associated neutropenic events is not fully understood. Inhibition of VEGF1 receptor has been shown to block hematopoietic stem cell cycling, differentiation and recovery after bone marrow suppression.[47] Various forms of VEGF blockade by inhibition of the receptor tyrosine kinase domains or through antibodies targeting VEGF ligand, may induce myelosuppression and delay bone marrow recovery. Despite this increased SAE rate in the combination treatment arm, there was no significant difference in the number of patients receiving full dose neoadjuvant treatment in our study. This is consistent with the findings in the NSABP-40 trial and Artemis trial, but in contradiction to the findings in GeparQuinto where a significant number of patients treated with the addition of bevacizumab received a delayed and reduced dose.[6,8]

In conclusion, the addition of bevacizumab to neoadjuvant chemotherapy increased the rate of good responders among patients with large HER 2-negative breast cancers, with the most pronounced effect in the ER-positive tumors. The improved response did not translate into a benefit in the DFS, but a significantly improved DFS was detected in the good responders receiving the addition of bevacizumab. An elevated immune response activity seems to predict good response to antiangiogenic therapy in ER-positive tumors. The association with activated immune response pathways is particularly interesting, in relation to the growing interest for testing immunological checkpoint inhibition in breast cancer. The use of an immune response signature along with other molecular markers should be further investigated to identify patients more likely to benefit from antiangiogenic therapy.

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CONFLICT OF INTEREST

The main sponsor for the study was Oslo University Hospital. The study was co-sponsored by Roche Norway and Sanofi-Aventis, contributing to funding of the study. Bjørn Naume and Erik Wist have received honoraria from Roche and GlaxoSmithKline.

DATA ACCESSIBILITY

Microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4439. Other data are available on request from the corresponding author. The data are not publicly available due to privacy and ethical restrictions.

ETHICS STATEMENT

Written informed consents were obtained from all patients prior to inclusion. The study protocol was approved by the institutional protocol review board, the regional ethics committee, the Norwegian Medicines Agency and carried out in accordance with the Declaration of Helsinki, International Conference on Harmony/Good Clinical practice. The NeoAva study is registered in the http://www.ClinicalTrials.gov/ database with the identifier NCT00773695.

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SUPPORTING INFORMATION

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