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

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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

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

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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. Angiogenesis as well as altered metabolism and tumor-related inflammation are cancer hallmarks, 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. We recently demonstrated that anti-VEGF-A therapy in breast cancer affects the tumor immune contexture.

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

Breast tumors have distinct metabolic profiles 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. Metallammary, metabolically induced low-grade and chronic inflammation, has mostly been studied in relation to metabolic diseases, but may also play an important role in breast cancer development and progression. 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.

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).
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.

| Markers for | Protein full name | Protein short name |
|-------------|-------------------|--------------------|
| Vascular inflammation | Osteoprotegrin | OPG |
|  | Pentraxin 3 | PTX3 |
|  | Tyrosine-protein kinase receptor | Axl |
|  | CX-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.sulphone | |
|  | Glucose | |
|  | Glycine | |
|  | Lactate | |
|  | Tyrosine | |
|  | Histidine | |
|  | Phenylalanine | |

**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 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 microliter serum were gently mixed with 150 μl of buffer (D2O with 0.075 mM Na2HPO4, 5 mM NaN, 3,5 mM TSP, pH 7.4). Data acquisition was fully automated using a SampleJet with ICON-NMR 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. Spectra were further baseline corrected using the asymmetric least squares method. 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. 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. 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 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:

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

where g is the target (molecule), n is the number of targets, \(\beta_g\) 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
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.

**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. 1a).

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/C6 Bev, (iii) 25 weeks: after the second regimen of chemotherapy: taxane/C6 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 1b 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

### Table 2. Clinical characteristics of the NeoAva cohort

|                      | Screening Chemotherapy | Combination (+Bev) | 12 weeks Chemotherapy | Combination (+Bev) | 25 weeks Chemotherapy | Combination (+Bev) | 31 weeks 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.
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 varying 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. 2a–2c) 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. 2a and 2b).

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. 2c and 2d). We also found that levels of CatS significantly increased at 31 weeks for Bev treated patients (Fig. 2e).

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

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

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\textsuperscript{19}; Supplementary Figs. S1a–S1c) and endothelial cell protein C receptor (ePCR) levels (Figs. 3d–3f), a gene associated with risk of breast cancer\textsuperscript{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
marker of TNF activity (Fig. 3i), delta-like protein 1 (DLL1; Supplementary Fig. S1f), involved in promoting luminal breast cancer21 and CRP (Supplementary Fig. S1i) 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. 3j–3l), 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 in 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% 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. 4e and 4f). 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. 4d and 4f). 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, 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. 4c). GDF15 was inversely correlated (ρ = −0.33, FDR p-value = 0.0007) with M1-type of macrophages (Fig. 4d); interestingly GDF15 has been reported to inhibit macrophages surveillance during early tumor development. In Figure 4e, 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 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. S3a). However, at 12 weeks, we identified a set of 10 molecules, the levels of which were associated to response (Supplementary Fig. S3b), 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 × 10−45 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 (scorei = \( \sum_{g=1}^{n} \beta_g \times X_{gi} \); 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. 5a).

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. 5b). 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. 5c), 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. 5d). 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. 5e).

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-positive31 and ER-negative37 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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