Apoptosis-related Biomarkers sFAS, MIF, ICAM-1 and PAI-1 in Serum of Breast Cancer Patients Undergoing Neoadjuvant Chemotherapy

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Abstract. *Background: Neoadjuvant chemotherapy improves surgical options and prognosis in patients with operable breast cancer. Predictive biomarkers are needed to choose the most effective therapy and to avoid unnecessary toxicity. Patients and Methods: We analyzed the courses of apoptosis-related serum biomarkers macrophage migration-inhibitory factor (MIF), soluble cell death receptor sFAS, soluble intercellular adhesion molecule (sICAM), plasminogen activator inhibitor 1 (PAI-1) as well as the oncological biomarkers carcino-embryonic antigen (CEA) and carbohydrate antigen 15-3 (CA15-3) in prospectively collected sera of 51 patients with locally confined breast cancer undergoing preoperative chemotherapy. As controls 31 healthy women, 13 patients with benign breast disease and 28 patients with metastasized breast cancer were included. Results: sFAS, MIF, CEA and CA15-3 showed significantly higher serum concentrations in patients with metastasized breast cancer than in healthy and benign controls. Additionally, sFAS and MIF discriminated between locally confined breast cancer and healthy controls with an area under the curve (AUC) in receiver operating characteristic (ROC) curves of 73.4% and 70.7%. After neoadjuvant chemotherapy, 38 patients achieved complete (N=9) or partial (N=29) remission, while 13 patients had no change of disease. Pretherapeutic levels of MIF were considerably higher in non-responsive patients (p=0.082). In addition, post-therapeutic sICAM and CA15-3 levels were higher in patients without complete remission. Conclusion: Apoptosis-related biomarkers are valuable markers in breast cancer patients and show potential for early estimation of the efficiency of neoadjuvant chemotherapy.*

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death among women worldwide with an estimated 1.4 million new breast cancer and 458.000 deaths per year (1). Screening programs in many Western countries have led to increased rates of early-stage detection of potentially curative breast cancer (2). Today, breast cancer is considered a systemic disease with a locoregional component. Based on an increasing amount of clinical evidence, neoadjuvant chemotherapy is used for women with locally advanced breast cancer in order to increase rates of breast-conserving surgery and to gain *in vivo* information on chemotherapy response and tumor biology (3). A pathologically complete remission following neoadjuvant chemotherapy is associated with improved disease-free and overall survival (4). Recent studies indicate that patients with early or mid-course response are more likely to reach a complete pathological remission (3).

Currently, therapy response is evaluated using clinical examination, ultrasound and mammography (3). Unfortunately, these methods do not indicate the response before application of several therapy cycles and do not represent the biochemical changes in the tumor very well. An early-in-the-course prediction of response or non-response to the chosen chemotherapeutic regimen is important in order to allow switching to another protocol in the case of non-response to reach the best response and thereby avoiding unnecessary toxicity (5). For predicting response and non-response as early as possible, the

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identification of new predictive peripheral blood-based biomarkers is necessary especially as classical serum tumor markers such as carcino-embryonic antigen (CEA) and carbohydrate antigen 15-3 (CA15-3) are known to be less effective in locally confined breast cancer (6).

Apoptotic cell death plays a crucial role in the development of cancer (7). Chemotherapeutic drugs aim at increasing the rate of apoptosis in tumor and reducing the number of tumor cells (8). Under physiological conditions, cellular and even nuclear products that are released during apoptosis are phagocytized by macrophages and neighboring cells and are detected in peripheral blood only at very low concentrations (9). If the recycling systems are impaired or overloaded, apoptotic products can accumulate in the circulation (10).

The cell-surface receptor FAS/CD 95/APO-1 is part of the tumor necrosis factor receptor superfamily (11). Ligation of FAS by FAS ligand (FASL) or agonistic anti-FAS antibody leads to trimerization of the FAS receptor and induces apoptosis in various kinds of cells. Dysregulation of FAS mediated apoptosis is known to play an important role in the development and progression of cancer (7, 11). For example, the FAS/FASL system is involved in the escape of cancer cells from the immune system as cancer cells can either express FASL on their surface or produce soluble FASL, both resulting in apoptosis of the immune cells (11, 12). In addition, sFAS is formed by cleavage of the external part of extracellular FAS (13). sFAS can inhibit FAS-mediated apoptosis by neutralising FASL (14). While the properties of sFAS have not been completely elucidated yet, there are diverse theories about the possible origin of sFAS in the peripheral blood. sFAS could derive from tumor cells themselves (15), from peripheral blood lymphocytes (16), or from the surrounding stromal tissue in response to the tumor or the immune system (17).

Macrophage migration-inhibitory factor (MIF) was originally described as a T-cell-derived cytokine that exhibits a broad range of immunostimulatory and proinflammatory activities (18, 19). For example, it promotes mitogen-activated protein-kinase (MAPK) signaling, secretion of tumor necrosis factor α (TNF-α) and activity of cyclooxygenase-2 (COX-2) (18-21). However, MIF is not only secreted by T-lymphocytes, but also by various parenchymal and tumor cells (18). It is known that MIF plays a pivotal role in regulation of cell homeostasis and therefore in carcinogenesis and tumor angiogenesis (22). For instance, it has been supposed that tumor cells secrete MIF to enhance their proliferation by autocrine amplification. Concerning its role in cell death, it is known that MIF can inhibit p53-dependent gene expression and thus inhibit apoptosis (23).

The intercellular adhesion molecule ICAM (CD54) is a member of the immunoglobulin superfamiliy (24). Its expression on various types of cells is stimulated by pro-inflammatory cytokines (25). It can be found on the cell surface or as soluble form in serum (sICAM). ICAM is essential for the migration of leukocytes to a focus of inflammation (26). Furthermore, ICAM plays a role in the progression of tumors (27). Tumor cells are believed to attach to the endothelium *via* ICAM and spread out in blood and lymph vessels (28). On the other hand, it has been suggested that shedding of sICAM by tumor cells is a basic mechanism by which they evade the anti-tumor immune response (29). In addition, it has been supposed that sICAM can be released into the circulation by proteolytic cleavage during apoptosis (30). sICAM in serum of patients with cancer is believed to derive either from the cancer cells or from tumor-associated stroma. Thus, sICAM can reflect the volume of a tumor or the loss of diffusion barriers (28, 31, 32).

Plasminogen activator inhibitor 1 (PAI-1) is a glycoprotein with a central regulating function in the plasminogen activator system (33). The plasminogen activator system induces the production of plasmin, another trigger of apoptosis and subsequently enhances degradation of extracellular matrix, cell migration and invasion (33). However, PAI-1 can also inhibit apoptosis by repressing the production of plasmin *via* binding to vitronectin resulting in the impairment of cell adhesion and by directly suppressing caspase-3. Therefore PAI-1 plays a major role in the ability of tumors to invade, metastasize and to grow new blood vessels (34). In locally advanced breast cancer, high expression levels of tissue PAI-1 are associated with unfavorable prognosis (35).

Apoptosis-associated markers relate to various aspects of pathophysiological processes during tumorigenesis and response to cytotoxic treatment and therefore may deliver complementary information concerning prognosis and prediction of therapy efficiency.

Patients and Methods

Patients. In total, 51 patients suffering from locally advanced breast cancer who underwent preoperative, neoadjuvant chemotherapy between 2007 and 2009 at the Oncological Outpatient Speciality Center Munich were prospectively and consecutively included in the study. Chemotherapy consisted in general of four cycles of epirubicin (90 mg/m^2) and cyclophosphamide (600 mg/m^2) followed by four cycles of docetaxel (75 mg/m^2) or paclitaxel (175 mg/m^2). Herceptin was added in HER2/neu overexpressing cases to taxane treatment. After completing chemotherapy, definitive surgery either breast-conserving or as mastectomy and additional axillary lymph node surgery followed.

In all patients, blood was taken before start of the first and second cycles and at the end of the therapy. As controls, serum samples from 31 healthy women, 13 patients with benign breast diseases and from 28 patients with metastasized breast cancer were used. The study was approved by the local Ethics Committee and written informed consent was obtained from all patients. Detailed characteristics of patients and controls are shown in Table I.

Evaluation of response to chemotherapy. Response to neoadjuvant chemotherapy was evaluated by histopathological means at the time of surgery. These results were compared with the results of the pretherapeutic examination of the breast by ultrasound.

According to the RECIST criteria, complete remission (CR) was defined as disappearance of invasive tumor in the breast and also in axillary lymph nodes. Partial remission (PR) was defined as reduction of the longest tumor diameter by ≥30%. Progressive disease (PD) was defined as the increase of the longest tumor diameter by ≥20% or the appearance of new tumor metastases. All other cases were defined as no change (NC) (36).

Methods. Blood samples were centrifuged at 3,000 × *g* for 15 minutes within one to two hours after venipuncture. Subsequently, serum samples were aliquoted and stored at –80˚C.

Concentrations of MIF, sFAS, sICAM and PAI-1 were quantified using a bead-based multiplex enzyme linked immunosorbent assay (ELISA; Human Sepsis/Apoptosis Lincoplex Kit, Millipore, Billerica, MA, USA). The bead-based technology allows a simultaneous and quantitative analysis of multiple biomarkers in one single blood sample. The beads are microscopic spherical polystyrol particles that bind to the analyte in a specific and concentration-dependent manner. They are labeled with analyte-specific different fluorescent dyes which emit light in the red region of the optical spectrum. Bead-bound analytes are detected by a reporter molecule that carries an additional fluorescent dye emitting in the green region of the optical spectrum. Thus, it is possible to perform the classification of the beads and the quantification of bound analytes simultaneously.

Initially, all reagents and working standards were prepared following the manufacturer's instructions. Serum samples were diluted 1:10 using serum matrix. A 96-well filterplate was pre-wetted by pipetting 200 µl of assay buffer into each well followed by subsequent sealing and mixing on a plate shaker at room temperature for 10 minutes. The assay buffer was then removed by vacuum extraction and 25 µl of standards and controls (each with additional 25 µl serum matrix) were added to the appropriate wells. In the sample wells, 25 µl of the assay buffer and 25 µl of the patient serum sample were added. Subsequently 25 µl of vortexed mixed beads were added into all wells. The plate was then sealed and incubated on a plate shaker at 4˚C overnight. The next day, fluid was gently removed by vacuum extraction and the plate was washed twice with 200 µl of wash buffer. After this, 25 µl of detection antibodies were added to every well and the plate was incubated on a plate shaker at room temperature for one hour. After adding 25 µl of the fluorescent dye streptavidin-phycoerythrin, the plate was sealed, covered and incubated on a plate shaker for 30 minutes. Once again, the fluid was removed by vacuum and the plate was washed twice with 200 µl of wash buffer. Before running the plate on a cytometric reader, 100 µl of sheath fluid were pipetted into each well. The fluorescence intensities were analyzed and the concentrations of the different markers were calculated using a 5-parameter curve fitting.

CEA and CA15-3 were measured by enzymatic chemiluminescent immunoassay (ECLIA) on an ElecSys 2010 immunoassay analyzer of Roche Diagnostics, Germany.

The sample series of single patients were determined within one run of the ELISA assays, respectively, to minimize method variance.

Statistics. Concerning differential diagnosis, pretherapeutic biomarker levels of the group of patients with locally confined breast cancer were compared with those of healthy controls and patients with benign breast diseases. Furthermore, biomarker levels of patients with metastasized breast cancer were compared with healthy and benign controls, as well as with patients with locally confined breast cancer.

Concerning therapy response, concentrations of all measured markers before the first cycle, eight days after the first cycle, before the second cycle and at the end of the therapy as well as their percentage changes (kinetics) from cycle one to two and to the end of the therapy, were considered for statistical evaluation.

As the main intention of the study was the early identification of non-responders, patients with complete and partial histopathological response were combined into a 'responder' group. This group was compared with patients classified as having no change and progressive disease (setting 1). Given that complete histopathological remission is associated with more favorable prognosis, those patients were compared with others who did not achieve complete remission in a second evaluation (setting 2)

Table I. *Characteristics of patients and controls.*

Groups	N	Age (years, median)
Locally confined breast cancer	51	46.2
Metastasized breast cancer	28	64.4
Benign breast disease	13	44.7
Healthy women	31	41.9
Characteristics of patients with locally confined breast cancer		
UICC stage before therapy	N	%
I	1	2.0
II	39	76.4
III	10	19.6
Not known	1	2.0
UICC stage after therapy		
No residual tumor detectable	9	17.6
I	14	27.4
II	19	37.2
III	9	17.6
Histology		
Invasive ductal carcinoma	42	82.4
Invasive lobular carcinoma	3	5.9
Adenocarcinoma	2	3.9
Unknown	4	7.8
Neoadjuvant chemotherapy		
Epirubicin+cyclophosphamide	6	11.8
Epirubicin+cyclophosphamide+docetaxel	29	56.9
Epirubicin+cyclophosphamide+docetaxel+paclitaxel	2	3.9
Epirubicin+cyclophosphamide+paclitaxel	12	23.5
Epirubicin+cyclophosphamide+5-fluorouracil+docetaxel	2	3.9
Response to the therapy		
Complete remission	9	17.7
Partial remission	29	56.9
No change	13	25.5
Progressive disease	0	0

Table II. *Distribution of pretherapeutic values of apoptosis-related markers in various groups.*

Marker	Group	N	Median	Min	Max	Comparison (*p*-Value) with		
						Benign breast disease	Localized breast cancer	Metastasized breast cancer
sFAS (pg/ml)	Healthy	31	1.7	0.5	3.6	0.3672	**0.0007**	**<0.0001**
	Benign breast disease	13	1.9	0.4	5.5		0.2009	**0.0014**
	Localized breast cancer	42	2.5	0.3	22.2			**<0.0001**
	Metastasized breast cancer	28	4.0	0.5	20.5			
MIF (pg/ml)	Healthy	31	0.8	0.1	6.0	**0.0014**	**0.0026**	**<0.0001**
	Benign breast disease	13	2.5	0.3	9.3		0.0881	**0.0033**
	Localized breast cancer	42	1.5	0.1	21.5			**<0.0001**
	Metastasized breast cancer	28	8.1	0.4	50.0			
PAI-1 (pg/ml)	Healthy	31	37.5	14.7	50.0	0.3033	0.9355	**0.0045**
	Benign breast disease	13	33.7	1.5	50.0		0.3129	0.1374
	Localized breast cancer	42	42.2	4.6	50.0			**0.0199**
	Metastasized breast cancer	28	26.0	17.2	50.0			
sICAM (pg/ml)	Healthy	31	51.6	16.4	250.0	0.1534	0.1291	**0.0006**
	Benign breast disease	13	76.4	5.0	247.0		0.5656	0.1199
	Localized breast cancer	42	61.5	23.3	163.0			**0.0008**
	Metastasized breast cancer	28	105.0	28.4	250.0			

sFAS: soluble cell death receptor sFAS; MIF: macrophage migration-inhibitory factor; PAI-1: plasminogen activator inhibitor 1; sICAM: soluble intercellular adhesion molecule.

Comparison of biomarker concentrations between the diagnostic groups and between groups with different therapy response was carried out by the Mann-Whitney *U*-test. Results are presented in tables as medians, percentiles and respective *p*-values, as well as graphically in form of dot plots. Discriminative power is further demonstrated in receiver operating characteristic (ROC) curves. Method comparison of the various parameters was made by calculating Spearman's rank correlation coefficients. A *p*-value of <0.05 was considered statistically significant. Calculations were carried out using software of Mathworks and of SAS (version 9.2; SAS Institute Inc.,Cary, N.C., USA).

Results

Diagnosis of breast cancer patients. In patients with metastasized breast cancer, serum concentrations of sFAS and MIF, and also of CEA and CA15-3 (data not shown), were significantly higher than those in healthy controls, patients with benign breast diseases and in patients with locally confined breast cancer. In addition sICAM was higher in patients with metastasized breast cancer than in healthy controls and those with locally confined breast cancer, while the reverse was true for PAI-1 (Table II, Figure 1).

In addition, concentrations of MIF and sFAS were significantly higher in serum of patients with locally confined breast cancer than of healthy controls but not than in patients with benign breast disease. There was no significant difference between patients with locally confined breast cancer and control groups for any other marker, nor for CEA and CA15-3 (37) (Table II, Figure 1).

Discrimination of patients with locally confined breast cancer from healthy controls was best for sFAS and MIF, which reached areas under the curve (AUC) of 73.3% and 70.7% in ROC curves, respectively (Figure 2A). In comparison, discriminative accuracy by established breast cancer biomarkers CEA and CA15-3 was lower with AUCs of 59.9% and 60.0%, respectively (37). For the discrimination of patients with metastasized breast cancer from all other groups, AUCs were 84.0% for sFAS and 87.6% for MIF (Figure 2B).

Among the various biomarkers there was a correlation in all patient groups between MIF and sFAS (r=0.431, $p<0.001$), sICAM (r=0.253, $p=0.005$), CA15-3 (r=0.324, $p<0.001$), and CEA (r=0.345, $p<0.001$); between sFAS and sICAM (r=0.390, $p<0.001$), CA15-3 (r=0.256, $p=0.007$), and CEA (r=0.381, $p<0.001$); between sICAM and CA15-3 (r=0.271, $p=0.004$) and CEA (r=0.287, $p=0.003$), while there was no correlation between PAI-1 and any other biomarker.

Early estimation of response to neoadjuvant chemotherapy. From 51 patients with locally confined breast cancer receiving neoadjuvant chemotherapy 9 achieved CR, 29 PR, and 13 were classified as NC (Table I). No patient was registered as having PD.

Setting 1 (CR+PR versus NC): Pretherapeutic levels of MIF tended to be higher in non-responders as compared with responders, but the difference was only of borderline significance (medians 2.0 pg/ml *versus* 1.2 pg/ml; $p=0.082$).

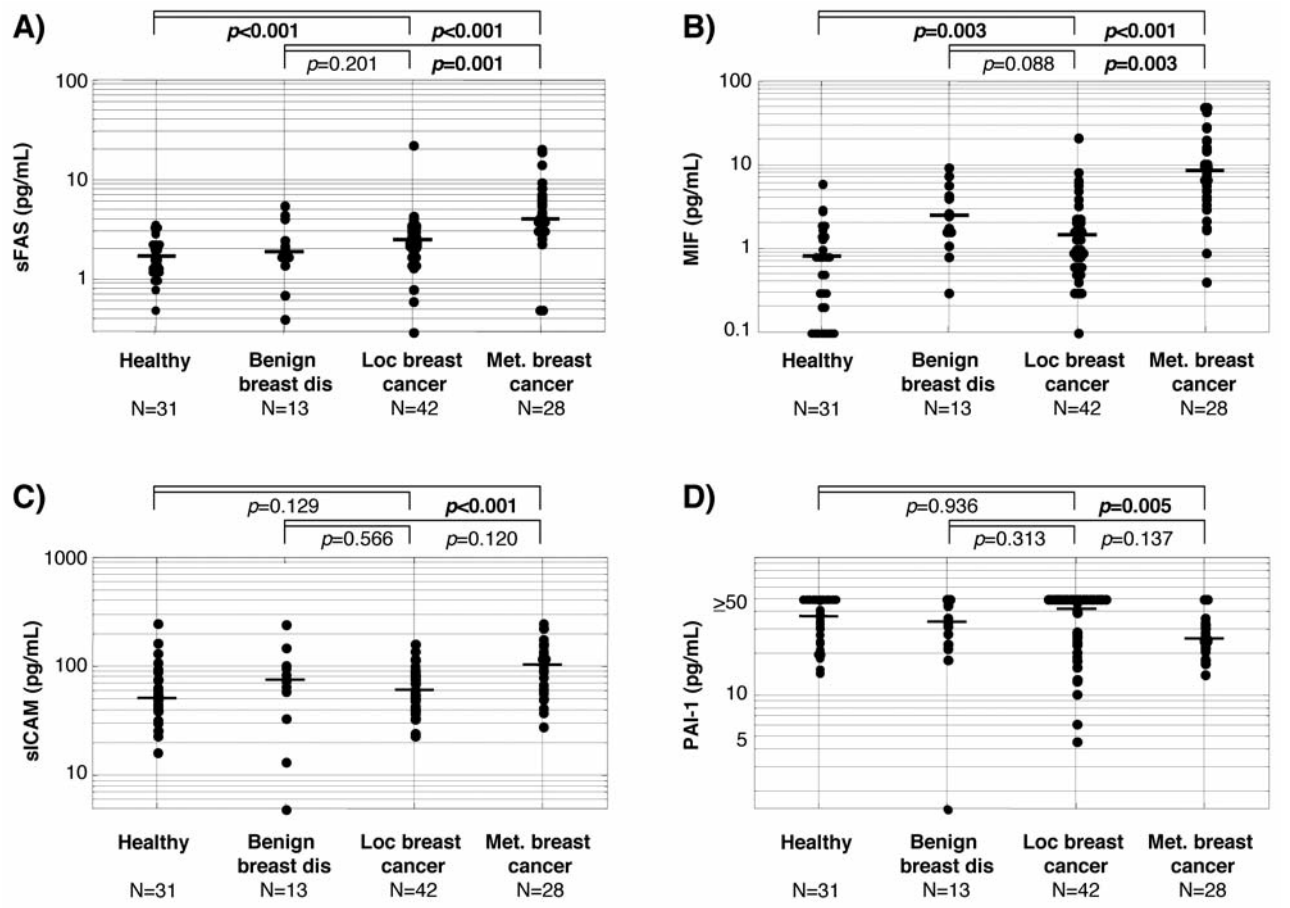

Figure 1. *Value distribution and medians of serum levels of soluble cell death receptor sFAS (A), macrophage migration-inhibitory factor (MIF) (B), soluble intercellular adhesion molecule (sICAM) (C) and plasminogen activator inhibitor 1 (PAI-1) (D) in healthy persons, patients with benign breast diseases, patients with locally confined breast cancer and patients with metastasized breast cancer.*

For all other markers, including CEA and CA15-3 (37), there were no differences of pretherapeutic values between responders and non-responders (Table III). Best discrimination of response groups was obtained by MIF with an AUC of 65.7% in ROC curves for detection of non-responding patients (Figure 3A).

During therapy the courses of apoptosis-related markers, and also of CEA and CA15-3 (37), were heterogeneous in both response groups. Measurements at several points of time after starting the therapeutic sequence (day 8 cycle 1, day 1 cycle 2, end of therapy) did not clearly reveal correlations between marker levels and reponse (Table III).

Setting 2 (CR versus PR+NC): Pretherapeutic levels of sICAM and PAI-1 tended to be higher in responders than in non-responders, while pretherapeutic levels of all other markers were similar in both groups (Table IV). Best discrimination of patients with CR and those without was obtained by sICAM (AUC=68.7%) and PAI-1 (AUC=69.8%), respectively (Figure 3B).

During therapy, the courses of apoptosis-related markers, as well as of CEA and CA15-3 (37), were heterogeneous in both response groups. Most markers did not discriminate between response groups, neither at day 8 of the first cycle, nor before the start of the second cycle, nor at the end of the therapy, nor for the kinetics.

However, the post-therapeutic concentrations of sICAM (median 97.6 pg/ml *versus* 80.3 pg/ml; p=0.026) and CA15-3 (23.0 U/ml *versus* 32.8 U/ml; p=0.035; 37) showed significant differences between responders and non-responders. Furthermore, the kinetics of PAI-1 from cycle one to two tended to discriminate between the response groups (p=0.056; Table IV)

Discussion

Neoadjuvant chemotherapy is an effective new therapy option for patients with locally advanced breast cancer by reducing tumor size and stage and thereby enhancing rates

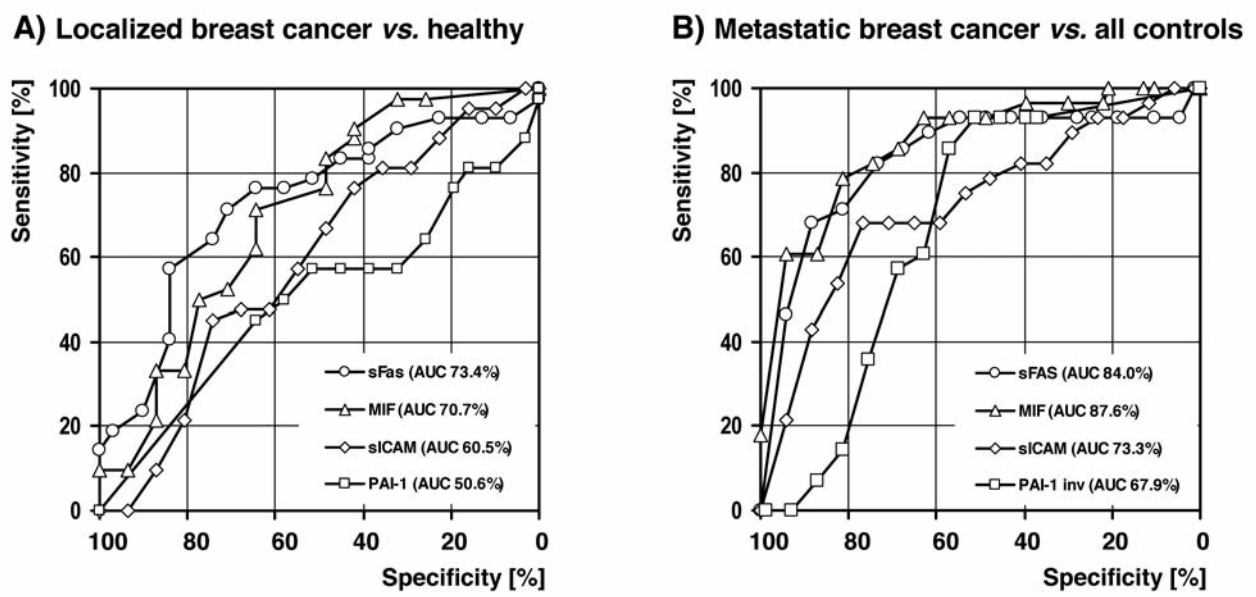

Figure 2. *Receiver operating characteristic (ROC) curves for the discrimination between patients with locally confined breast cancer and healthy controls (A), as well as between patients with metastatic breast cancer and all other controls (B).*

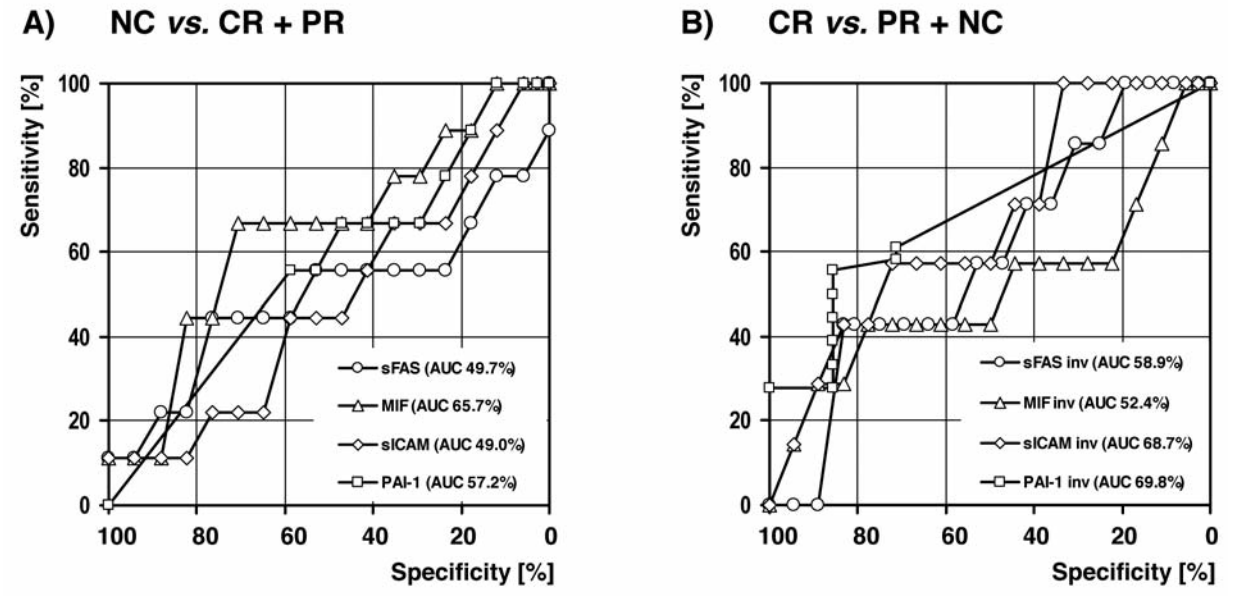

Figure 3. *Receiver operating characteristic (ROC) curves for the discrimination between patients with response and those with no response to neoadjuvant chemotherapy. Setting 1: NC versus CR+PR (A); Setting 2: NC+PR versus CR (B).*

of breast-conserving surgery and perhaps a patient's long-term outcome (38, 39). Currently used antineoplastic drugs are able to inhibit tumor growth by inhibition of tumor cell division and proliferation, or by induction of tumor cell death (8). In order to monitor the individual efficacy of neoadjuvant therapies, serial measurements of treatment-associated serum biomarkers, such as apoptosis-related markers, would be most valuable (5, 40).

MIF which is known mainly as pro-inflammatory cytokine, is also up-regulated in breast cancer and other kinds of cancer (22). Positive correlations between MIF concentration in serum and MIF expression in epithelial cells has been demonstrated

Table III. *Discrimination of breast cancer patients with response versus those without response to neoadjuvant chemotherapy by apoptosis-related markers (Setting 1: NC versus PR+CR).*

Marker	Response	Time	N	Median	Min	Max	*P*-Value
sFAS (pg/ml)	NC	Cy 1 D 0	12	2.8	0.3	22.2	0.845
	PR+CR		30	2.4	0.8	4.4	
	NC	Cy 1 D 8	3	3.1	2.8	28.3	0.946
	PR+CR		13	3.5	2.5	5.3	
	NC	Cy 2 D 0	11	2.4	0.9	27.4	0.587
	PR+CR		32	3.2	1.0	7.0	
	NC	End	11	2.7	0.5	25.6	0.452
	PR+CR		32	3.2	1.1	21.3	
	NC	Cy 2-1 (%)	10	22.2	–42.9	433.3	0.623
	PR+CR		24	20.9	–30.8	268.4	
	NC	End-Cy1 (%)	10	20.2	–38.5	500.0	0.547
	PR+CR		25	33.3	–19.4	508.6	
MIF (pg/ml)	NC	Cy 1 D 0	12	2.0	0.5	21.5	0.082
	PR+CR		30	1.2	0.1	8.2	
	NC	Cy 1 D 8	3	1.5	1.5	2.2	0.500
	PR+CR		13	0.8	0.1	15.6	
	NC	Cy 2 D 0	11	1.4	0.5	10.0	0.486
	PR+CR		32	1.4	0.2	8.4	
	NC	End	11	2.9	0.3	46.3	0.330
	PR+CR		32	1.4	0.2	11.2	
	NC	Cy 2-1 (%)	10	–11.2	–68.4	376.2	0.650
	PR+CR		24	5.6	–84.1	900.0	
	NC	End-Cy1 (%)	10	28.2	–87.9	660.0	0.476
	PR+CR		25	40.0	–85.4	1766.7	
PAI-1 (pg/ml)	NC	Cy 1 D 0	12	45.0	18.0	50.0	0.630
	PR+CR		30	35.8	4.6	50.0	
	NC	Cy 1 D 8	3	48.1	40.4	50.0	0.943
	PR+CR		13	50.0	7.5	50.0	
	NC	Cy 2 D 0	11	50.0	18.9	50.0	0.636
	PR+CR		32	50.0	6.6	50.0	
	NC	End	11	40.1	20.6	50.0	0.736
	PR+CR		32	48.7	4.5	50.0	
	NC	Cy 2-1 (%)	10	0.0	–60.7	58.3	0.510
	PR+CR		24	0.0	–16.7	987.0	
	NC	End-Cy1 (%)	10	–6.9	–39.4	85.6	0.346
	PR+CR		25	0.0	–61.2	987.0	
sICAM (pg/ml)	NC	Cy 1 D 0	12	69.1	33.9	163.0	0.878
	PR+CR		30	61.5	23.3	138.0	
	NC	Cy 1 D 8	3	89.6	43.8	236.0	0.501
	PR+CR		13	79.2	43.0	154.0	
	NC	Cy 2 D 0	11	69.5	24.0	178.0	0.550
	PR+CR		32	72.0	31.1	184.0	
	NC	End	11	80.3	56.2	145.0	0.967
	PR+CR		32	87.4	31.3	250.0	
	NC	Cy 2-1 (%)	10	6.9	–70.6	58.4	0.777
	PR+CR		24	11.2	–8.2	91.6	
	NC	End-Cy1 (%)	10	19.0	–11.0	83.5	0.250
	PR+CR		25	20.4	–11.1	155.3	

NC: no change; PR: partial remission; CR: complete remission; Cy 1: cycle 1; Cy 2: cycle 2; D 0: day 0; D 8: day 8; End: end of therapy; Cy 2-1 (%): percentage changes from cycle 1 to 2; End-Cy1 (%):percentage changes from cycle 1 to end of therapy; sFAS: soluble cell death receptor sFAS; MIF: macrophage migration-inhibitory factor; PAI-1: plasminogen activator inhibitor 1; sICAM: soluble intercellular adhesion molecule.

(41). Elevated serum MIF levels have been described in patients with prostate (42), gastric (43) and breast cancer (44).

In line with those results, serum MIF concentrations were increased in patients with breast cancer in the present study as well. It was particularly remarkable that MIF was not only elevated in patients with metastasized breast cancer as compared with healthy and benign controls but already in patients with early, locally confined tumor stages when

Table IV. *Discrimination of breast cancer patients with response versus those without response to neoadjuvant chemotherapy by apoptosis-related markers (Setting 2: NC + PR versus CR).*

Marker	Response	Time	N	Median	Min	Max	*P*-Value
sFAS (pg/ml)	CR	Cy 1 D 0	7	2.7	1.7	3.7	0.437
	PR+NC		35	2.4	0.3	22.2	
	CR	Cy 1 D 8	2	3.9	2.5	5.3	0.936
	PR+NC		14	3.4	2.8	28.3	
	CR	Cy 2 D 0	8	3.9	2.4	4.7	0.104
	PR+NC		35	2.8	0.9	27.4	
	CR	End	7	3.6	1.7	21.3	0.479
	PR+NC		36	2.9	0.5	25.6	
	CR	Cy 2-1 (%)	6	18.7	8.3	41.2	0.651
	PR+NC		28	24.2	–42.9	433.3	
	CR	End-Cy1 (%)	6	15.3	0	508.6	0.555
	PR+NC		29	33.3	–38.5	500	
MIF (pg/ml)	CR	Cy 1 D 0	7	1.4	0.4	8.2	0.853
	PR+NC		35	1.5	0.1	21.5	
	CR	Cy 1 D 8	2	2.4	2	2.7	0.233
	PR+NC		14	1.1	0.1	15.6	
	CR	Cy 2 D 0	8	1.9	0.3	8.4	0.502
	PR+NC		35	1.4	0.2	10	
	CR	End	7	1.5	0.4	11.2	0.469
	PR+NC		36	1.5	0.2	46.3	
	CR	Cy 2-1 (%)	6	–25	–84.1	300	0.527
	PR+NC		28	0	–83.7	900	
	CR	End-Cy1 (%)	6	9.8	–85.4	1766.7	0.793
	PR+NC		29	44.4	–87.9	862.5	
PAI-1 (pg/ml)	CR	Cy 1 D 0	7	50	23.3	50	0.103
	PR+NC		35	40.2	4.6	50	
	CR	Cy 1 D 8	2	50	50	50	0.203
	PR+NC		14	46.2	7.5	50	
	CR	Cy 2 D 0	8	50	35.4	50	0.367
	PR+NC		35	50	6.6	50	
	CR	End	7	50	19.2	50	0.349
	PR+NC		36	41.7	4.5	50	
	CR	Cy 2-1 (%)	6	0	–16.7	0	0.056
	PR+NC		28	0.5	–60.7	987	
	CR	End-Cy1 (%)	6	0	–44.6	0	0.521
	PR+NC		29	0	–61.2	987	
sICAM (pg/ml)	CR	Cy 1 D 0	7	84.2	52	138	0.121
	PR+NC		35	58.8	23.3	163	
	CR	Cy 1 D 8	2	114	87	141	0.177
	PR+NC		14	78.2	43	236	
	CR	Cy 2 D 0	8	76.5	57.1	146	0.502
	PR+NC		35	70.1	24	184	
	CR	End	7	97.6	87.1	153	**0.026**
	PR+NC		36	80.3	31.3	250	
	CR	Cy 2-1 (%)	6	4.2	–8.2	16.9	0.268
	PR+NC		28	11.2	–70.6	91.6	
	CR	End-Cy1 (%)	6	10	–5.1	133	0.983
	PR+NC		29	20.4	–11.1	155.3	

NC: no change; PR: partial remission; CR: complete remission; Cy 1: cycle 1; Cy 2: cycle 2; D 0: day 0; D 8: day 8; End: end of therapy; Cy 2-1 (%): percentage changes from cycle 1 to 2; End-Cy1 (%):percentage changes from cycle 1 to end of therapy; sFAS: soluble cell death receptor sFAS; MIF: macrophage migration-inhibitory factor; PAI-1: plasminogen activator inhibitor 1; sICAM: soluble intercellular adhesion molecule.

compared with healthy controls. Furthermore, MIF tended also to be higher in those patients who did not respond to neoadjuvant chemotherapy and to predict early poor therapy efficacy.

Nolen *et al*. analyzed the predictive value of MIF and 54 other serum biomarkers by multiplexed bead-based assays in 44 patients with breast cancer (UICC stages IIB and III) who were treated by neoadjuvant chemotherapy consisting of four

cycles of doxorubicin and paclitaxel, each in combination with whole-breast hyperthermia before surgery (45). In that study, blood was drawn prior to each therapy cycle and prior to surgery. While pretherapeutic MIF levels did not differ between response groups, serum MIF levels prior to the second cycle were significantly higher in patients with pathological PR or CR than in patients without adequate therapeutic response (45). Our results did not support those findings as we did not observe differences in MIF levels or kinetics during therapy in the various response groups. However, pretherapeutic levels of MIF tended to be higher in non-responders as compared with responders. Interestingly, Xia *et al*. found high pretherapeutic serum concentrations of MIF in 97 patients with gastric adenocarcinoma having unfavourable prognosis (43).

In line with earlier studies (46, 47), we found significantly higher serum sFAS levels in patients with breast cancer than in healthy individuals or patients with benign diseases. By means of sFAS levels, we were even able to discriminate between patients with locally confined breast cancer and healthy controls. The combination of sFAS and MIF further improved the discriminative performance. It has to be emphasized that established biomarkers for breast cancer such as CEA and CA15-3 were not comparably useful in this setting (37). Unfortunately, neither sFAS values before nor during neoadjuvant therapy were predicitive or indicative of the efficacy of the treatment. However, this corresponds with a report of Pichon *et al*. who found no predictive value of serum sFAS levels in 42 patients with various kinds of cancer under chemotherapy. However, they stated that low pretherapeutic sFAS levels were associated with a better prognosis (48). Ueno *et al*. also showed such prognostic value for sFAS in 162 patients with primary and 71 patients with recurrent breast cancer. Patients with high levels had significantly worse prognosis for overall and disease-free survival (14). In our setting, the prognostic aspect will be evaluated after a sufficient postsurgical observation time has been reached.

Similarly to the above-mentioned markers, elevated levels of sICAM have been found in serum of patients with early breast cancer by various groups (49, 50). Patients with metastasized breast cancer exhibited higher serum ICAM levels than patients with locally confined disease (28). Moreover, high sICAM levels were associated with poor prognosis in patients with metastasized breast cancer (51). In line with these results, we found significantly higher levels of sICAM in serum of patients with metastasized breast cancer than in that of healthy controls and patients with locally confined breast cancer, confirming results of previous studies by Köstler *et al*. (52). However, there are conflicting data from others who did not find differences in serum ICAM values between breast cancer patients (stages not specified) and healthy controls (53).

The predictive and prognostic relevance of sICAM is still under debate. A study on 49 patients with metastasized breast cancer reported higher pretherapeutic sICAM levels in non-responders and those with poor survival (27). A further study in 98 patients with metastasized breast cancer revealed prognostic value of sICAM for a treatment with cyclophosphamide and carboplatin/vinblastine but not for a cyclophosphamide and paclitaxel treatment combination (51). In our setting, using a sequential epirubicin/cyclophosphomide and taxane combination, pretherapeutic sICAM values were not predictive of therapeutic response. However, high post-therapeutic levels of sICAM correlated with CR after neoadjuvant chemotherapy.

PAI-1 is known as a prognostic tissue biomarker for breast cancer. While patients without lymph node metastases and low levels of PAI-1 have a favourable prognosis, patients with high levels have an unfavourable prognosis and benefit from adjuvant chemotherapy (35). PAI-1 can inhibit apoptosis in tumor cells. Higher levels of PAI-1 in serum of cancer patients as compared to healthy individuals have been described for various types of malignant diseases (54-58). But current results are conflicting and valuable data on serum PAI-1 in patients with breast cancer are lacking. In our study, patients with metastasized breast cancer had lower levels than did healthy controls. This result is confirmed by other studies about PAI-1 in serum of patients with breast (53) and prostate cancer (58). Furthermore, we revealed that kinetics of PAI-1 from therapy cycle one to two in patients with locally confined breast cancer nearly significantly differentiated between responders and non-responders. It should be mentioned that PAI-1 measurement in the multiplex bead-based array was not optimal because the concentrations of the diverse antibodies within the assay were not well balanced for the present patient sample. Therefore, many PAI-1 values were measured at the upper limit of detection thus limiting the information of this parameter.

In the present study, we analyzed the biomarker concentration at four different time points, as well as the proportional changes. Former studies showed that courses of various apoptosis-related markers during the early hours and days after application of chemotherapeutic drugs could be even more suitable for monitoring the therapy response. For example, in 212 patients with inoperable non-small cell lung cancer, nucleosome levels during the first week of chemotherapy revealed significant differences between responders and non-responders (59). Responders exhibited a slight increase of nucleosome levels followed by a notable decrease, while non-responders had strongly increasing levels followed by less dropping levels during the first week (59). Similar results were found in patients with colorectal (60) and pancreatic (61) cancer. For technical reasons, in our current study it was not possible to analyze the marker courses during the first week. However, there have not been many studies published on this subject so far.

Herein, we present an explorative hypothesis-generating study. Preanalytics as well as analytics were very carefully carried out and the response to the therapy was clearly defined and evaluated. Moreover, we compared new promising markers with established tumor markers CA15-3 and CEA, and demonstrated the higher sensitivity of the apoptosis-related markers for the detection of locally confined breast cancer. Concerning prediction of response to neoadjuvant, the established markers were not as meaningful as expected, which reveals the necessity for identifying new approaches and markers that can fill this need.

Conclusion

In conclusion we demonstrated that apoptosis-related biomarkers in serum of patients with breast cancer are a low-cost and non-invasive option for the detection of breast cancer and partly for the early prediction of response to neoadjuvant chemotherapy. Their value should be further explored in prospective trials with more patients.

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