Plasma and tissue proteomic prognostic factors of response in primary breast cancer patients receiving neoadjuvant chemotherapy

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Abstract. A pathological complete response (pCR) after neoadjuvant chemotherapy is observed in approximately 20% of breast cancer patients. A proteomic analysis was performed on plasma and tumor tissue before treatment to evaluate its potential impact on the prediction of response. One hundred and forty-nine breast cancer patients eligible for neoadjuvant chemotherapy were included in the study between February 2004 and January 2009 at three centers. The proteomic analysis was performed using SELDI Technology (ProteinChip CM10 pH4, IMAC-Cu and H50). Three acquisition protocols were used according to the mass range. Plasma and tumor proteomic signatures were generated using generalized ROC criteria and cross-validation. Twenty-eight (18.8%) patients out of 149 experienced a pCR according to Sataloff criteria. In the cytosol analysis, respectively 4, 2 and 8 proteins had significantly different levels of expression in the responders and non-responders using IMAC-Cu, H50 and CM10 pH4. Among the 8 proteins of interest on CM10 pH4, 2 (C1 and C7) were selected and were validated in 95.0 and 85.6% of the models. In the plasma analysis, respectively 12, 6 and 2 proteins had different levels of expression using the same ProteinChips. Among the 12 plasma proteins of interest on IMAC-Cu, 2 (P1 and P7) were selected and were validated in 94.8 and 97.6% of the models. A combined proteomic signature was generated, which remained statistically significant when adjusted for hormone receptor status and Ki-67. Our results show that proteomic analysis can differentiate complete pathological responders in breast cancer patients after neoadjuvant chemotherapy.

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Introduction

Breast cancer is currently the most common female cancer. There are 183,000 new cases each year and 41,000 women succumb to this disease in the USA in 2000 (1). In 85% of cases the tumor remains localized and the treatment consists of surgery, radiotherapy ± chemo and/or hormonotherapy. Despite this treatment, local or distant relapses occur in approximately 40% of patients. Predictive risk factors are being used to a greater extent to identify subgroups of patients who will most likely benefit from adjuvant treatment. The use of the St. Gallen guidelines (2) has led to the treatment of approximately 50% of patients after surgery for early breast cancer with anthracyclin-based chemotherapy sequenced with taxanes (3). This probabilistic criterion has resulted in many patients being submitted to aggressive treatments.

Neoadjuvant chemotherapy is widely used in the management of patients with locally advanced breast cancer. In addition to increasing the rates of breast conservation, this treatment strategy allows the use of pathologic response as an early surrogate marker for overall survival. The uncertain benefit, the toxicity of chemotherapy and the existence of alternative treatments calls for the development of methods to select patients most likely to benefit.

Using conventional two-dimensional electrophoresis and mass spectrometry, we previously identified markers of potential clinical interest in human breast cancer, such as the molecular chaperone 14-3-3σ which is downregulated in breast cancer cells as compared with normal breast epithelial cells (4). Surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry (MS) coupled with appropriate bio-informatic tools have been used to identify protein patterns related to various stages and types of solid tumors and serum (5-12). This technology combines chromatographic fractionation of the proteome using protein biochips and TOF MS analysis that can be applied to various clinical samples, such as serum and tissue (13); it allows relatively high-throughput protein analysis of complex biological samples, with limited preprocessing steps.

To date, most proteomic-based studies are largely performed *in vitro* for the identification of differential expression levels between parental and chemotherapy-resistant cell

sublines. Only a few small studies are based on fresh breast cancer tissue samples (14,15) and plasma proteomic evaluations in this situation have not yet been published.

Herein, we define plasma and tumor proteomic profiles of primary breast cancer patients and provide evidence that such an approach may have a significant impact in predicting response to neoadjuvant chemotherapy. The primary objective of this study was to define proteomic signatures correlated with a complete pathological response after neoadjuvant chemotherapy. Secondary objectives included the study of correlations between proteomic signatures and nodal involvement, pathological subtypes, clinical and ultrasound response and menopausal status.

Materials and methods

Patients. Main inclusion criteria were: female patients over 18 years of age, with a histologically proven breast adenocarcinoma, eligible for neoadjuvant chemotherapy with anthracyclines and taxanes with no previous chemotherapy for malignant disease. Information and informed consent were obtained according to the French law. Exclusion criteria were metastatic disease, non-adenocarcinoma breast tumors, contraindication of anthracyclines/taxanes, patient's refusal of storage of blood and tumor biopsy and inclusion in another clinical research study.

Treatment. The chemotherapy protocol included a sequence of FEC100 (epirubicin, 100 mg/m^2; cyclophosphamide, 500 mg/m^2 and fluorouracil, 500 mg/m^2) followed by docetaxel, 100 mg/m^2 on Day 1 every 3 weeks, provided the neutrophil cell count was over 1,500/mm^3. At the beginning of the study, all patients received 4 cycles of each regimen. After the publication of the results of the PACS 01 adjuvant trial (3), it was decided to administer 3 courses of each for a total of 6.

Pathological analysis. The pathological diagnosis included the following variables: histological type (essentially ductular or lobular), histoprognostic grading, estradiol and progesterone receptor status, HER2 positivity on immunohistochemistry or FISH, and Ki-67 hyperexpression. In triple-negative tumors (TNBC), the basal phenotype was assessed by determining c-Kit and R EGF hyperexpression.

Inprints of the frozen part of the tumor were performed to be sure that the tissue extract proteomic analysis was performed on tumor tissue. The pathological diagnosis, using the same criteria, was also performed after surgery. The pathological tumor and nodal responses were assessed according to Sataloff (16) criteria.

Protein expression profiling

Tumor samples. All tumor samples were processed within 1 h after collection and rapidly frozen at -80˚C.

Plasma samples. Plasma (10 ml of blood) was obtained before the initiation of neoadjuvant chemotherapy and processed within 1 h after collection and frozen at -80˚C.

Preparation of cytosols. The frozen tissues were weighed, then disrupted and homogenized in 200 μl of 50 mM Tris-HCl buffer (pH 9.0) containing 7 M urea, 2 M thiourea, 2% CHAPS using a Potter Homogenizer and a Rotor-Stator homogenizer (Ribolyser, Hybaid). The homogenate was ultracentrifuged at 105,000 x g for 60 min at 4˚C.

The protein concentration of the cytosols was determined using the Quick Start Bradford Dye Reagent (Bio-Rad Laboratories Inc., France) based on the method of Bradford (17).

ProteinChip array analysis. Three types of ProteinChips (Bio-Rad Laboratories) with a surface chemistry of hydrophobic (H50), cationic (CM10) and metal affinity (IMAC-Cu) were tested to determine which might provide the best plasma and tumor cytosol profiles.

Plasma samples were aliquoted (50 μl) and thawed at -80˚C. Each sample (10 μl) was denaturated by adding 90 μl of 50 mM Tris-HCl buffer (pH 9.0) containing 7 M urea, 2 M thiourea and 2% CHAPS [(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid). The mixture was vortex-mixed and shaken for 20 min at room temperature. Tumor cytosols (100 μg proteins) were diluted in 200 μl of 50 mM Tris-HCl buffer pH 9.0 containing 7 M urea, 2 M thiourea and 2% CHAPS.

The denatured plasma samples (20 μl) and the diluted tumor cytosol samples (20 μl) were then diluted (1:10) with the adequate binding buffer (acetonitrile 10 ml/l, trifluoroacetic acid 1 ml/l, NaCl 150 mM for H50, sodium acetate 100 mM pH 4.0 for CM10 and phosphate-buffer saline (PBS) pH 7.4 for IMAC-Cu). Then 100 μl was spotted on the ProteinChip array in a 96-well bioprocessor (Bio-Rad Laboratories). IMAC-Cu ProteinChips were precharged for activation with 50 mM $CuSO_4$ for 10 min according to the manufacturer's instructions (Bio-Rad Laboratories).

After the samples were allowed to bind at room temperature for 45 min on a platform shaker (Heidolph Titramax 100), the arrays were washed twice with 200 μl adequate binding buffer for 5 min, followed by two quick rinses with 200 μl deionized water. After air-drying, 1 μl of saturated sinapinic acid [5 mg dissolved in 400 μl of acetonitrile/trifluoroacetic acid (50%/0.5%)] was applied twice to each spot, allowing the array surface to air-dry 10 min between each application. Proteins bound to the ProteinChips arrays were detected with the ProteinChip System Series 4000 (Bio-Rad Laboratories).

Time of flight spectra were generated by averaging 530 laser shots collected at a laser intensity of 2,500 with a focus mass of 7,000 (for 1,800-10,000 Da proteins), at a laser intensity of 3,500 with a focus mass of 16,000 (for 10,000-20,000 Da proteins), and finally at a laser intensity of 5,000 with a focus mass of 40,000 (for 20,000-150,000 Da proteins). External mass calibration was performed using the All-In-One Peptide molecular mass standard. Spectra analyses (peak detection, mass calibration, baseline substraction and total ion current normalization) were performed using Ciphergen ProteinChip Data Manager DE Software 4.1.

Reproducibility was estimated using one pool of plasma and one pool of tumor cytosols. The mean of the CV, estimated on all the detected peaks both at each laser intensity tested and on each type of ProteinChip array, ranged from 10 to 20%.

Statistical methods. Clinical and echographic assessments of tumor size, node involvement and tissue diagnosis were

recorded at baseline and at the end of treatment. Patients with a pathological complete response (pCR) were considered responders: tumor A (TA)/node A (NA) or TA/NB. All other categories were considered non-responders.

On the basis of an expected pCR rate of 20%, the inclusion of 100 breast cancer patients treated with neoadjuvant chemotherapy allowed a 95% confidence interval width of ±8%. Initial demographic and tumor characteristics for categorical variables are presented as frequencies and percentages. Continuous variables are presented as medians and range. For the receiver operating characteristic curve (ROC) analyses, cytosol and plasma variables were transformed to the logarithmic scale if deemed necessary in order to stabilize the variance.

The search for candidate proteins was first performed by univariate analysis using the non-parametric Wilcoxon test comparing responders and non-responders. Multivariate analyses using a generalized ROC criterion were then performed to obtain a proteomic signature separately for cytosol and plasma and then in combination (18). This technique selects the variable combinations which maximize the area under the curve (AUC). Each cytosol and plasma model was then validated internally on 500 random samples with replacement on the whole dataset. Logistic regression models were then applied selecting variables according to the Akaike Information Criterion (AIC). The percentage of times each variable was selected was extracted. Only those variables which were selected in >80% of models were retained. Results of statistical tests were considered significant at the 5% level. The final model was adjusted for significant clinical variables.

Results

Demographic and clinical characteristics. One hundred and forty-nine breast cancer patients were enrolled between February 2004 and January 2009 from 3 centers. A total of 8 patients were excluded: 4 were ineligible (prior chemotherapy in one and metastatic disease in 3); and 4 were non-evaluable (one patient was untreated, one patient received only one cycle due to toxicity, one had no tumor nor blood plasma sample and one patient developed a carcinomatous meningitis while receiving neoadjuvant chemotherapy).

Clinical characteristics for the 141 patients analyzed are presented in Table I. Median age was 46 years (range, 26-74). Median tumor size was 40 mm (range, 10-130). All patients received a minimum of three cycles of chemotherapy. Overall, 82 (58%) patients received 3 cycles of FEC and 3 cycles of docetaxel; 38 patients received eight cycles. The reason for not receiving 6 or 8 cycles were side effects which prevented the continuation of the same regimen.

Overall, 89 patients (63%) had conservative surgery, and 136 (96%) had an axillary clearance; 98% of patients had surgery within 6 weeks after the last chemotherapy cycle.

The pathological complete response rate according to Sataloff was 18.8% (95% CI, 12.6-25.7). Negative hormone receptor tumors showed a significantly higher pCR rate then positive ones. In addition, in spite of many missing data for Ki-67, not a single patient with Ki-67 <15% had a pCR (Table II).

Cytosols. One hundred and eight patients (77%) were evaluable for cytosol analysis. Using IMAC-Cu and H50 arrays, 150 and 131 different proteins were observed respectively and their levels were statistically different between responders and non-responders for 4 proteins (IMAC-Cu) and for 2 proteins (H50), respectively. Using CM10 pH4 arrays, 180 different proteins were observed. For 8 proteins, the levels were statistically different between responders and non-responders (Table III,

Table I. Characteristics of the breast cancer patients before chemotherapy.

Characteristics	n=141 n (%)
Site of tumor	
Right	76 (54)
Left	65 (46)
Tumor stage	
T1	9 (6)
T2	83 (59)
T3	30 (21)
T4	19 (14)
Nodal involvement	
N0	73 (52)
N1	62 (45)
N2	4 (3)
Missing	2
Histology	
Ductal	127 (90)
Lobular	8 (6)
Other	6 (4)
Missing	2
Differentiation	
Well	8 (7)
Medium	33 (28)
Poor	75 (65)
SBR grade	
I	10 (9)
II	74 (67)
III	26 (24)
NP	17
Missing	15
Hormone receptor status	
ER^+ PR^+	63 (45)
ER^+ PR^-	23 (16)
ER^- PR^+	3 (2)
ER^- PR^-	52 (37)
Ki-67 index	
<15	10 (7)
≥15	40 (93)
Missing	91

NP, non-gradable; SBR, Scarff-Bloom-Richardson; ER, estrogen receptor; PR, progesterone receptor.

Table II. Complete pathological response according to clinical variables.

Characteristics	Non-response (n=114), n (%)	Response (n=27), n (%)	P-value
Surgery			
Conservative	68 (60)	21 (78)	0.079
Mastectomy	46 (40)	6 (22)	
Side			
Right	65 (57)	11 (41)	0.127
Left	49 (43)	16 (59)	
Tumor stage			
T1	7 (6)	2 (7)	0.783
T2	66 (58)	17 (63)	
T3	24 (21)	6 (23)	
T4	17 (15)	2 (7)	
Nodal involvement			
N0	59 (52)	14 (56)	0.700
N^+	55 (48)	11 (44)	
SBR stage			
I	8 (9)	2 (10)	0.304
II	63 (70)	10 (53)	
III	19 (21)	7 (37)	
ER status			
Negative	36 (32)	18 (69)	0.0004
Positive	78 (68)	8 (31)	
PR status			
Negative	54 (47)	20 (77)	0.0064
Positive	60 (53)	6 (23)	
HER2 status			
Negative	70 (75)	16 (73)	0.867
Positive	24 (25)	6 (27)	
Ki-67 index			
<15	10 (24)	0	0.098
≥15	31 (76)	9 (100)	

Fig. 1). The generalized ROC criterion identified cytosolic proteins C1, C7 and C8 as the best three variables. However, only C1 and C7 were selected since they were subsequently validated in 95.0 and 85.6% of models, respectively. On the other hand, C8 was only present in 59.2% of the models and was not retained. The AUC for log(C1) and log(C7) was equal to AUC = 0.768 (95% CI, 0.623-0.858) (Fig. 2A).

Plasma. Eighty-eight (62%) patients were evaluable for plasma analysis. Using CM10 pH4 and H50 arrays, 85 and 107 different proteins were observed, respectively, and their levels were statistically different between responders and non-responders for 2 proteins (CM10 pH4) and 6 proteins (H50). Using IMAC-Cu arrays, 98 different proteins were observed. For 12 proteins, the levels were statistically different between the two populations (Table III, Fig. 1). The generalized ROC criterion identified plasma proteins P1, P2 and P7 as the best three variables. However, only P1 and P7 were selected since they were validated in 94.8 and 97.6% of models respectively. On the other hand, P2 was only present in 55.0% of the models and was not retained. The AUC for log(P1) and log(P7) was equal to AUC = 0.774 (95% CI, 0.631-0.865) (Fig. 2B).

Cytosol and plasma combination. In an effort to use both cytosol and plasma results for constructing a combined proteomic signature, the weighted combination variable Z = 2*Z_C + Z_P was generated from the integer values of the coefficients estimated from the mROC analysis (1.088 and 0.509, respectively). The AUC for this combination was equal to AUC = 0.843 (95% CI, 0.692-0.933) (Fig. 2C). Overall correct classification was 87.3% (Table IV). The proteomic signature remained statistically significant when adjusted for hormone receptor status. Moreover, in the population of patients with positive Ki-67, the proteomic signature remained statistically significant.

Table III. pCR according to the proteomic analysis of the cytosols and the plasma.

	Non-response, median (range)	Response, median (range)	P-value
Cytosols (protein molecular weight, m/z)			
C1 (3077)	**5.5 (1.1-83.2)**	**3.1 (0.1-23.0)**	**<0.0001**
C2 (4629)	301.7 (6.6-749.7)	164.7 (6.8-549.5)	0.035
C3 (44002)	20.6 (0.1-58.1)	14.0 (1.8-40.9)	0.024
C4 (116996)	22.0 (0.1-71.3)	13.1 (2.0-35.7)	0.0116
C5 (3556)	6.9 (0.1-133.7)	3.1 (0.6-51.8)	0.009
C6 (3348)	53.0 (3.8-139.1)	25.9 (2.8-149.9)	0.056
C7 (5071)	**2.9 (0.1-13.0)**	**2.0 (0.6-3.9)**	**0.006**
C8 (5793)	0.17 (0.00-0.46)	0.16 (0.05-0.23)	0.022
Z_C = log(C1)+log(C7)	1.39 (-0.16-2.59)	0.77 (-1.11-1.57)	<0.00001
Plasma (protein molecular weight, m/z)			
P1 (7948)	**5.4 (0.1-114.5)**	**2.9 (0.1-19.9)**	**0.022**
P2 (4752)	5.0 (0.1-32)	2.1 (0.1-11.1)	0.036
P3 (74986)	16.0 (0.1-98.5)	8.4 (0.1-31.5)	0.0398
P4 (89539)	33.9 (9.9-172.7)	17.7 (8.8-132.2)	0.012
P5 (116754)	14.0 (1.5-59.7)	8.3 (3.6-53.3)	0.026
P6 (115505)	2.8 (0.4-13.8)	4.1 (1.1-14.9)	0.092
P7 (101771)	**4.0 (0.2-11.0)**	**5.5 (0.9-8.8)**	**0.029**
P8 (8052)	5.4 (2.6-9.4)	6.4 (3.7-10.3)	0.021
P9 (7849)	1.7 (0.7-3.9)	2.3 (0.9-4.7)	0.080
P10 (7577)	1.6 (0.1-4.7)	2.4 (0.8-4.8)	0.048
P11 (100104)	0.9 (0.1-3.7)	1.4 (0.2-3.3)	0.039
P12 (43422)	0.8 (0.1-2.9)	1.3 (0.3-3.1)	0.035
Z_P = log(P1)-2*log(P7)	-0.41 (-2.49-1.67)	-0.90 (-2.85--0.29)	0.0004
Cytosol and plasma			
2*Z_C + Z_P	2.32 (-0.27-4.99)	0.53 (-3.14-2.36)	<0.0001

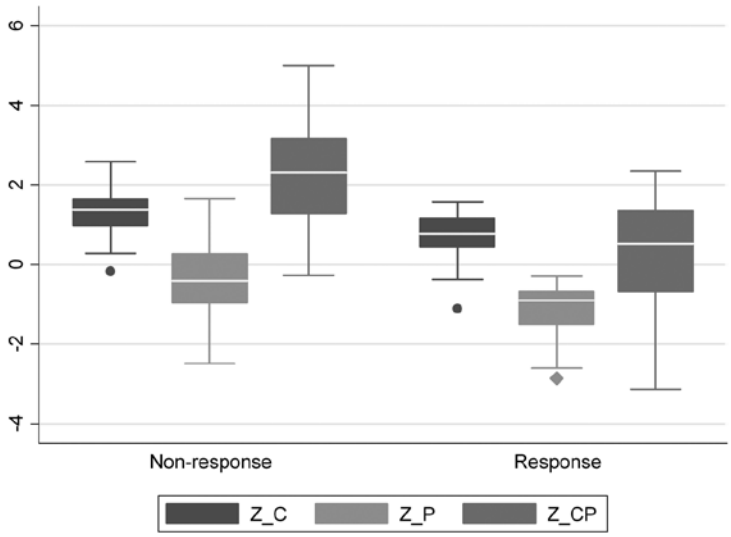

Figure 1. Box plot of Z_C, Z_P and the combination Z_CP according to the response.

Due to missing data, only 63 (45%) samples could be evaluated for the combination. However, no significant difference in patient and tumor characteristics were noted for patients included and excluded from the combination analysis (data not shown).

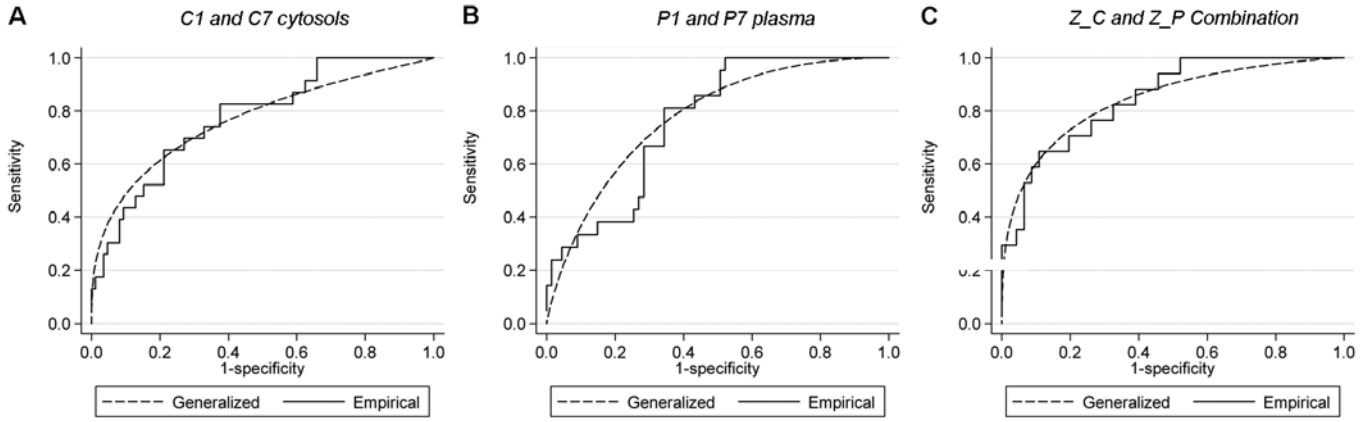

Figure 2. ROC curves for cytosols, plasma and the combination.

Table IV. ROC analysis of the cytosols and plasma combination.

Term	Result
AUC (95% CI)	0.84 (0.69-0.93)
Sensitivity (%)	89.1
Specificity (%)	82.4
Positive predictive value (%)	93.2
Negative predictive value (%)	73.7
Overall correct classification (%)	87.3

Discussion

Our results showed that proteomic analysis in the plasma, in the tumor and a combination of results in the plasma and the tumor could differentiate complete pathological responders and non-responders in breast cancer patients receiving neoadjuvant chemotherapy. Two plasma and two cytosolic proteins were selected and a combined plasma and tumor signature was generated. This signature remained statistically significant when adjusted for hormone receptor status and in Ki-67-positive patients.

Overall, the study population was similar to the patient population of other neoadjuvant studies. Ninety-four percent of patients had a tumor >2 cm (median tumor size, 4 cm). Nearly half of them had positive axillary nodes. Only 61% of the tumors had positive estradiol receptors and 47% were positive for progesterone receptors. Twenty-four percent of tumors were grade 3 and the median value of Ki-67 was 30%. Twenty-five percent of tumors were HER2-positive (either by immunohistochemistry or SISH). This population had a high tumor burden and aggressive tumor.

SELDI-TOF MS profiling appears to be a promising tool for diagnosis (6-7,11,19). The use of such a technique in relation to therapeutic response prediction has been limited (20). In the present study, we used this technology on both plasma and cytosols to predict the therapeutic response in a series of breast cancer patients receiving neoadjuvant chemotherapy.

In both plasma and cytosols, the assays were performed using three types of ProteinChip arrays (CM10 pH4, IMAC-Cu and H50). In cytosols, CM10 pH4 ProteinChip arrays appeared to be the best arrays to discriminate between responder and non-responder patients, whereas IMAC-Cu array was the best discriminator in plasma.

In the present study, no data was contained concerning the characterization of the proteins of the established signature. It is noteworthy that relevant biomarkers identified in SELDI-TOF profiling studies are in most cases non-specific host response-generated proteins at rather high levels (21). For example, in such studies, haptoglobin, transferrin, or the C3A or C3B complement have been evidenced. Furthermore, ubiquitin and ferritin light chain, corresponding to the two peaks of interest, were evidenced using SELDI-TOF-MS screening to identify differentially expressed cytosolic proteins with a prognostic impact in node-negative breast cancer patients with no relapse vs. patients with metastatic relapse (22).

We demonstrated that plasma and tumor proteomic profiles obtained prior to neoadjuvant chemotherapy in primary breast cancer may predict a complete pathological response to treatment. Comparable results were found by He *et al* (15) in a smaller population. In the entire group, a single peak at mass/charge ratio (m/z) of 16,906 correctly separated 88.9% of the tumors with pathological complete response and 91.7% of the resistant tumors. These data suggest that breast cancer protein biomarkers may be used to pre-select patients for optimal chemotherapeutic treatments. Other studies have identified potential markers related to neoadjuvant chemotherapy using SELDI-TOF MS by comparing the proteomic profiles before and after treatment. Relatively few changes were identified in plasma by Pusztai *et al* (23). They detected only a single chemotherapy-inducible SELDI-MS peak [mass/charge ratio (m/z), 2790] that was induced by paclitaxel and, to a lesser extent, by FAC chemotherapy. Recently, the intensities of eight different protein peaks were demonstrated to be higher in breast cancer tissue extracts after neoadjuvant chemotherapy than those before neoadjuvant chemotherapy (24). Although further experiments are needed to prove the reliability of these eight proteins, these results will help in the establishment of protein models based on drug resistance-related protein peaks

to screen whether a patient is suitable for adopting neoadjuvant chemotherapy and to improve cancer treatment.

The pCR according to Sataloff (TA NA, TA, NB) was in the lower range of the published results for anthracycline-taxane combining regimens (25). One reason for this relatively low rate could be the very restrictive definition of the pCR related to the surgical specimen process for pathological analysis as previously described (26). The prognostic factors of response were ER (P=0.0004) and PR (P=0.006) negativity and Ki-67 above the median value of 30% (P=0.003). No pCR was observed for Ki-67 below 15%. These factors have been previously reported in many neoadjuvant studies (27). Similarly, the pCR rate was higher in triple-negative tumors (28) and in ductular than in lobular cancer (29). Several molecular signatures are being tested in the neoadjuvant setting (30-33). However, the benefit of using genetic predictors over usual pathological biomarkers is not clear. Our study showed that a proteomic analysis of plasma and cytosol could also predict pCR in breast cancer patients treated with FEC and docetaxel. The role of a proteomic analysis in clinical practice remains to be defined.

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