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Serum levels of inflammation-related markers and metabolites predict response to neoadjuvant chemotherapy with and without bevacizumab in breast cancers

Marianne E. Nome[1], Leslie R. Euceda[2], Shakila Jabeen[1], Julia Debik[2], Tone F. Bathen[2], Guro F. Giskeødegård[2], Kristin A. Taskén[3,6], Gunhild M. Mælandsmo[3,4], Bente Halvorsen[5,6], Arne Yndestad[5,6], Elin Borgen[7], Øystein Garred[7], Pål Aukrust[5,6,8,9], Thor Ueland[5,6,9], Olav Engebraaten[6,10], Vessela N. Kristensen[1,6,11]* and Xavier Tekpli[1,11]*

[1]Department of Clinical Molecular Biology, Division of Medicine, Akershus University Hospital, Lørenskog, Norway
[2]Department of Circulation and Medical Imaging, NTNU - The Norwegian University of Science and Technology, Trondheim, Norway
[3]Department of Tumour Biology, Institute for Cancer Research, Radium Hospital, Oslo University Hospital, Oslo, Norway
[4]Institute of Medical Biology, Faculty of Health Sciences, Artic University of Norway – University of Tromsø, Tromsø, Norway
[5]Research Institute of Internal Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway
[6]Institute of Clinical Medicine, University of Oslo, Oslo, Norway
[7]Department of Pathology, Division of Laboratory Medicine, Oslo University Hospital, Oslo, Norway
[8]Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital Rikshospitalet, Oslo, Norway
[9]K.G. Jebsen – Thrombosis Research and Expertise Center (TREC), Department of Clinical Medicine, University of Tromsø – The Arctic University of Norway, Tromsø, Norway
[10]Department of Oncology, Oslo University Hospital, Oslo, Norway
[11]Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway

Angiogenesis is necessary for tumor growth and has been targeted in breast cancer; however, it is unclear which patients will respond and benefit from antiangiogenic therapy. We report noninvasive monitoring of patient response to neoadjuvant chemotherapy given alone or in combination with anti-vascular endothelial growth factor (bevacizumab) in a randomized clinical trial. At four time points during neoadjuvant chemotherapy ± bevacizumab of receptor tyrosine-protein kinase erbB-2-negative breast cancers, we measured metabolites and inflammation-related markers in patient's serum. We report significant changes in

*V.N.K. and X.T. jointly supervised the work

Additional Supporting Information may be found in the online version of this article.

Key words: breast cancer, metabolism, inflammation, bevacizumab, neoadjuvant therapy

Abbreviations: ALCAM: activated leukocyte cell adhesion molecule; ANOVA: analysis of variance; AUC: area under the curve; Bev: bevacizumab; CatS: Cathepsin S; CCL18: chemokine (C-C motif) ligand 18; CRP: C-reactive protein; CXCL16: chemokine (C-X-C motif) ligand 16; DLL1: delta-like protein 1; ePCR: endothelial cell protein C receptor; ER: estrogen receptor; FDR: false discovery rate; GDF15: growth differentiation factor-15; HER2: receptor tyrosine-protein kinase erbB-2; IL: interleukin; IP-10: inducible protein 10; minRT: minimal residual tumor; NMR: nuclear magnetic resonance; PARC: pulmonary and activation-regulated cytokine; PC: principal component; PTX3: pentraxin 3; sTNFR1: soluble tumor necrosis factor receptor 1; VEGF-A: vascular endothelial growth factor A; VWF: von Willebrand factor

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Correspondence to: Xavier Tekpli, Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, N-0310 Oslo, Norway, Tel.: +47-22-78-13-75, Fax: +47-22-78-13-95, E-mail: xavier.tekpli@medisin.uio.no; or Vessela Kristensen, Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, N-0310 Oslo, Norway, Tel.: +47-22-78-13-75, Fax: +47-22-78-13-95, E-mail: v.n.kristensen@medisin.uio.no

the levels of several molecules induced by bevacizumab, the most prominent being an increase in pentraxin 3 (PTX3) and von Willebrand factor (VWF). Serum levels of AXL, VWF and pulmonary and activation-regulated cytokine (PARC/CCL18) reflected response to chemotherapy alone or in combination with bevacizumab. We further analyzed serum cytokines in relation to tumor characteristics such as gene expression, tumor metabolites and tumor infiltrating leukocytes. We found that VWF and growth-differentiation factor 15 tumor mRNA levels correlated with their respective serum protein levels suggesting that these cytokines may be produced by tumors and outflow to the bloodstream while influencing the tumor microenvironment locally. Finally, we used binomial logistic regression which allowed to predict patient's response using only 10 noninvasive biomarkers. Our study highlights the potential of monitoring circulating levels of cytokines and metabolites during breast cancer therapy.

What's new?

How inflammatory markers and metabolites mold the tumor microenvironment, especially angiogenesis, is still poorly understood. Here the authors tested the response of patients with breast cancer to neoadjuvant chemotherapy with or without the angiogenesis inhibitor bevacizumab. They identified 10 non-invasive biomarkers that predict therapy response, including the acute phase protein pentraxin-3 and hemostasis regulator von Willebrand factor that were specifically upregulated by bevacizumab treatment. The authors suggest that these factors could be further targeted to potentiate antiangiogenic therapies in breast cancer and beyond.

Introduction

Inflammation and an appropriate immune response can limit tumor growth. On the other hand, chronic inflammation can enhance tumorigenesis and metastasis through angiogenesis and metabolic dysregulation.[1] Angiogenesis as well as altered metabolism and tumor-related inflammation are cancer hallmarks,[2] and these factors may interplay during tumor progression. Vascular endothelial growth factor A (VEGF-A), the main cytokine regulating angiogenesis, has been targeted in several cancer types.[3] We recently demonstrated that anti-VEGF-A therapy in breast cancer affects the tumor immune contexture.[4]

Immune infiltration influences prognosis and response to therapies.[5,6] Notably high CD8+ T cell counts associate with better overall survival in estrogen receptor (ER) negative breast cancer patients.[7,8] High immune infiltration has also been associated with an increased response to neoadjuvant and adjuvant chemotherapy.[9]

Breast tumors have distinct metabolic profiles[10] which may affect the differentiation and activity of immune cells and *vice versa*. How metabolites produced by anabolic and hypoxic tumors mold the tumor microenvironment and angiogenesis remains poorly understood. Metaflammation, metabolically induced low-grade and chronic inflammation,[11] has mostly been studied in relation to metabolic diseases, but may also play an important role in breast cancer development and progression.[12] In fact, this bidirectional interaction between inflammation and tumor metabolism could be an important pathway in the regulation of tumor progression.

Cytokines and metabolites secreted by tumors may outflow to the bloodstream and affect the levels of related molecule systemically. We hypothesized that serum levels of cytokines and related metabolic markers may reflect breast tumor characteristics and thereby the response to neoadjuvant chemotherapy.

In the NeoAva, a randomized clinical trial studying in a neoadjuvant setting the effects of chemotherapy ± bevacizumab (Bev; VEGF-A inhibition), we recently demonstrated that Bev decreased serum levels of interleukin (IL)-12, interferon-gamma inducible protein 10 (IP-10/CXCL10) and IL-10.[4]

In the present study, we extend our study of noninvasive biomarkers associated with response to neoadjuvant chemotherapy of breast cancers by analyzing upstream markers of endothelial and vascular inflammation and regulators of extracellular matrix remodeling (Table 1). Based on the concept of metabolically induced inflammation, we also collected the levels of 26 metabolites measured in serum of the same patients (Debik *et al*., submitted manuscript).

Cytokines serum levels were analyzed in perspective of clinicopathological characteristics and tumor phenotypes such as gene expression, tumor metabolism and immune infiltration. Our results demonstrate that noninvasive measurement of metabolites and cytokines could help to follow patient's response to neoadjuvant therapy.

Materials and Methods

Patients and treatment

Patients were recruited at Oslo University Hospital, Oslo, or St. Olav's University Hospital, Trondheim, between November 2008 and July 2012. Informed consents were obtained prior to inclusion. The study was approved by the institutional protocol review board, the regional ethics committee and the Norwegian Medicines Agency and was carried out in accordance with the Declaration of Helsinki, International Conference on Harmony/Good Clinical practice. The study is registered at http://www.ClinicalTrials.gov/ database with the identifier NCT00773695. Untreated receptor tyrosine-protein kinase erbB-2 (HER2)-negative mammary carcinomas with size ≥2.5 cm and with no sign of metastatic disease were included.

Patients were randomized to receive chemotherapy alone (chemo arm) or Bev and chemotherapy (combination arm).

Table 1. Markers included in the study

Markers for	Protein full name	Protein short name
Vascular inflammation	Osteoprotegrin	OPG
	Pentraxin 3	PTX3
	Tyrosine-protein kinase receptor	Axl
	C-X-C motif chemokine ligand 16	CXCL16
	Von Willebrand factor	VWF
	Endothelial cell protein C receptor	ePCR
Activated monocytes/macrophage markers	Activated leukocyte cell adhesion molecule	ALCAM (CD166)
	p53-associated parkin-like cytoplasmic protein	PARC
	Cluster of differentiation 163	sCD163
	Galectin-3-binding protein	Gal3BP
Extracellular matrix	Cluster of differentiation 147 (Basigin. EMMPRIN)	CD147
	Endostatin	Endostatin
	Growth differentiation factor-15	GDF15
	Cathepsin S (Chloramphenicol acetyl transferase)	Cats
General inflammation	Tumor necrosis factor receptor 1	sTNFR1
	C-reactive protein	CRP
Notch	Delta-like protein 1	DLL1
Metabolites	Leucine	
	Valine	
	Isoleucine	
	2.methylglutarate	
	Alanine	
	Lysine	
	Acetate	
	Glutamine. Glutamate	
	Acetoacetate	
	3.hydroxybutyrate	
	Glutamate	
	Pyruvate	
	Glutamine	
	Citrate	
	Methionine	
	Creatine	
	Creatinine	
	Ornithine	
	Proline.betaine	
	Dimethyl.sulfone	
	Glucose	
	Glycine	
	Lactate	
	Tyrosine	
	Histidine	
	Phenylalanine	

For more details on the treatment regimen, see Ref. 13. At surgery, patient response was determined and minimal residual tumor (minRT) was defined as at least 90% reduction of tumor size. Table 2 summarizes relevant clinical data of the patients included in our study.

Cytokine serum levels

Blood samples were collected at diagnosis (screening), 12 weeks (after the first regimen of chemotherapy: fluorouracil/epirubicin/cyclophosphamide ± Bev), 25 weeks (after the second regimen of chemotherapy: taxane ± Bev;

also, time of surgery) and 31 weeks (follow-up; 6 weeks after surgery). For an overview of data available for each sample, see Supplementary Table S1.

Serum levels of 17 inflammation-related markers were measured in duplicate using commercially available reagents by enzyme immunoassay using R&D Systems (Minneapolis, MN). For von Willebrand factor (VWF), the assay was performed with DakoCytomation (Glostrup, Denmark). Assays were performed in a 384-format using the combination of a SELMA (Jena, Germany) pipetting robot and a BioTek (Winooski, VT) dispenser/washer (EL406). Primary and secondary antibody concentrations were used according to manufacturer instructions (Coating 1–4 μg/ml; secondary 0.2–2 μg/ml). Assay volume was 25 μl and coating was performed in phosphate buffered saline. Subsequent assay buffer was with 1% bovine serum albumin in PBS while sample diluent was PBS with 25% heat inactivated fetal calf serum (Gibco, Thermo Fisher Scientific, Waltman, MA). Wash buffer was PBS with 0.05% tween-20 and three wash cycles were included per step. Samples were incubated overnight at 4°C. Absorption was read at 450 nm with wavelength correction set to 540 nm using an EIA plate reader (Synergy H1 Hybrid, Biotek, Vinooski, VT). Intra- and interassay coefficients of variation were <10% for all assays. The assays included a series of known concentrations to generate standard curves. The resulting data obtained were log transformed before further analyses. Log transformed serum levels are available in Supplementary Table S9.

Metabolite serum levels

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance III Ultrashield Plus spectrometer operating at 600 MHz (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a 5 mm QCI Cryoprobe. Serum samples were thawed at 4°C prior to the analysis. One hundred fifty microliter serum were gently mixed with 150 μl of buffer (D_2O with 0.075 mM Na_2HPO_4, 5 mM NaN, 3,5 mM TSP, pH 7.4). Data acquisition was fully automated using a SampleJet with IconNMR on TopSpin 3.1 (Bruker BioSpin). Carr-Puncell-Meiboom-Gill spectra were acquired at 37°C. Spectra were Fourier transformed to 128 K after 0.3 Hz exponential line broadening. Furthermore, spectral preprocessing was performed in Matlab R2017b. The left peak of the alanine doublet at 1.47 ppm was used as a chemical shift reference. Three spectra were removed from the analysis due to poor water suppression after a visual inspection. Spectral peaks were aligned to the peaks of the spectra with the highest correlation to the other spectra using the function iscoshift.[14] Spectra were further baseline corrected using the asymmetric least squares method.[15] Quantification was performed by integrating the spectral peaks, and for metabolites with more than one resonance, the average was used. Quantified peaks were normalized by mean normalization, giving the relative amounts of metabolites in each sample. The serum metabolites are analyzed in perspective of clinicopathological features in a separate study (Debik *et al.*, submitted).

CIBERSORT analysis

The algorithm CIBERSORT was used on normalized bulk tumor sample expression data to infer the relative proportions of 22 types of infiltrating immune cells. CIBERSORT uses a set of reference gene expression values (547 genes) to predict the proportions of 22 immune cell types using support vector regression.[16] The algorithm was obtained from the developers and analysis was performed by using the default signature matrix at 1,000 permutations.

Gene expression

Expression profiling of tumor biopsies was obtained from Ref. 13. Briefly, total RNA from bulk tumors was analyzed by GE 8 × 60 k Microarrays (Agilent Technologies, Santa Clara, CA). Arrays were scanned using Microarray Scanner with SureScan High Resolution Technology (Agilent Technologies). Images were processed using Feature Extraction software (v10.7.3.1; Agilent Technologies). The data were quantile normalized applying the Bioconductor package limma, and missing values were imputed using Bioconductor package pcaMethods.

Binomial logistic regression

Binomial logistic regression was performed through the glmnet R package.[17] We set up, based on levels of the molecules measured in the serum of the patients a logistic regression using the binomial distribution to predict categorical response of the two possible outcomes: minRT (less than 10% residual disease at 25 weeks) or residual disease (more than 10% residual disease). The levels of 17 inflammation-related cytokines, 26 metabolites and 27 previously published cytokines[4] were normalized together by *Z*-scores. The normalized levels of these 70 molecules were used to predict response at baseline (screening) or 12 weeks.

This approach was successful at 12 weeks and gave a signature of target molecules which captured the variation associated with the two categories. A patient could now be assigned to minRT or not according to the following score:

$$\text{score} = \sum_{g=1}^{n} \beta_g . X_{gi}$$

where g is the target (molecule), n is the number of targets, β_g is the Lasso coefficient for the target molecule and X_{gi} is the molecule serum levels in sample i. If score for patient i was higher than 0, a prediction of response to therapy was assumed.

Statistical analyses

All analyses were performed after natural log transformations of cytokines and metabolites levels in the R version 3.5.1.

Analysis of variance (ANOVA) was performed with time points, treatment arm or treatment response as factors to

Table 2. Clinical characteristics of the NeoAva cohort

	Screening		12 weeks		25 weeks		31 weeks	
	Chemotherapy	Combination (+Bev)	Chemotherapy	Combination (+Bev)	Chemotherapy	Combination (+Bev)	Chemotherapy	Combination (+Bev)
Age (mean)	48	51	47	48	47	51	47	49
Tumor stage								
T2	15 (43%)	6 (24%)	14 (37%)	12 (33%)	13(35%)	11 (34%)	8 (32%)	5 (22%)
T3	18 (51%)	17 (68%)	21 (55%)	22(59%)	21 (57%)	17 (53%)	15 (60%)	17 (74%)
T4	2 (6%)	2 (8%)	3 (8%)	3 (8%)	3 (8%)	4 (13%)	2(8%)	1 (4%)
Nodal status								
pN0	13 (37%)	11 (44%)	13 (34%)	14 (38%)	12 (32%)	16 (50%)	9(36%)	9 (39%)
pN1	10 (28%)	3 (12%)	9 (24%)	7 (19%)	9 (24%)	4 (13%)	7 (28%)	5 (22%)
pN2	3 (9%)	1 (4%)	5 (13%)	2 (5%)	4 (11%)	2 (6%)	3 (12%)	3 (13%)
pN3	3 (9%)		3 (8%)		4 (11%)		2(8%)	
NA	6 (17%)	10 (40%)	8 (21%)	14 (38%)	8 (22%)	10 (31%)	4 (16%)	6 (26%)
Treatment	35(58%)	25 (42%)	38 (51%)	37 (49%)	37 (54%)	32 (46%)	25 (52%)	23 (48%)
Response								
minRT	6 (17%)	11 (44%)	7 (18%)	14 (38%)	8 (21%)	11 (34%)	6 (24%)	7 (30%)
RT	28 (80%)	11 (44%)	30 (79%)	20 (54%)	28 (76%)	19 (60%)	19 (76%)	14 (61%)
NA	1 (3%)	3 (12%)	1(3%)	3 (8%)	1 (3%)	2 (6%)	0	2(9%)
Estrogen receptor status								
Positive	29 (83%)	19 (76%)	31 (82%)	29 (78%)	34 (92%)	24 (75%)	22 (88%)	20 (87%)
Negative	6 (17%)	6 (24%)	7 (18%)	8 (22%)	3 (8%)	8 (25%)	3 (12%)	3 (13%)

Abbreviations: Bev, bevacizumab; minRT, minimal residual tumor; NA, not available; RT, residual tumor.

identify molecules with global significant differences in log molecule expression levels for any of the factors (the results are shown in Supplementary Tables S2–S5, respectively). *p*-Values were adjusted for multiple testing by controlling the false discovery rate (FDR) according to the method of Benjamini–Hochberg.[18] FDR-corrected *p*-values less than 0.05 were considered significant.

Mann–Whitney *U* or Kruskal–Wallis tests were used to assess statistical significance within boxplots.

Pearson correlations were assessed with R and were visualized with the corrplot package version 0.84 or scatterplot.

Principal component (PC) analysis was performed with R. Scatter plot of PC1 and PC2 are represented, where each dot represents a sample projected onto the two main principal components (PC1 and PC2); the dots are colored according to time points.

Unsupervised clustering was performed using the pheatmap package with correlation distance and average linkage.

Data availability

Log transformed serum levels of the 17 inflammation-related markers are available in Supplementary Table S9. The levels of 26 metabolites measured in serum of the same patients are available in an upcoming article (Debik *et al.*, submitted manuscript). Gene expression profiling of tumor biopsies can be obtained at Ref. 13.

Results

Serum levels of inflammation-related markers during neoadjuvant therapy

Seventeen inflammation-related markers were measured by enzyme immunoassay in serum of breast cancer patients at different time points of neoadjuvant chemotherapy. PC analysis of the longitudinal log transformed serum levels suggested differences according to time points (Fig. 1*a*).

We therefore sought for significant changes in serum levels between the different time points, at (*i*) diagnosis: screening, (*ii*) 12 weeks: after first regimen of chemotherapy: fluorouracil/epirubicin/cyclophosphamide ± Bev, (*iii*) 25 weeks: after the second regimen of chemotherapy: taxane ± Bev (also time of surgery) and (*iv*) 31 weeks: 6 weeks after surgery (follow-up).

To identify inflammation-related markers with significant changes throughout the different time points, we computed an ANOVA FDR corrected *p*-value. Cytokines with corrected *p*-value <0.05 (Supplementary Table S2) are presented in Figure 1*b* which precises the changes between two consecutive time points. We found that most of the significant changes occurred between screening and 12 weeks. We interpret this as a result of the potent effects of chemotherapy locally at the tumor site but also systemically as we previously reported.[4]

The most prominent and significant changes in inflammation-related markers between baseline and 12 weeks were the increase in the levels of CXCL16, VWF and growth differentiation factor-15 (GDF15), cytokines which may reflect

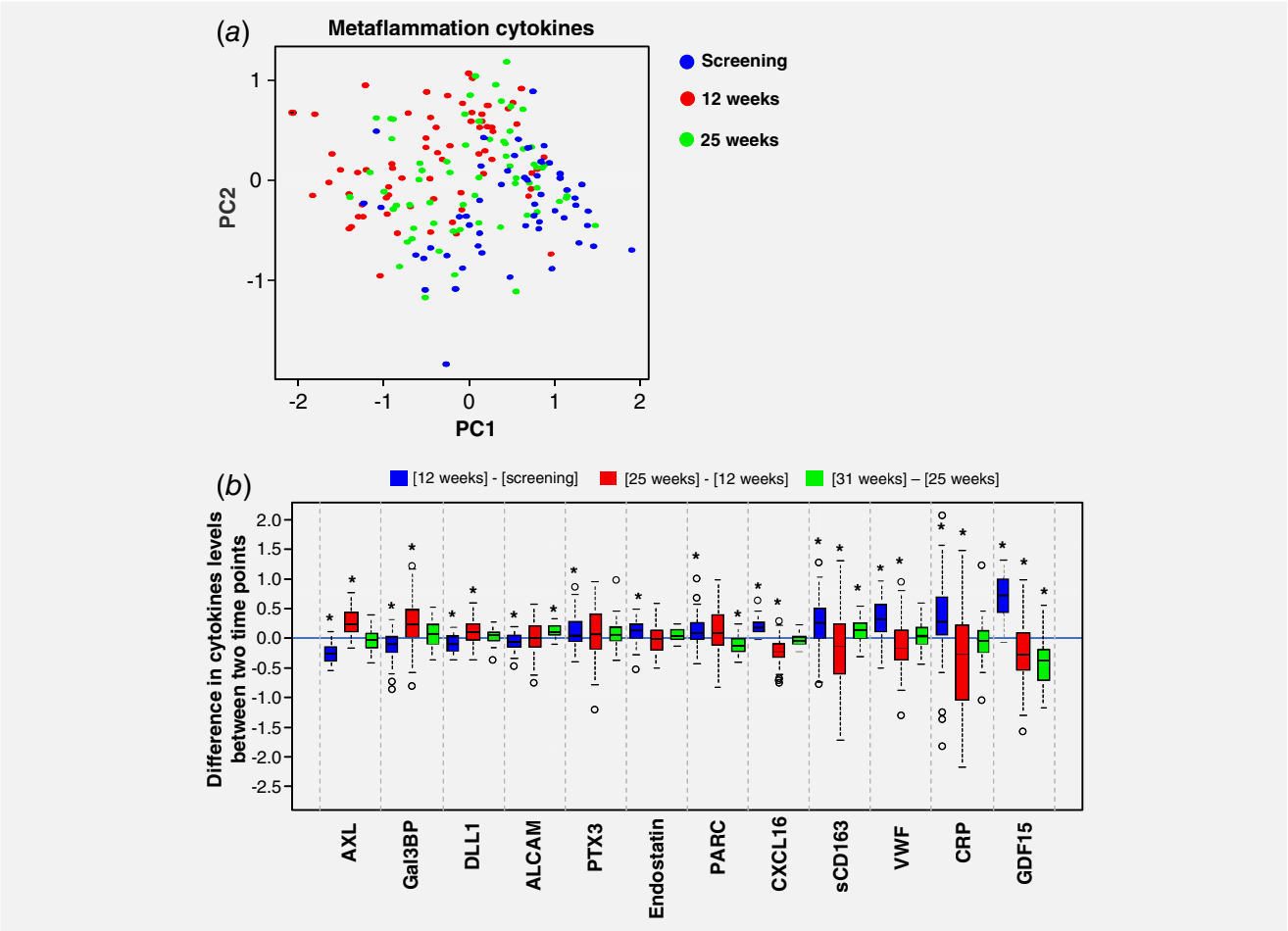

Figure 1. Changes in serum inflammation-related markers according to time points. (*a*) Principal component analysis was performed using the serum levels of the 17 inflammation-related markers. Scatter plot of principal components 1 and 2 are represented, each dot represents a sample projected in the two main principal components (PC1 and PC2) and are colored according to the time point they belong. (*b*) Differences in serum inflammation-related markers between two successive time points are presented in boxplots. Only 12 molecules for which significant changes between time points were observed according to ANOVA FDR corrected $p < 0.05$ (Supplementary Table S2) are presented. In the box-and-whiskers plots, the line within each box represents the median. Upper and lower edges of each box represent 75th and 25th percentile, respectively. Boxplots over the horizontal blue line crossing the *y*-axis at 0 indicates an increased in serum levels between two successive time points. *$p < 0.05$ *t*-test, significant differences.

vascular inflammation and extra cellular matrix remodeling. Between 12 and 25 weeks, the levels of these molecules tended to go back to baseline levels. We also identified a prominent decrease in AXL levels between baseline and 12 weeks.

These initial analyses identified the molecules with the most variating serum levels during therapy independently of the treatment arm or response.

Bevacizumab-induced changes in serum levels of inflammation-related markers

We next focused on the effects of Bev on serum levels of inflammation-related markers by comparing cytokine serum levels according to the two randomized arms of the clinical trial: chemotherapy only *vs.* chemotherapy + Bev.

We identified five inflammation-related markers: ALCAM, AXL, VWF, pentraxin 3 (PTX3) and Cathepsin S (CatS) (Figs. 2*a*–2*e*) with significantly different levels according to treatment arm (ANOVA FDR corrected *p*-value <0.05; Supplementary Table S3). We previously described a Bev-induced significant decrease in VEGF-A levels accompanied by a decrease in IL-12 and IP-10.[4] Here, we show that levels of ALCAM and AXL followed a similar pattern with marked decrease in serum levels induced by Bev (Figs. 2*a* and 2*b*).

Inversely, levels of VWF and PTX3 significantly increased in the Bev arm at 12 weeks and remained high throughout therapy and until 6 weeks after surgery (week 31) (Figs. 2*c* and 2*d*). We also found that levels of CatS significantly increased at 31 weeks for Bev treated patients (Fig. 2*e*).

Altogether, these results indicate prominent changes in systemic levels of inflammation-related cytokines induced by Bev. Such changes could have important repercussions regarding response.

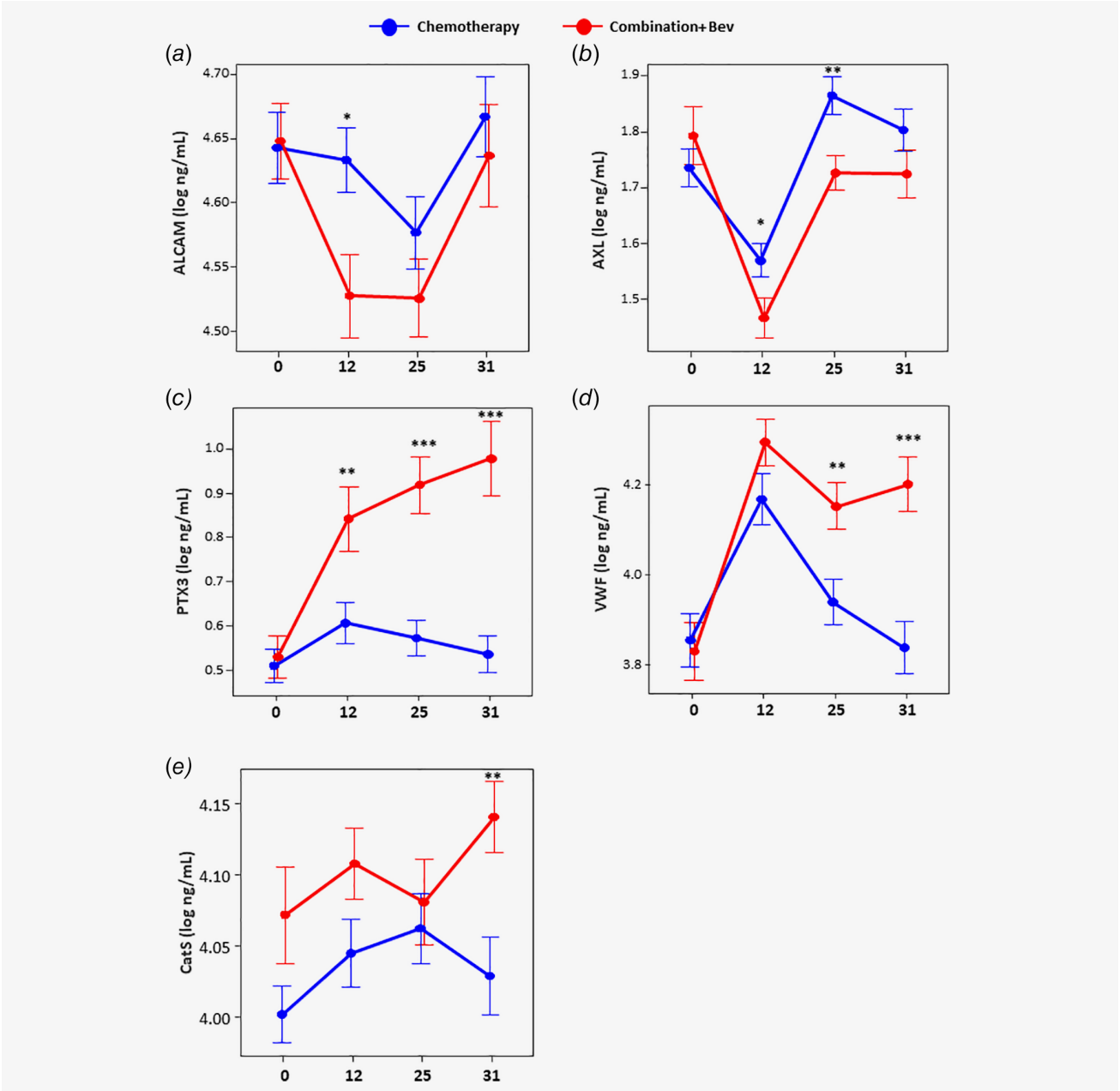

Figure 2. Effect of Bevacizumab on serum cytokines levels. Line plots of the log transformed serum levels of ALCAM (*a*), AXL (*b*), PTX3 (*c*), VWF (*d*), CatS (*e*) in the two treatment arms: chemotherapy (blue), chemotherapy + Bev (red), at four different time points: 0, 12, 25 and 31 weeks. Dots represent the averaged serum levels in each arm and error bars represent standard error to the mean. The significant differences between the two arms are first tested for global differences at all time points using ANOVA and FDR corrected *p*-value (see Supplementary Table S3, for summary statistics). The molecules with a significant ANOVA FDR *p*-value are further tested for significant difference at each time point using *t*-test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. [Color figure can be viewed at wileyonlinelibrary.com]

Serum inflammation-related markers associated with response

We next sought to identify noninvasive biomarkers associated with response to treatment. Response to treatment was defined by minRT which means reduction of at least 90% of tumor size at 25 weeks (time of surgery). Through computation of ANOVA FDR-corrected *p*-value <0.05 at the different time points between responders (minRT) and patients with residual disease (more than 10% residual disease), we identified cytokines associated with response (Fig. 3 and Supplementary Fig. 1). Summary statistics for the cytokine levels in relation to response are shown in Supplementary Table S4.

According to ANOVA FDR-corrected *p*-value <0.05, only in the combination arm (Bev treatment), lower VWF levels were

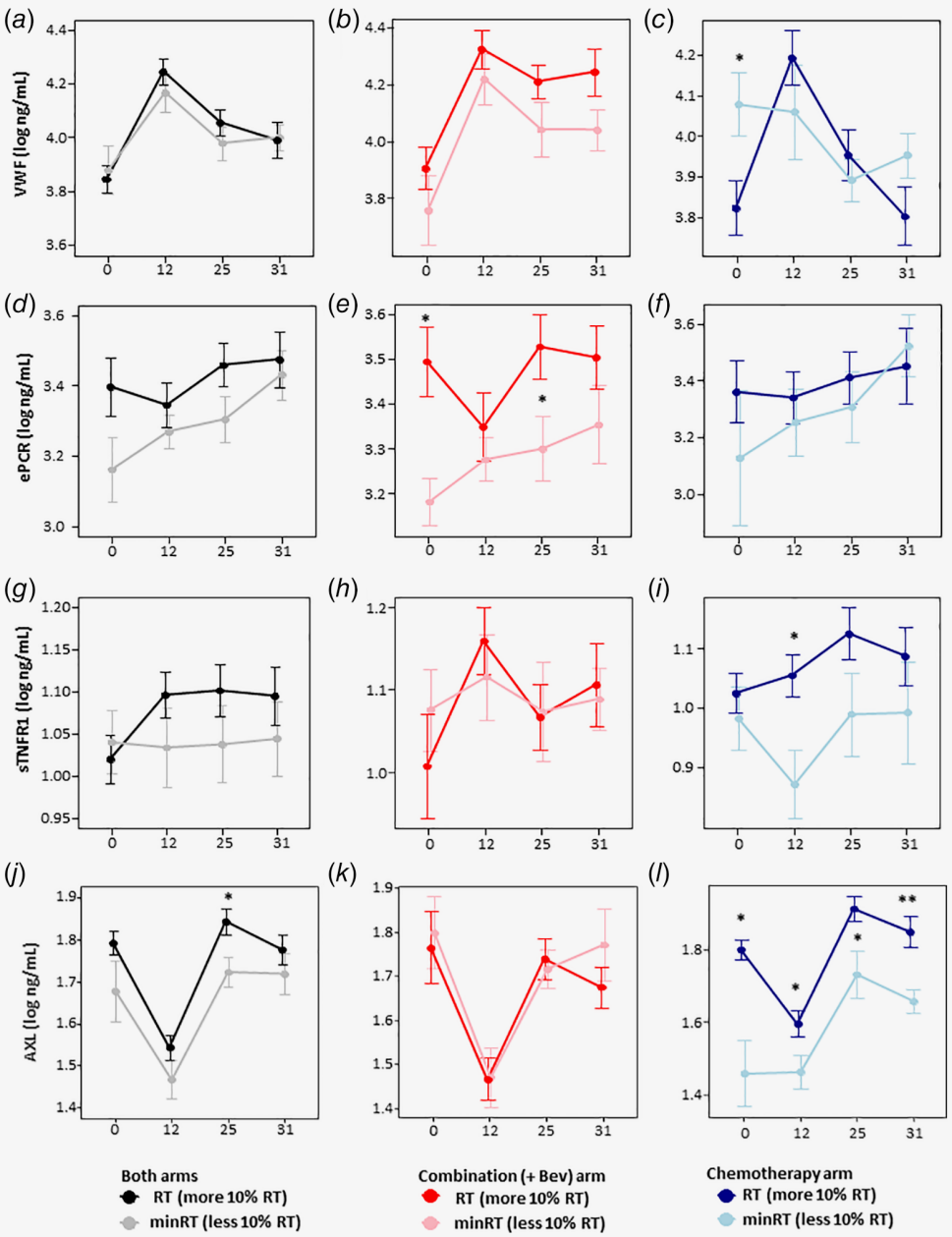

Figure 3. Differences in molecule levels according to response to chemotherapy. Comparison using line plots of the serum levels of VWF (*a-c*), ePCR (*d-f*), sTNFR1 (*g-i*), AXL (*j-l*) in responders (minRT, less than 10% residual disease) *vs*. nonresponders (residual tumor [RT]) in both treatment arms. The differences in responders *vs*. nonresponders are shown in the left panels (black and gray lines) independently of the treatment arm, middle panels combination arm (pink and red lines) and chemotherapy only arm (right panels, blue and light blue lines). Dots represent the averaged serum levels and error bars show standard error to the mean. Summary statistics between responders and nonresponders in the different arms are shown in Supplementary Table S4. Significant difference at each time point are assessed using *t*-test $*p < 0.05$, $**p < 0.01$. [Color figure can be viewed at wileyonlinelibrary.com]

found throughout the treatment period. However, when considering each time point separately, no significant difference between responders and nonresponder was observed (Fig. 3*b*).

Similarly, in the Bev arm, pulmonary and activation-regulated cytokine (PARC) serum levels (chemokine [C-C motif] ligand 18 [*CCL18*], a cytokine involved in breast cancer cell invasion[19]; Supplementary Figs. S1*a*–S1*c*) and endothelial cell protein C receptor (ePCR) levels (Figs. 3*d*–3*f*), a gene associated with risk of breast cancer[20] were lower for responders throughout treatment.

For patients in the chemotherapy only arm, at 12 weeks, lower levels of three cytokines were associated with response. Soluble tumor necrosis factor receptor 1 (sTNFR1), a soluble

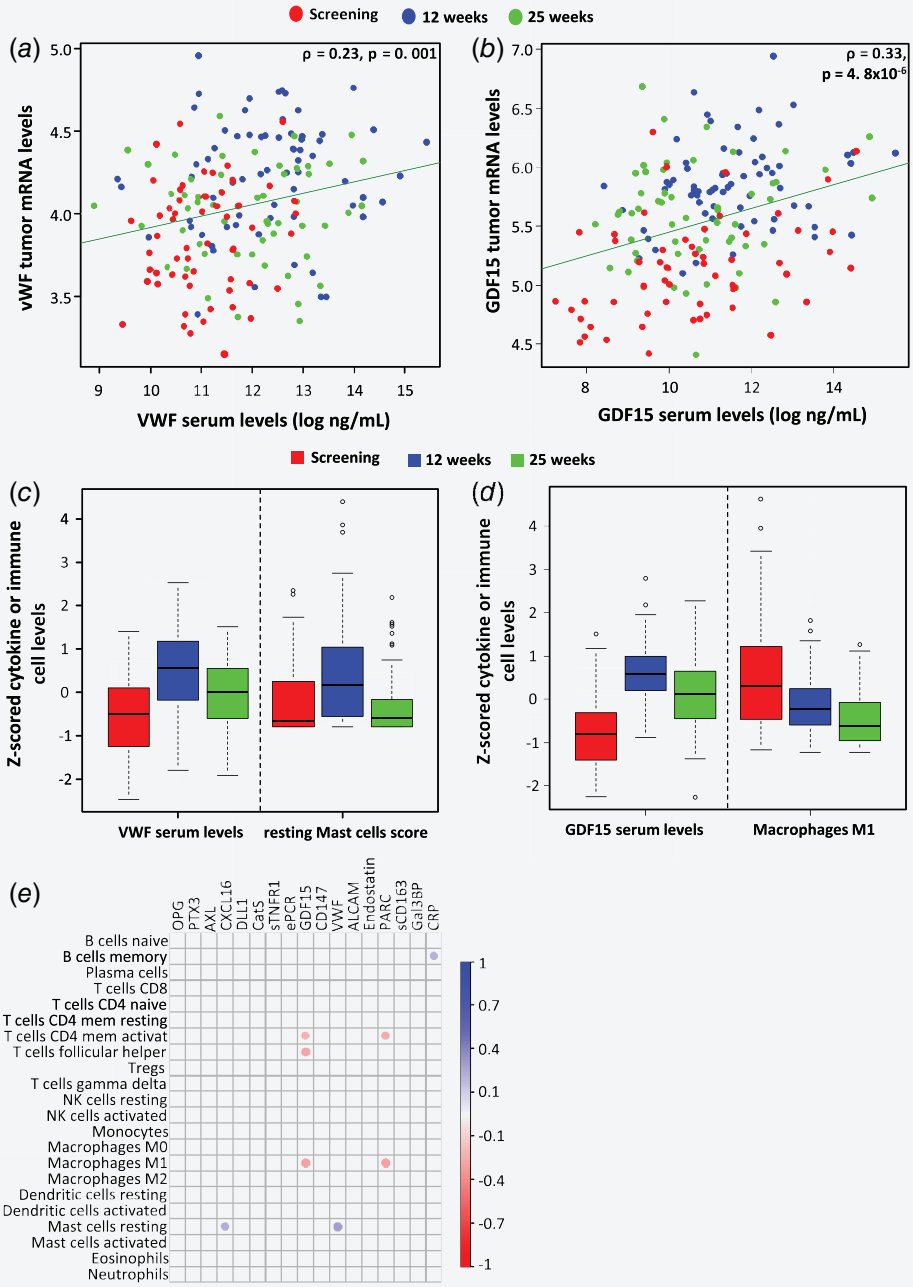

Figure 4. Association between serum levels and tumor characteristics. Correlation among (*a*) VWF, (*b*) GDF15 serum (x axis) and mRNA levels at the tumor site (y axis) across time points. Time points are denoted using different colors in the scatter plots. Pearson correlation ρ and *p*-values are denoted. The green line shows the best fit. To show serum levels and the predicted immune cell infiltration on the same scale, *Z*-scored serum levels of (*c*) VWF and (*d*) GDF15 and inferred mast cells (*c*) and M1-type macrophages infiltration at the tumor site are plotted across three time points (0, 12 and 25 weeks) using boxplots. (*c*) VWF serum levels correlate with mast cell infiltration (ρ = +0.33, FDR *p*-value = 0.0007). (*d*) GDF15 serum levels are negatively correlated with M1-type macrophages infiltration (ρ = −0.33, FDR *p*-value = 0.0007). (*e*) Correlation plot represents all the significant (FDR *p*-value ‹0.05) Pearson correlations between inflammation-related markers serum levels (x axis) and inferred immune infiltration at the tumor site (y axis). Color of the dots indicate positive (blue) or negative correlations (red).

marker of TNF activity (Fig. 3*i*), delta-like protein 1 (DLL1; Supplementary Fig. S1*f*), involved in promoting luminal breast cancer[21] and CRP (Supplementary Fig. S1*i*) which has previously been monitored during neoadjuvant breast cancer therapy.[22,23] In addition, in the chemotherapy arm, lower AXL levels throughout the treatment period (Figs. 3*j*–3*l*), a molecule associated with aggressive breast cancers,[24] were associated with better response.

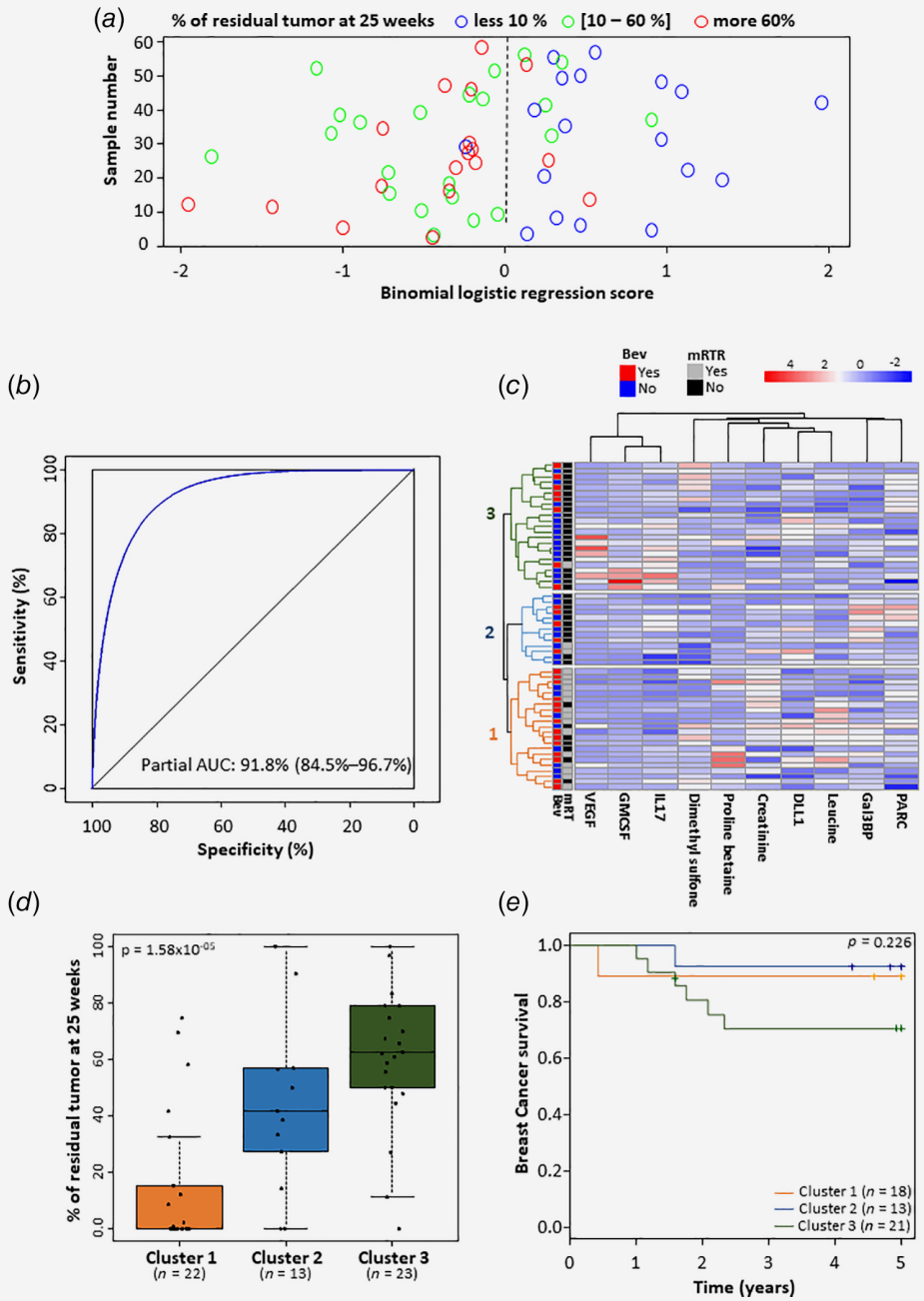

Figure 5. Predicting response using serum phenotype and binomial logistic regression. (*a*) The score, output of the binomial logistic regression penalized by the lasso method, is plotted and colored according to categories reflecting percentage of residual disease at surgery (blue dot: less than 10%, green dot: between 10 and 60%, red dot: more than 60%). The vertical line crossing the x-axis at 0 indicates the threshold for the samples predicted to have a good *vs*. bad response. (*b*) The efficiency of the binomial logistic regression penalized by the lasso method at predicting the percentage of residual tumor was assessed using ROC curves. The model showed good performances with an AUC of 91.8% (with 95% of CI between 84.5.5 and 96.7%). Confidence intervals are constructed using 100 bootstrapping. (*c*) Unsupervised clustering by correlation distance and average linkage using the serum levels of the 10 molecules identified to predict response. We identify three clusters of patients. Patient annotations on the y-axis indicate treatment received (+/- Bev) and minRT. (*d*) Boxplot represents the percentage of residual tumor at surgery according to the three clusters identified in the heatmap (*c*). Kruskal–Wallis test *p*-value is denoted on the top left. In the box-and-whiskers plots, the line within each box represents the median. Upper and lower edges of each box represent 75th and 25th percentile, respectively. (*e*) Kaplan–Meier survival curves for breast cancer survival for the three clusters are depicted. The *p*-value is from log-rank tests.

Overall, our results indicate that the inflammation-related markers associated with response are different in the two treatment arms.

Associating circulating molecule levels to tumor phenotype

We next assessed whether the measured inflammation-related markers were related to tumor characteristics.

Serum levels of VWF and GDF15 were significantly positively correlated with their mRNA levels at the tumor site (Figs. 4*a* and 4*b*). For both markers, the positive correlation was stronger in the combination (Bev) arm (Supplementary Fig. S2). In addition, serum and mRNA levels of VWF and GDF15 were higher at 12 weeks (Figs. 4*c* and 4*d*). All rho and corrected *p*-values for the correlation between cytokines and their respective tumor mRNA expression levels are given in Supplementary Table S5.

As VWF and GDF15 have previously been reported to interact with immune cells,[25,26] we investigated whether their serum levels were correlated with the presence of specific tumor infiltrating immune cells.

We used the tumor mRNA expression to infer for the infiltration of 22 immune cell types using the algorithm CIBERSORT.[4,16] Resting mast cell infiltration was positively correlated (ρ = +0.33, FDR *p*-value = 0.0007) with serum levels of VWF (Fig. 4*c*). GDF15 was inversely correlated (ρ = −0.33, FDR *p*-value = 0.0007) with M1-type of macrophages (Fig. 4*d*); interestingly GDF15 has been reported to inhibit macrophages surveillance during early tumor development.[25] In Figure 4*e*, all the significant Pearson correlations (FDR corrected) between cytokine serum levels and the predicted immune infiltration using CIBERSORT are shown: the color of the dots indicates positive (blue) or negative correlations (red) and the size of the dots reflects the strength of the correlation. We further identified GDF15 serum levels correlated with antitumorigenic immune infiltration (Macrophages M1, T helper and activated T cells).

These analyses suggest a continuum between the tumor and systemic patients' characteristics.

Predicting response to chemotherapy using levels of circulating molecules

We aimed at predicting response to chemotherapy ± Bev using only noninvasive biomarkers. We used serum levels of the inflammation-related cytokines measured in our study (n = 17) as well as a previously published set of 27 cytokines[4] and 26 serum metabolites measured in the serum of the patients of the same cohort. In total, a set of 70 molecules for the time-series was obtained: at screening (n = 45), 12 weeks (n = 58) and 25 weeks (n = 50). Due to relatively small sample sizes for each time point, we combined both treatment arms to fit a model predicting response to treatment.

Binomial logistic regression penalized by the lasso method was used to predict response minRT *vs.* residual tumor.

At baseline, it was not possible to build a model in which the levels of multiple molecules could accurately predict response at this sample size (Supplementary Fig. S3*a*). However, at 12 weeks, we identified a set of 10 molecules, the levels of which were associated to response (Supplementary Fig. S3*b*), these molecules and their lasso coefficient are reported in Supplementary Table S6. We found a significant negative correlation (Spearman $r = -0.5$, $p = 5.5 \times 10^{-05}$ and Pearson $r = -0.46$, $p = 0.0002$) between the percentage of residual tumor at 25 weeks (time of surgery) and the score obtained from the 10 weighted molecules selected by the binomial logistic regression ($\text{score}_i = \sum_{g=1}^{n} \beta_g . X_{gi}$; see Materials and Methods section). To illustrate the relationship between response to treatment and the binomial logistic regression score, we plotted the score as a function of the percentage of residual disease (Fig. 5*a*).

To assess the specificity and sensitivity of the binomial logistic regression at predicting the percentage of residual tumor, we used receiver operating characteristic (ROC) curve and area under the curve (AUC) analysis (Fig. 5*b*). Our model predicted response to therapy with an AUC = 91.8% (84.5–96.7%).

We compared this AUC to AUCs obtained when using the levels of each individual inflammation-related markers at each time point (Supplementary Table S7) which highlights the predictive value of the score (combining the levels of 10 cytokines) when compared to individual cytokine. Similarly, the score obtained from the binomial logistic regression showed better correlation with the percentage of minimal residual disease than any of the inflammation-related marker at any time point (Supplementary Table S8). This result indicated that for the NeoAva cohort, the measurement of 10 molecules at 12 weeks could help predict which patients are likely to be responders at 25 weeks.

Further using the levels of the 10 selected molecules in unsupervised clustering (Fig. 5*c*), we identified a cluster (Cluster 1) which contained 83% of the responders regardless of the treatment arm. The three clusters obtained were significantly associated with the percentage of residual disease (Fig. 5*d*). Interestingly, while Cluster 1 points to responders, Cluster 3 was mainly composed of nonresponders with a worse survival according to the "early" 5 years follow-up data for this cohort (Fig. 5*e*).

Altogether, our analyses demonstrate the relevance of monitoring the serum of breast cancer patients during neoadjuvant chemotherapy.

Discussion

In our study, we assessed the relevance of measuring levels of markers involved in vascular inflammation, angiogenesis, extra cellular matrix remodeling and cell-to-cell interaction in the serum of patients from a phase II randomized clinical trial of HER2-negative breast cancer patients receiving neoadjuvant chemotherapy with or without antiangiogenic therapy.

At different time points, we measured 17 inflammation-related markers using enzyme-immunoassay and examined them in perspective of treatment response and tumor molecular

characteristics. Based on our results, we propose that a set of 10 molecules measured at 12 weeks (after the first regimen of chemotherapy) could predict which are the responders.

Bev administration significantly affected the systemic levels of several molecules, that is, PTX3, VWF, AXL, ALCAM and CatS.

Of these, PTX3 has previously been described as an inhibitor of the proangiogenic effect of fibroblast growth factor 2.[27] Such an antiangiogenic effect of PTX3 has been described to be especially effective for hormone dependent tumors.[28] We found increased PTX3 levels in the Bev arm compared to the chemotherapy only arm at all measured time points. These results support the role of PTX3 in angiogenesis which may be modulated by VEGF inhibition. PTX3 could therefore be an actionable target when fibroblast growth factor 2 and angiogenesis play a pathogenic role, as recently suggested.[29]

AXL is a receptor tyrosine kinase which has been associated with an aggressive breast cancer phenotype.[30] In HER2-positive breast cancers, AXL is an important factor driving metastasis.[24] We found that serum levels of the soluble form of AXL were decreased by Bev. Furthermore, low AXL levels were associated with better response in the chemotherapy only arm consistent with the known oncogenic role of AXL in cancers.[31] We suggest that monitoring AXL in the serum of breast cancer patients could be indicative of aggressive tumor phenotype and a promising measurement of treatment response.

We found several inflammation-related molecules associated with treatment response, in the combination arm: VWF, ePCR and PARC and in the chemotherapy only arm: AXL, DLL1, sTNFR1 and CRP.

Of these, in the combination arm (+Bev), lower levels of PARC and VWF were associated with better response, but notably, the levels of these two inflammation-related markers were also increased by Bev. It is therefore conceivable that increased levels of VWF and PARC may counteract the effects of Bev or reflect adverse secondary effects of the drug. Bev treatment in neoadjuvant setting has been documented to increase adverse events.[32,33] In the NeoAva cohort, we observed secondary effects associated with Bev treatment, such as hemorrhage, febrile neutropenia, infections and hypertension. Taking into account that VWF and PARC have been associated with hemorrhage/hemostasis, endothelial dysfunction and neutropenia/infections,[34,35] we may hypothesize that the levels of these two cytokines may be related to adverse effects induced by Bev and therefore with worse patient overall condition and response.

In the chemotherapy-only arm, lower levels of four cytokines were associated with response (i.e., AXL, DLL1, sTNFR1 and CRP). Of these, four AXL has been associated with epithelial mesenchymal transition and resistance to therapy,[30,36] DLL1, a Notch ligand, promotes ER-positive[21] and ER-negative[37] breast cancer carcinogenesis, while sTNFR1 has been associated with the risk of breast cancer.[38] These results suggest that these known oncogenic molecules at the tumor site could be monitored systemically and still relate to response.

Using tumor characteristics such as metabolites, gene expression and inferred immune cell infiltration, we found two cytokines, VWF and GDF15, for which the serum levels correlated with the mRNA expression of their corresponding gene from the bulk tumor, but also with infiltration of specific immune cells. VWF expression correlated with mast cell infiltration, while GDF15 was negatively correlated with M1 type of macrophages, T helper cells and CD4 activated cells. This is in agreement with a link between VWF expression and mast cells adhesion/recruitment which has previously suggested.[26] Our results therefore indicate that measuring the serum levels of VWF and GDF15 cytokines can reflect the characteristics of the tumor microenvironment. GDF15 has been associated with the process of epithelial mesenchymal transition in breast cancer.[39] Therefore, our results highlight a potential link between cytokine production and immune infiltration at the tumor site which may be monitored systemically. However, based on the lack of protein data on these cytokine-related markers at the tumor site, our data should be interpreted with caution.

Our findings deserve further investigations on a larger scale for noninvasive monitoring of breast cancer treatment. Recently, Hart *et al.* demonstrated that serum metabolomics profiles measured by NMR could be prognostic of recurrences.[40] Here, we suggest that a set of 10 molecules (cytokines and metabolites) could be useful to predict response to neoadjuvant chemotherapy ± Bev. It is important to keep in mind that since the model leading to selection of these 10 markers was produced with a relatively small sample size, validation in an independent data set is required. Furthermore, it would be interesting to investigate whether serum levels of the inflammation-related markers investigated here could be used as biomarkers to detect the presence of breast cancers by comparing the patients' serum levels to those of healthy individuals.

In conclusion, we demonstrate the importance of measuring serum molecules by systematic assessment of cytokines and metabolites in breast cancer. In the NeoAva cohort in which patients have been randomized for chemotherapy or chemotherapy + Bev, we suggest that such serum levels could reflect adverse effect of therapy affecting patient fitness and response to neoadjuvant chemotherapy. Our analyses of serum levels of inflammation-related markers and metabolites in this cohort point also to actionable targets to potentiate the effects of antiangiogenic therapy applied today in many cancer types.[41,42]

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References

1. Khan KA, Kerbel RS. Improving immunotherapy outcomes with anti-angiogenic treatments and vice versa. *Nat Rev Clin Oncol* 2018;15:310–24.
2. Hanahan D, Weinberg R. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
3. Pierga JY, Petit T, Delozier T, et al. Neoadjuvant bevacizumab, trastuzumab, and chemotherapy for primary inflammatory HER2-positive breast cancer (BEVERLY-2): an open-label, single-arm phase 2 study. *Lancet Oncol* 2012;13:375–84.
4. Jabeen S, Zucknick M, Nome M, et al. Serum cytokine levels in breast cancer patients during neoadjuvant treatment with bevacizumab. *Oncoimmunology* 2018;7:e1457598.
5. Manuel M, Tredan O, Bachelot T, et al. Lymphopenia combined with low TCR diversity (divpenia) predicts poor overall survival in metastatic breast cancer patients. *Oncoimmunology* 2012;1:432–40.
6. Papatestas AE, Lesnick GJ, Genkins G, et al. The prognostic significance of peripheral lymphocyte counts in patients with breast carcinoma. *Cancer* 1976;37:164–8.
7. Ali HR, Provenzano E, Dawson SJ, et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. *Ann Oncol* 2014;25:1536–43.
8. Mahmoud SM, Paish EC, Powe DG, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol* 2011;29: 1949–55.
9. Pruneri G, Vingiani A, Denkert C. Tumor infiltrating lymphocytes in early breast cancer. *Breast* 2018;37:207–14.
10. Haukaas TH, Euceda LR, Giskeodegard GF, et al. Metabolic clusters of breast cancer in relation to gene- and protein expression subtypes. *Cancer Metab* 2016;4:12.
11. Gregor M, Hotamisligil G. Inflammatory mechanisms in obesity. *Annu Rev Immunol* 2011;29: 415–45.
12. Iyengar NM, Hudis CA, Dannenberg AJ. Obesity and inflammation: new insights into breast cancer development and progression. *Am Soc Clin Oncol Educ Book* 2013;33:46–51.
13. Silwal-Pandit L, Nord S, von der Lippe Gythfeldt H, et al. The longitudinal transcriptional response to neoadjuvant chemotherapy with and without bevacizumab in breast cancer. *Clin Cancer Res* 2017;23:4662–70.
14. Savorani F, Tomasi G, Engelsen SB. Icoshift: a versatile tool for the rapid alignment of 1D NMR spectra. *J Magn Reson* 2010;202:190–202.
15. Eilers PH. Parametric time warping. *Anal Chem* 2004;76:404–11.
16. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* 2015;12:453–7.
17. Friedman J, Hastie T, Tibshirani R. Regularization paths for generalized linear models via coordinate descent. *J Stat Softw* 2010;33:1–22.
18. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc B* 1995;57:289–300.
19. Song X, Liu W, Yuan X, et al. Acetylation of ACAP4 regulates CCL18-elicited breast cancer cell migration and invasion. *J Mol Cell Biol* 2018;10:559–72.
20. Tinholt M, Viken MK, Dahm AE, et al. Increased coagulation activity and genetic polymorphisms in the F5, F10 and EPCR genes are associated with breast cancer: a case-control study. *BMC Cancer* 2014;14:845.
21. Kumar S, Srivastav RK, Wilkes DW, et al. Estrogen-dependent DLL1-mediated Notch signaling promotes luminal breast cancer. *Oncogene* 2018; 38:2092–107.
22. Chan DS, Bandera EV, Greenwood DC, et al. Circulating C-reactive protein and breast cancer risk-systematic literature review and meta-analysis of prospective cohort studies. *Cancer Epidemiol Biomarkers Prev* 2015;24:1439–49.
23. Reinertsen KV, Engebraaten O, Loge JH, et al. Fatigue during and after breast cancer therapy—a prospective study. *J Pain Symptom Manage* 2017; 53:551–60.
24. Goyette MA, Duhamel S, Aubert L, et al. The receptor tyrosine kinase AXL is required at multiple steps of the metastatic cascade during HER2-positive breast cancer progression. *Cell Rep* 2018;23:1476–90.
25. Ratnam NM, Peterson JM, Talbert EE, et al. NF-kappaB regulates GDF-15 to suppress macrophage surveillance during early tumor development. *J Clin Invest* 2017;127:3796–809.
26. Swystun LL, Lai JD, Notley C, et al. The endothelial cell receptor stabilin-2 regulates VWF-FVIII complex half-life and immunogenicity. *J Clin Invest* 2018;128:4057–73.
27. Inforzato A, Baldock C, Jowitt T, et al. The angiogenic inhibitor long pentraxin PTX3 forms an asymmetric octamer with two binding sites for FGF2. *J Biol Chem* 2010;285:17681–92.
28. Leali D, Alessi P, Coltrini D, et al. Long pentraxin-3 inhibits FGF8b-dependent angiogenesis and growth of steroid hormone-regulated tumors. *Mol Cancer Ther* 2011;10:1600–10.
29. Presta M, Foglio E, Churruca Schuind A, et al. Long pentraxin-3 modulates the angiogenic activity of fibroblast growth factor-2. *Front Immunol* 2018;9: 2327.
30. Gjerdrum C, Tiron C, Hoiby T, et al. Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc Natl Acad Sci U S A* 2010; 107:1124–9.
31. Antony J, Huang RY. AXL-driven EMT state as a targetable conduit in cancer. *Cancer Res* 2017;77: 3725–32.
32. Bear HD, Tang G, Rastogi P, et al. Neoadjuvant plus adjuvant bevacizumab in early breast cancer (NSABP B-40 [NRG oncology]): secondary outcomes of a phase 3, randomised controlled trial. *Lancet Oncol* 2015;16:1037–48.
33. von Minckwitz G, Eidtmann H, Rezai M, et al. Neoadjuvant chemotherapy and bevacizumab for HER2-negative breast cancer. *N Engl J Med* 2012; 366:299–309.
34. Franchini M, Lippi G. The role of von Willebrand factor in hemorrhagic and thrombotic disorders. *Crit Rev Clin Lab Sci* 2007;44:115–49.
35. Hector A, Kroner C, Carevic M, et al. The chemokine CCL18 characterises pseudomonas infections in cystic fibrosis lung disease. *Eur Respir J* 2014; 44:1608–15.
36. Jin G, Wang Z, Wang J, et al. Expression of Axl and its prognostic significance in human breast cancer. *Oncol Lett* 2017;13:621–8.
37. Sales-Dias J, Silva G, Lamy M, et al. The Notch ligand DLL1 exerts carcinogenic features in human breast cancer cells. *PLoS One* 2019;14: e0217002.
38. Xu F, Zhou G, Han S, et al. Association of TNF-alpha, TNFRSF1A and TNFRSF1B gene polymorphisms with the risk of sporadic breast cancer in northeast Chinese Han women. *PLoS One* 2014;9:e101138.
39. Peake BF, Eze SM, Yang L, et al. Growth differentiation factor 15 mediates epithelial mesenchymal transition and invasion of breast cancers through IGF-1R-FoxM1 signaling. *Oncotarget* 2017;8: 94393–406.
40. Hart CD, Vignoli A, Tenori L, et al. Serum metabolomic profiles identify ER-positive early breast cancer patients at increased risk of disease recurrence in a multicenter population. *Clin Cancer Res* 2017;23:1422–31.
41. Saltz LB, Clarke S, Diaz-Rubio E, et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. *J Clin Oncol* 2008;26:2013–9.
42. Skelton WP 4th, Castagno J, Cardenas-Goicoechea J, et al. Bevacizumab eligibility in patients with metastatic and recurrent cervical cancer: a retrospective review. *Clin Med Insights Oncol* 2018;12:1–5.