8

CIRCULATING APOPTOSIS-RELATED PARAMETERS ARE INCREASED IN LONG-TERM DISEASE-FREE BREAST CANCER SURVIVORS AFTER ANTHRACYCLINE CHEMOTHERAPY AND CHEST WALL IRRADIATION

PATRICK J. PERIK [1,2]
WINETTE T.A. VAN DER GRAAF [1]
ELISABETH G.E. DE VRIES [1]
FRANS BOOMSMA [3]
JUERGEN MESSERSCHMIDT [4]
DIRK J. VAN VELDHUISEN [2]
DIRK TH. SLEIJFER [1]
JOURIK A. GIETEMA [1]

[1] DEPARTMENT OF MEDICAL ONCOLOGY,
[2] DEPARTMENT OF CARDIOLOGY,
UNIVERSITY MEDICAL CENTER GRONINGEN, THE NETHERLANDS
[3] DEPARTMENT OF INTERNAL MEDICINE, ERASMUS MEDICAL CENTER, ROTTERDAM, THE NETHERLANDS
[4] INSTITUTE OF SPECTROCHEMISTRY AND APPLIED SPECTROSCOPY, DORTMUND, GERMANY

SUBMITTED FOR PUBLICATION

ABSTRACT

Background

To evaluate whether circulating soluble (s) apoptosis-related proteins and inflammation markers are increased in long-term disease free breast cancer survivors and associated with cardiotoxicity, and if subgroups could be identified based on the applied treatments.

Patients and methods

Circulating tumor necrosis factor (TNF) α, sTNF-receptor (sTNF-R) 1 and 2, sFas, sFas ligand, sTNF-related apoptosis inducing ligand (sTRAIL) and serum HER2 were measured with immunoassay (ELISA) in long-term disease-free breast cancer survivors after adjuvant anthracyclines and chest wall irradiation. High-sensitivity C-reactive protein (HS-CRP), fibrinogen, plasma B-type and N-terminal atrial natriuretic peptide (NT-ANP and BNP) were also determined. Serum platinum was analyzed in subjects who had received carboplatin.

Results

34 patients with a median follow-up of 6.0 years, and 12 healthy age-matched women were enrolled. Chemotherapy, consisting of standard-dose (5x fluorouracil, epirubicin (450 mg/m^2), cyclophosphamide (FEC), n=14) or high-dose (4x FEC, epirubicin (360 mg/m^2), and myeloablation with 1600 mg/m^2 carboplatin, 6 g/m^2 cyclophosphamide, thiotepa 480 mg/m^2, n=20) with hematopoietic stem cell rescue, preceded irradiation and tamoxifen. Although no associations with natriuretic peptides or clinical cardiotoxicity (n=2) were observed, circulating apoptosis markers were higher in breast cancer survivors than in controls. SFas ligand and sTRAIL were higher in the high-dose than in the standard-dose group. Circulating platinum levels did not correlate with apoptosis marker levels.

Conclusions

Circulating apoptosis marker levels were increased in long-term disease-free breast cancer survivors, treated with adjuvant chemoradiotherapy, and were higher after myeloablative than after standard-dose chemotherapy. The potential relation with late cardiotoxicity of antineoplastic therapy deserves further study.

shed into the circulation and can be quantified in serum with an immunosorbent assay.[19]

The neurohormones B-type natriuretic peptide (BNP) and N-terminal atrial natriuretic peptide (NT-ANP) are elevated in patients with low LVEF values and clinical heart failure, and increase with the NYHA class.[20-22] Furthermore, increased natriuretic peptide levels have been related to decreased left ventricular ejection fraction (LVEF) values in patients with left ventricular dysfunction or coronary artery disease.[23,24] As a consequence, elevated natriuretic peptide values are considered to be indicative of left ventricular dysfunction. In patients who received adjuvant anthracycline-based chemotherapy followed by chest wall irradiation and oral tamoxifen maintenance treatment for early stage breast cancer, we previously observed that plasma natriuretic peptides increased during the first year after the start of treatment.[25]

To date, no data is available regarding circulating levels of TNF-related soluble apoptotic proteins, acute phase proteins and serum HER2 in relation to the occurrence of late cardiotoxicity, during follow-up of breast cancer survivors who received potentially cardiotoxic treatment. Therefore, in this cross-sectional study, we determined whether circulating levels of sTNF-related apoptotic proteins, HER2 and acute phase proteins are increased and associated with cardiac dysfunction, in long-term disease-free breast cancer survivors after adjuvant anthracycline chemotherapy and chest wall irradiation. Additionally, we evaluated whether subgroups could be identified, based on the applied treatment regimens.

PATIENTS AND METHODS

Breast cancer survivors and treatment

Between February 2003 and June 2004, breast cancer patients visiting the oncology outpatient clinic of the University Medical Center Groningen, The Netherlands for routine follow-up, were asked to participate in this study. Eligible patients were those who were treated successfully in a nation wide randomized trial comparing efficacy of two adjuvant anthracycline-containing chemotherapy regimens.[26] In this randomized study, patients aged 18-55 years received either 5 three-weekly cycles of standard dose 5-fluorouracil (5-FU) (500 mg/m^2), epirubicin (90 mg/m^2) and cyclophosphamide (500 mg/m^2) (FEC) or 4 courses of FEC followed by high-dose cyclophosphamide (1500 mg/m^2), thiotepa (120 mg/m^2) and carboplatin (400 mg/m^2), daily for 4 days (4x FEC+CTC). Hematopoietic stem cells were reinfused 7 days after the start of high-dose CTC. In both treatment arms, chemotherapy was followed by locoregional irradiation, which was started after hematological recovery from the last chemotherapy cycle. The dose applied to the supraclavicular and axillary lymph node area was between 46 and 50 Gy in 23-25 fractions. Chest wall irradiation was performed in 20-25 fraction to a dose of 40 to 50 Gy. In the case of breast conserving surgery, an additional boost of 16 to 20 Gy was

INTRODUCTION

The use of chemotherapy in the treatment of early breast cancer has lead to improved prognosis and survival. As a result, long-term complications related to chemotherapeutic agents become more and more of an issue in clinical practice. Several antineoplastic agents, anthracyclines in particular, are known to have adverse effects on the cardiovascular system (for review see [1]. Anthracycline-induced cardiotoxicity mostly consists of cumulative dose-dependent cardiomyopathic alterations that can ultimately lead to heart failure. Besides anthracyclines, other antineoplastic agents can also induce cardiotoxicity. For instance, acute symptomatic cardiotoxicity occurs in 5-28% of the patients treated with high-dose cyclophosphamide.[2-4]

Currently, left ventricular ejection fraction (LVEF) measurement is generally accepted as the diagnostic tool for the detection of anthracycline-related cardiotoxicity. However, with this technique only functional loss can be detected. When a drop in LVEF occurs, the myocardial damage has already occurred. Therefore, many investigators have searched for alternative methods for earlier detection or prediction of chemotherapy-induced cardiac injury, to enable medical intervention that limits myocardial injury and symptomatic cardiac dysfunction.

In patients with heart failure unrelated to anticancer treatment, cardiomyocyte apoptosis is increased.[5,6] Plasma levels of the soluble forms of several members belonging to the tumor necrosis factor (TNF)-superfamily of apoptosis-related proteins (TNFα, TNF receptor (TNF-R) 1 and 2, Fas and Fas ligand) are increased in heart failure and correlate positively with New York Heart Association (NYHA) functional class.[7-10] Increased TNF-related apoptosis-inducing ligand (TRAIL) cDNA expression is present in peripheral blood mononuclear cells of heart failure patients.[11]

Next to plasma markers of apoptosis, serum levels of the acute phase reactants high-sensitivity C-reactive protein (HS-CRP) and fibrinogen are elevated in heart failure patients and positively associated with the NYHA class. Both HS-CRP and fibrinogen are independent negative prognostic factors in these patients.[12,13] Increased plasma fibrinogen may also be suggestive of a hypercoagulable state in heart failure.[14]

The human epidermal growth factor receptor 2 (HER2; also known as erbB-2) is predominantly known for its role in breast cancer, in which it is overexpressed/amplified in 25-30% of patients and related to worse prognosis.[15] The anti-HER2 antibody trastuzumab is associated with an increased risk of cardiac dysfunction, especially if administered with concurrent anthracycline treatment.[16] HER2 is hypothesized to play a role in the pathogenesis of heart failure, predominantly based on the observation that mice lacking the intracardiac gene coding for HER2 develop severe dilated cardiomyopathy shortly after birth.[17,18] The proteolytically cleaved extracellular domain of the transmembrane HER2 is

administered to the tumor bed. Tamoxifen (40 mg/day orally) was started in both treatment arms after bone marrow recovery for 2-5 years.
To be eligible for the current study, patients were required to be free of disease, with the last course of anthracycline-containing chemotherapy at least 6 months prior to inclusion.
A history and physical examination with special attention to signs and symptoms related to heart failure was performed. If present, the severity of heart failure was classified according to the NYHA functional scale.
Age-matched, healthy female individuals, with no history of malignancy or cardiovascular disease, recruited through an advertisement, served as controls.
The study protocol was approved by the local ethics committee. Written informed consent was obtained from all participants.

Blood sampling
Peripheral blood samples were drawn and transferred in 10 mL disposable tubes containing either 2-natrium-ethylenediamine tetra-acetic acid (EDTA), heparin or no additive for serum collection. After sampling, tubes containing EDTA or heparin as an additive were placed on ice immediately. In tubes containing no additive, blood was allowed to clot at room temperature until centrifugation. Serum and plasma were separated by centrifugation at 4ºC, and stored at –80ºC until determination.

Soluble apoptosis-related proteins
Plasma levels of TNFα, sTNF-R1, sTNF-R2, sFas, sFas ligand and sTRAIL were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D systems, Minneapolis, MN, USA) following the manufacturer's instructions. Serum HER2 was determined with a sandwich ELISA (HER-2/ECD assay; Oncogene Sciences, Cambridge, MA, USA). TNFα was measured in EDTA plasma, sTNF-R1 and sTNF-R2 in heparin plasma. Serum was used for sFas, sFas ligand, sTRAIL and HER2.

Neurohormones
BNP and NT-ANP were analyzed as markers for cardiac dysfunction. BNP plasma levels were measured with an immunoradiometric assay (Shionora, Osaka, Japan), with a normal range of 1-10 pmol/L. NT-ANP levels were assessed with a radioimmunoassay (Biotop, Oulu, Finland), with a normal range between 150 and 500 pmol/L.

Acute phase reactants
Serum HS-CRP and fibrinogen were determined as markers for inflammation. HS-CRP was assayed with the BNII Nephelometer (Dade Behring, Brussels, Belgium). Lower detection limit 0.16 mg/L (normal values 0.16-10 mg/L). Plasma fibrinogen

was measured with the Clauss functional assay on an STA coagulation analyzer (normal range 1.7-3.5 g/L).

Platinum

Serum platinum concentrations were analyzed in the patients who had received carboplatin-based chemotherapy (4x FEC+CTC). This analysis was performed using a highly sensitive procedure in which high-pressure decomposition of plasma is followed by adsorptive voltammetric determination of platinum with a detection limit of 6 pg/g.[27]

HER2 tumor status

Formalin-fixed, paraffin-embedded tumor samples were stained with antibodies against HER2 [28]. Staining for HER2/*neu* was scored as follows: a score of 0, no staining; a score of 1, more than 10 percent of cells were weakly positive; a score of 2, moderate homogeneous staining; and a score of 3, strong homogeneous staining. A score of 0-1 was considered as HER2 negative and a score of 2-3 was considered HER2 positive.

Statistics

Quantitative variables were compared between two groups using an unpaired *t* test for normally distributed variables or a Mann-Whitney-U test for skewed distributed variables. Normally distributed variables are reported as mean ±SD, skewed distributed variables are reported as median and range. Correlations between variables were calculated using Pearson's correlation coefficient or Spearman rank sum test. All P values were two-sided and $P<0.05$ was considered statistically significant.

RESULTS

Thirty-four breast cancer survivors, with a median age of 52 years and a median follow-up duration of 6.0 years since the start of chemotherapy, were enrolled. None of the patients had evidence of cardiac disease at the start of breast cancer treatment. 5x FEC had been the applied chemotherapy regimen in 14 (41%) patients, 20 (59%) had received 4x FEC+CTC. All breast cancer survivors had received locoregional irradiation to the chest wall and axillary lymph nodes with a median dose of 50 (range 32-50) Gy and oral tamoxifen maintenance therapy; 27 for the duration of 2 years and 7 for the duration of 5 years. Sixteen patients had received left sided chest wall irradiation and right sided chest wall irradiation had been performed in the remaining 18 patients.

Second primary breast cancers were diagnosed in three breast cancer survivors during follow-up after adjuvant treatment; one after 5x FEC and two after 4x FEC+CTC. All three underwent surgery followed by adjuvant chemotherapy (6x

CMF; cyclophosphamide, methotrexate and 5-fluorouracil), one patient underwent chest wall irradiation. At the time of enrollment in the current study, the patients were free of malignant disease. For the analysis of the influence of the applied antineoplastic treatment on circulating soluble apoptosis marker levels the three patients with a second breast cancer were excluded.
The control group consisted of 12 healthy women with a median age of 51 (35-63) years.

Cardiac evaluation
Symptoms of heart failure (NYHA II) were present in two breast cancer survivors (6%), both after 4x FEC+CTC. Plasma natriuretic peptide values median 6.0 years after treatment, did not differ between breast cancer survivors and controls. Plasma BNP >10 pmol/L was found in eight of 34 breast cancer survivors, but in none of the controls. In one of these eight patients, elevated plasma BNP coincided with symptoms of heart failure. Plasma NT-ANP values above the upper limit of normal were not observed in the control group, whereas two breast cancer survivors, both without heart failure symptoms, had plasma NT-ANP levels above normal values.

Circulating proteins
Circulating soluble apoptosis-related proteins Figure 1 and table 1 represent circulating plasma apoptosis marker concentrations in breast cancer survivors compared to controls. Median plasma TNFα, serum sFas, sFas ligand and sTRAIL levels were higher in breast cancer survivors than in controls (Table 1). A trend was observed for higher sTNF-R2 values in patients. Plasma sTNF-R1 levels did not differ between patients and controls. SFas correlated with sFas ligand ($R = 0.497$, $P < 0.001$), sTNF-R2 ($R = 0.648$, $P < 0.001$) and sTRAIL ($R = 0.555$, $P < 0.001$). STNF-R2 correlated with sTNF-R1 ($R = 0.444$, $P < 0.001$), sFas ligand ($R = 0.448$, $P = 0.002$) and sTRAIL ($R = 0.568$, $P < 0.001$). Furthermore, sTRAIL correlated with sFas ligand ($R = 0.440$, $P = 0.002$) No correlations were observed between follow-up duration and plasma apoptosis marker concentrations. No subgroups could be identified based on high serum and plasma levels (> mean +1 or 2SD) of more than one of the circulating apoptosis markers.
Serum HER2 Serum HER2 was higher in breast cancer survivors than controls. For 30 of the 34 patients, primary tumor HER2 status had been determined; eight patients had a HER2-positive primary tumor, whereas the remaining 22 had HER2-negative primary tumors. Serum HER2 did not differ between patients with a primary HER2-positive tumor and those with HER2-negative tumors. Furthermore, patients with HER2-negative primary tumors had higher serum HER2 levels than controls ($P = 0.035$), as well as patients with a HER2-positive primary tumor ($P = 0.004$) (data not shown).

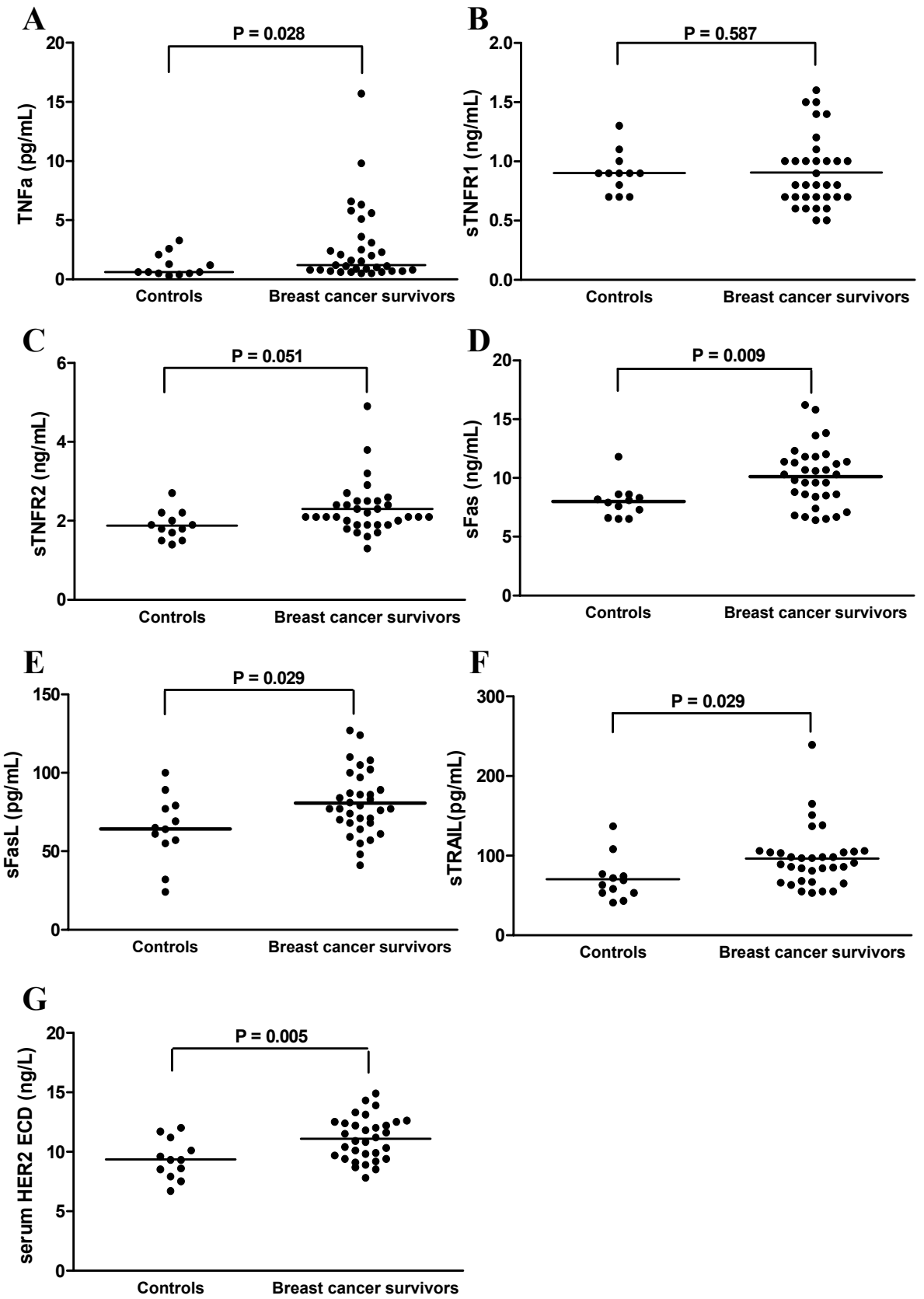

Figure 1. Dot plots of circulating soluble apoptosis-related proteins of the TNF-superfamily and HER2 in breast cancer survivors and healthy age-matched controls. Lines represent median values for TNFα and mean for sTNF-R1, sTNF-R2, sFas, sFas ligand, sTRAIL and HER2. (**A**) TNFα (pg/mL), (**B**) sTNF-R1 (ng/mL), (**C**). sTNF-R2 (ng/mL), (**D**) sFas (ng/mL), (**E**) sFas ligand (pg/mL), (**F**) sTRAIL (pg/mL), (**G**) HER2 ECD (pg/mL).

Acute phase reactants Serum HS-CRP and plasma fibrinogen levels did not differ between breast cancer survivors and controls. HS-CRP and fibrinogen were positively associated (R = 0.385, P = 0.008). HS-CRP correlated positively with sTNF-R2 (R = 0.520, P < 0.001). In breast cancer survivors with high HS-CRP and/or fibrinogen (> mean +1 or +2 SD), TNF-related apoptotic protein concentrations were not different from subjects with low HS-CRP and/or fibrinogen (< mean + 1 or 2SD).

Table 1. Circulating soluble apoptosis markers of the whole population (median follow-up 6.0 years)

	Controls (n=12)	Breast cancer Survivors (n=34)	P-value
TNFα (pg/mL)	0.6 (0.3-3.3)	1.4 (0.5-15.7)	**0.028**
sTNF-R1 (ng/mL)	0.9 (0.7-1.3)	0.8 (0.5-1.6)	0.587
sTNF-R2 (ng/mL)	1.9 (±0.4)	2.3 (±0.7)	0.051
sFas (ng/mL)	8.0 (6.5-11.8)	10.1 (6.4-16.2)	**0.009**
sFas ligand (pg/mL)	64 (±22)	80 (±20)	**0.029**
STRAIL (pg/mL)	71 (±28)	97 (±37)	**0.029**
HER2 ECD (ng/mL)	9.4 (±1.7)	11.1 (±1.8)	**0.005**
HS-CRP (mg/L)	1.0 (0.2-4.8)	1.6 (0.2-6.8)	0.341
Fibrinogen (g/L)	3.0 (±0.5)	3.3 (±0.5)	0.119
BNP (pmol/L)	4.0 (1.0-9.5)	5.6 (0.8-25.5)	0.476
BNP >10 pmol/L	-	8	
NT-ANP (pmol/L)	255 (118-426)	273 (121-557)	0.532
NT-ANP >500 pmol/L	-	2	

Data are expressed as median (range) or mean (±SD)

Serum and plasma markers in relation to cardiac dysfunction

In breast cancer survivors with plasma NT-ANP and/or BNP values above normal values, circulating apoptosis marker, HER2, nor acute phase protein levels were different from subjects with plasma natriuretic peptide concentrations within normal ranges. No correlations were observed between plasma soluble apoptosis markers and natriuretic peptides. HS-CRP and fibrinogen plasma levels did not correlate with plasma BNP or NT-ANP.

Serum and plasma markers in relation to treatment

We evaluated whether subgroups could be identified based on the applied chemotherapy regimens with regard to the circulating levels of the soluble proteins. For this analysis, the three breast cancer survivors with a second primary tumor were excluded. In the remaining 31 patients, subjects who had received high-dose CTC followed by hematopoietic stem cell reinfusion had markedly higher serum sFas ligand and sTRAIL levels than women treated with standard-dose

chemotherapy (Table 2). A trend for higher sFas levels was observed in patients who had received high-dose chemotherapy. Acute phase reactants HS-CRP and fibrinogen were not different in women who had received high-dose chemotherapy, compared to patients after standard-dose chemotherapy.

Tabel 2. Circulating soluble apoptosis markers in relation to treatment (median follow-up 6.0 years)

		Treatment		P-values		
	Cotnrols	**5x FEC (n=13)**	**4x FEC+CTC (n=18)**	**5x FEC vs. controls**	**4x FEC+CTC vs. controls**	**5x FEC vs. 4x FEC+CTC**
TNFα (pg/mL)	0.6 (0.3-3.3)	2.4 (0.5-15.7)	1.2 (0.5-9.80	**0.041**	0.087	0.622
sTNF-R1 (ng/mL)	0.9 (0.7-1.3)	0.8 (0.5-1.4)	0.8 (0.6-1.6)	0.252	0.819	0.258
sTNF-R2 (ng/mL)	1.9 (±0.4)	2.0 (±0.5)	2.4 (±0.7)	0.368	**0.027**	0.131
sFas (ng/mL)	8.0 (6.5-11.8)	8.6 (6.5-12.0)	10.5 (6.4-16.2)	0.271	**<0.001**	0.051
sFas ligand (pg/mL)	64 (±22)	71 (±16)	86 (±22)	0.332	**0.013**	**0.043**
sTRAIL (pg/mL)	71 (±28)	82 (±21)	112 (±420	0.323	**0.005**	**0.024**
Serum HER2 (ng/mL)	9.4 (±1.7)	10.8 (±2.1)	11.4 (±1.7)	0.056	**0.003**	0.381
HS-cRP (mg/L)	1.0 (0.2-4.8)	1.8 (0.3-4.9)	1.1 (0.2-6.4)	0.225	0.632	0.441
Fibrinogen (g/L)	3.0 (±0.5)	3.3 (±0.3)	3.3 (±0.5)	0.131	0.113	0.793
BNP (pmol/L)	4.0 (1.0-9.5)	6.6 (3.2-15.0)	3.8 (0.8-25.5)	0.087	0.917	0.125
NT-ANP (pmol/L)	255 (118-426)	313 (158-577)	268 (121-557)	0.266	0.983	0.185
Platinum (pg/g plasma)	-	-	64 (17-223)		-	-

Radiation therapy No differences were detected between patients who had received left versus right-sided chest wall irradiation, with regard to the circulating apoptosis-related proteins, serum HER2 or acute phase proteins. In addition, natriuretic peptide values did not differ between subjects after left sided compared to women after right-sided chest wall irradiation.
Tamoxifen Serum and plasma apoptosis marker, serum HER2 and acute phase protein levels were not different in the patients who had received 2 years tamoxifen compared to patients who had received tamoxifen for 5 years. In addition, the duration of tamoxifen use did not correlate with levels of the measured circulating proteins.
Platinum In the subgroup of 18 women who had received chemotherapy containing carboplatin (4x FEC+CTC), we evaluated if persisting circulating platinum could explain the higher circulating plasma soluble apoptosis markers. Circulating platinum concentrations ranged from 17 to 223 pg/g, with a median of 64 pg/g plasma at a median follow-up of 6.5 (range 4.1-9.8) years. Serum platinum levels decreased with longer follow-up duration (Figure 2). Partial Spearman correlation coefficients, with adjustment for follow-up duration, revealed no associations between circulating platinum concentrations and any of the serum and plasma apoptosis markers.

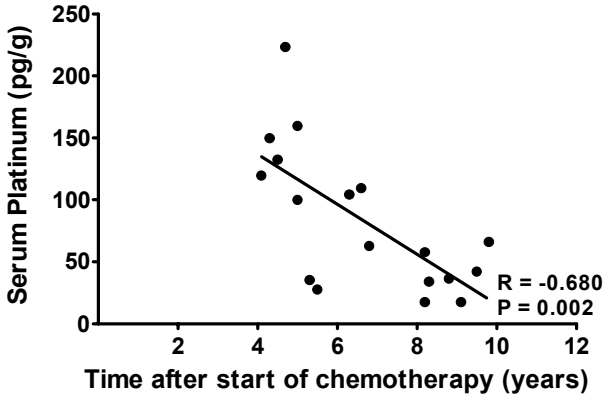

Figure 2.
Dot plot of serum platinum concentrations (pg/g) in relation to the time since the start of chemotherapy in months after the start of 4x FEC+CTC treatment (n=18). Line, R and P value represent Pearson correlation coefficient.

DISCUSSION

In this cross-sectional study, we observed for the first time that circulating levels of the apoptosis-related proteins TNFα, sFas, sFas ligand, sTRAIL and HER2 were higher in disease-free breast cancer survivors, at a median follow-up of 6 years after anticancer treatment, than in age-matched healthy controls. No association was observed between the increased circulating apoptosis marker levels and cardiac dysfunction, based on the existence of symptomatic heart failure, which was observed in two breast cancer survivors, or elevated plasma BNP and NT-ANP. Remarkably however, after a median follow-up of more than 6 years, sFas ligand and sTRAIL were higher in the breast cancer survivors who had received high-dose

chemotherapy, followed by hematopoietic stem cell rescue, compared to breast cancer survivors after standard-dose chemotherapy.

Plasma BNP was elevated in eight of the breast cancer survivors, with symptomatic heart failure being present in one of these patients. NT-ANP was elevated in two patients, both without heart failure symptoms. Since, natriuretic factors can become elevated before the development of cardiac functional loss,[29] patients with an asymptomatic elevation of plasma natriuretic peptides after anticancer treatment may be at increased risk for developing cardiac dysfunction and may require more intensive cardiac follow-up.

Although no direct association with cardiac dysfunction was observed in our breast cancer survivors after potentially cardiotoxic treatment, the increase in plasma soluble apoptotic protein levels might be indicative of subtle ongoing cardiac damage and as such, may be associated with the development of cardiac dysfunction later on. Recently, a population-based study showed that in women who participated in a large prospective cohort study and were free of cardiovascular disease, increased baseline sTNF-R1, sTNF-R2 and CRP plasma levels were found to be related to a higher risk of developing cardiovascular disease during follow-up.[30]

Atherosclerosis is considered to be an inflammatory process and increased serum HS-CRP levels are present in patients with heart failure based on coronary artery disease and non-ischemic causes.[31-33] In addition, elevated serum fibrinogen levels were shown in heart failure patients, and were associated with higher NYHA class.[34] We previously reported that serum HS-CRP and plasma fibrinogen were higher in cured testicular cancer patients at a median follow-up of 7 years since the start of cisplatin-based chemotherapy, than in controls.[35] In our breast cancer survivor population however, serum HS-CRP and plasma fibrinogen concentrations were not different from controls. Furthermore, we observed no associations between the acute phase reactants and plasma natriuretic peptides or apoptotic proteins.

To our knowledge, the results presented in this report provide the first evidence of increased circulating apoptosis-related protein levels during long-term follow-up after antineoplastic therapy for breast cancer, especially after high-dose chemotherapy and hematopoietic stem cell rescue.

A possible explanation for the increased plasma apoptosis marker concentrations long after high-dose chemotherapy may be the prolonged retention of circulating platinum in the body after carboplatin-containing chemotherapy. Retention of circulating platinum was observed in 61 testicular cancer patients and 21 patients with an ovarian malignancy cured with cisplatin-based chemotherapy. It was suggested that persisting circulating platinum is associated with long-term sequelae of chemotherapy.[36,37] In the testicular cancer patients, the mean plasma platinum concentration was 65 pg/g, after a median follow-up of 14 years following platinum-containing chemotherapy with cisplatin doses ranging from 350-950 mg/m^2.[38] In the current study, we observed a median serum platinum concentration

of 64 pg/g, at a median of 6.5 years after carboplatin-based chemotherapy at a dose of 1600 mg/m^2. We found no associations between circulating serum platinum concentrations and serum and plasma TNF-related apoptotic protein levels. This suggests that the platinum concentration per se is at least not the only risk factor for late toxicity.

Prior tamoxifen treatment may also have influenced circulating apoptosis marker levels. To date, no data are available with regard to the *in vivo* effects of tamoxifen on plasma circulating apoptosis marker levels. Only limited evidence, derived from *in vitro* studies in breast cancer cells, indicate that the effects of tamoxifen on Fas ligand membrane expression may depend on the tamoxifen dose.[39,40] All patients in the current study had received tamoxifen for the duration of two or more years. Although the duration of tamoxifen use was not associated with circulating levels of the soluble apoptosis markers, the effects of tamoxifen treatment on soluble apoptosis markers remains cannot be excluded based on these results.

Next to tamoxifen, radiation therapy to the chest wall may also have played a role in the increased circulating apoptosis marker concentrations in our study population. In patients treated with total body irradiation prior to bone marrow transplantation for hematological malignancies, plasma TNFα concentrations were increased at 4 hours after irradiation, compared to pre-treatment levels.[41] To our knowledge however, no data exist regarding plasma levels of circulating apoptosis markers in patients with long-term follow-up after radiotherapy to the chest wall. Since all patients in the current study underwent chest wall irradiation, the influence of radiotherapy on the plasma soluble apoptosis marker levels could not be determined.

Serum HER2 levels were higher in our population of breast cancer survivors in comparison to control subjects, but was not associated with cardiac dysfunction or treatment regimen. In patients with HER2-positive active breast cancer, serum levels of HER2 are increased and correlate positively with the number of metastatic sites.[42] In our study, the HER2 status of the primary tumor did not explain the higher serum HER2 levels in the breast cancer survivors. The higher serum HER2 concentrations may be related to an altered basal apoptotic state. Several studies have linked HER2 to protection from apoptosis. In ovarian cancer cells for instance, endogenous HER2 overexpression is associated with resistance to TNF-induced cytotoxicity.[43] The increased soluble apoptosis marker and HER2 levels, observed in the current study, could suggest an interaction between HER2 shedding and cleavage of ligands and receptors involved in the death receptor apoptotic pathways.

Considerations for interpreting the results

Our breast cancer survivor population had received a relatively low cumulative epirubicin dose of either 450 mg/m^2 in the standard-dose arm (5x FEC) or 360 mg/m^2 in the high-dose arm (4x FEC+CTC). Both the standard-dose and the high-dose regimen contained cyclophosphamide, 2.5 g/m^2 and 8.0 g/m^2 respectively,

which has also been related to cardiotoxicity.[44,45] Mild clinical cardiac dysfunction was observed in only two breast cancer survivors after moderate dose chemotherapy and chest wall irradiation. The limited number of breast cancer survivors who experienced (mild) symptomatic heart failure at the time of evaluation, could explain for the fact that we did not observe a relation between the circulating apoptosis marker levels and cardiac dysfunction in our study population. The small sample size of this cross-sectional study may also have limited the statistical significance of the findings. In addition, because multiple circulating apoptosis-related proteins were investigated, multiple testing had to be performed while analyzing our data, which could theoretically have increased the type I (α) error.

The cytokines TNFα and sTRAIL are known to have pro-apoptotic effects,[46,47] whereas the soluble forms of both death-receptors (Fas and TNF-R2) are linked to inhibition of apoptosis, by binding their specific ligands after which the pro-apoptotic signal is lost.[48,49] It is remarkable that we observed higher plasma levels of both pro- and anti-apoptotic proteins in breast cancer survivors compared to controls. An explanation for the elevated plasma levels of sFas and sTNF-R2 may be the proteolytic cleavage and subsequent release in the circulation of increased membrane-expressed receptor.

The pathogenetic role of the increase in both pro- and anti-apoptotic circulating proteins in this population of breast cancer survivors remains to be determined. The improved diagnostic and therapeutic modalities in recent years, make the early detection and prediction of late sequelae caused by anticancer therapy of increasing interest. Increased serum pro-inflammatory cytokine (TNF-R2 and interleukin-1) levels were found to be associated with fatigue, in 40 breast cancer survivors at 5 years after diagnosis.[50] Whether the higher levels of circulating soluble apoptosis markers in breast cancer survivors compared to controls are associated with long-term sequelae deserves further study.

In summary, we observed that long-term breast cancer survivors treated with anthracyclines and irradiation had higher circulating levels of TNFα, sFas, sFas ligand, sTRAIL and sHER2 than healthy age-matched women, especially when they received high-dose chemotherapy followed by hematopoietic stem cell rescue. In this small cross-sectional study, no clear associations were observed between circulating apoptosis markers and the existence of cardiac dysfunction, measured with natriuretic peptides. It remains to be determined whether the higher circulating soluble apoptosis markers play a pathogenetic role in the long-term sequelae of anticancer therapy for breast cancer, particularly in relation to high-dose chemotherapy regimens. Nevertheless, these results could indicate that basal apoptotic rates remain increased during long-term follow-up after antineoplastic therapy for breast cancer.

REFERENCES

1. Meinardi MT, Gietema JA, van Veldhuisen DJ, et al. Long-term chemotherapy-related cardiovascular morbidity. Cancer Treat Rev 2000; 26: 429-447.
2. Ayash LJ, Wright JE, Tretyakov O, et al. Cyclophosphamide pharmacokinetics: correlation with cardiac toxicity and tumor response. J Clin Oncol 1992; 10: 995-1000.
3. Bearman SI, Petersen FB, Schor RA, et al. Radionuclide ejection fractions in the evaluation of patients being considered for bone marrow transplantation: risk for cardiac toxicity. Bone Marrow Transplant 1990; 5: 173-177.
4. Cazin B, Gorin NC, Laporte JP, et al. Cardiac complications after bone marrow transplantation. A report on a series of 63 consecutive transplantations. Cancer 1986; 57: 2061-2069.
5. Narula J, Haider N, Virmani R, et al. Apoptosis in myocytes in end-stage heart failure. N Engl J Med 1996; 335: 1182-1189.
6. Olivetti G, Abbi R, Quaini F, et al. Apoptosis in the failing human heart. N Engl J Med 1997; 336: 1131-1141.
7. Torre-Amione G, Kapadia S, Benedict C, et al. Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD). J Am Coll Cardiol 1996; 27: 1201-1206.
8. Torre-Amione G, Kapadia S, Lee J, et al. Tumor necrosis factor-alpha and tumor necrosis factor receptors in the failing human heart. Circulation 1996; 93: 704-711.
9. Nishigaki K, Minatoguchi S, Seishima M, et al. Plasma Fas ligand, an inducer of apoptosis, and plasma soluble Fas, an inhibitor of apoptosis, in patients with chronic congestive heart failure. J Am Coll Cardiol 1997; 29: 1214-1220.
10. Yamaguchi S, Yamaoka M, Okuyama M, et al. Elevated circulating levels and cardiac secretion of soluble Fas ligand in patients with congestive heart failure. Am J Cardiol 1999; 83: 1500-3.
11. Yndestad A, Kristian DJ, Geir EH, et al. Increased gene expression of tumor necrosis factor superfamily ligands in peripheral blood mononuclear cells during chronic heart failure. Cardiovasc Res 2002; 54: 175-182.
12. Gibbs CR, Blann AD, Watson RD, Lip GY. Abnormalities of hemorheological, endothelial, and platelet function in patients with chronic heart failure in sinus rhythm: effects of angiotensin-converting enzyme inhibitor and beta-blocker therapy. Circulation 2001; 103: 1746-1751.
13. Yin WH, Chen JW, Jen HL, et al. Independent prognostic value of elevated high-sensitivity C-reactive protein in chronic heart failure. Am Heart J 2004; 147: 931-938.
14. Lip GY, Gibbs CR. Does heart failure confer a hypercoagulable state? Virchow's triad revisited. J Am Coll Cardiol 1999; 33: 1424-1426.
15. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987; 235: 177-182.
16. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001; 344: 783-792.
17. Crone SA, Zhao YY, Fan L, et al. ErbB2 is essential in the prevention of dilated cardiomyopathy. Nat Med 2002; 8: 459-465.
18. Ozcelik C, Erdmann B, Pilz B, et al. Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. Proc Natl Acad Sci U S A 2002; 99: 8880-8885.
19. Harris L, Luftner D, Jager W, Robertson JF. c-erbB-2 in serum of patients with breast cancer. Int J Biol Markers 1999; 14: 8-15.
20. Maisel AS, Krishnaswamy P, Nowak RM, et al. Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. N Engl J Med 2002; 347: 161-167.
21. McDonagh TA, Robb SD, Murdoch DR, et al. Biochemical detection of left-ventricular systolic dysfunction. Lancet 1998; 351: 9-13.
22. Omland T, Aakvaag A, Bonarjee VV, et al. Plasma brain natriuretic peptide as an indicator of left ventricular systolic function and long-term survival after acute myocardial infarction. Comparison with plasma atrial natriuretic peptide and N-terminal proatrial natriuretic peptide. Circulation 1996; 93: 1963-1969.
23. Davidson NC, Naas AA, Hanson JK, et al. Comparison of atrial natriuretic peptide B-type natriuretic peptide, and N-terminal proatrial natriuretic peptide as indicators of left ventricular systolic dysfunction. Am J Cardiol 1996; 77: 828-831.
24. Omland T, Aakvaag A, Vik-Mo H. Plasma cardiac natriuretic peptide determination as a screening test for the detection of patients with mild left ventricular impairment. Heart 1996; 76: 232-237.
25. Meinardi MT, van Veldhuisen DJ, Gietema JA, et al. Prospective evaluation of early cardiac damage induced by epirubicin-containing adjuvant chemotherapy and locoregional radiotherapy in breast

cancer patients. J Clin Oncol 2001; 19: 2746-2753.
26. Rodenhuis S, Bontenbal M, Beex LV, et al. High-dose chemotherapy with hematopoietic stem-cell rescue for high-risk breast cancer. N Engl J Med 2003; 349: 7-16.
27. Gelevert T, Messerschmidt J, Meinardi MT, et al. Adsorptive voltametry to determine platinum levels in plasma from testicular cancer patients treated with cisplatin. Ther Drug Monit 2001; 23: 169-173.
28. van de Vijver MJ, Peterse JL, Mooi WJ, et al. Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med 1988; 319: 1239-1245.
29. Suzuki T, Hayashi D, Yamazaki T, et al. Elevated B-type natriuretic peptide levels after anthracycline administration. Am Heart J 1998; 136: 362-363.
30. Pai JK, Pischon T, Ma J, et al. Inflammatory markers and the risk of coronary heart disease in men and women. N Engl J Med 2004; 351: 2599-2610.
31. Kaneko K, Kanda T, Yamauchi Y, et al. C-Reactive protein in dilated cardiomyopathy. Cardiology 1999; 91: 215-219.
32. Pietila KO, Harmoinen AP, Jokiniitty J, Pasternack AI. Serum C-reactive protein concentration in acute myocardial infarction and its relationship to mortality during 24 months of follow-up in patients under thrombolytic treatment. Eur Heart J 1996; 17: 1345-1349.
33. Yin WH, Chen JW, Jen HL, et al. Independent prognostic value of elevated high-sensitivity C-reactive protein in chronic heart failure. Am Heart J 2004; 147: 931-938.
34. Gibbs CR, Blann AD, Watson RD, Lip GY. Abnormalities of hemorheological, endothelial, and platelet function in patients with chronic heart failure in sinus rhythm: effects of angiotensin-converting enzyme inhibitor and beta-blocker therapy. Circulation 2001; 103: 1746-1751.
35. Nuver J, Smit AJ, Sleijfer DT, et al. Microalbuminuria, decreased fibrinolysis, and inflammation as early signs of atherosclerosis in long-term survivors of disseminated testicular cancer. Eur J Cancer 2004; 40: 701-706.
36. Gietema JA, Meinardi MT, Messerschmidt J, et al. Circulating plasma platinum more than 10 years after cisplatin treatment for testicular cancer. Lancet 2000; 355: 1075-1076.
37. de Vos FY, Nuver J, Willemse PH, et al. Long-term survivors of ovarian malignancies after cisplatin-based chemotherapy; cardiovascular risk factors and signs of vascular damage. Eur J Cancer 2004; 40: 696-700.
38. Gietema JA, Meinardi MT, Messerschmidt J, et al. Circulating plasma platinum more than 10 years after cisplatin treatment for testicular cancer. Lancet 2000; 355: 1075-1076.
39. Mor G, Kohen F, Garcia-Velasco J, et al. Regulation of fas ligand expression in breast cancer cells by estrogen: functional differences between estradiol and tamoxifen. J Steroid Biochem Mol Biol 2000; 73: 185-194.
40. Nagarkatti N, Davis BA. Tamoxifen induces apoptosis in Fas+ tumor cells by upregulating the expression of Fas ligand. Cancer Chemother Pharmacol 2003; 51: 284-290.
41. Girinsky TA, Pallardy M, Comoy E, et al. Peripheral blood corticotropin-releasing factor, adrenocorticotropic hormone and cytokine (interleukin beta, interleukin 6, tumor necrosis factor alpha) levels after high- and low-dose total-body irradiation in humans. Radiat Res 1994; 139: 360-363.
42. Colomer R, Montero S, Lluch A, et al. Circulating HER2 extracellular domain and resistance to chemotherapy in advanced breast cancer. Clin Cancer Res 2000; 6: 2356-2362.
43. Lichtenstein A, Berenson J, Gera JF, et al. Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-activated killer cells: correlation with expression of HER2/neu oncogenes. Cancer Res 1990; 50: 7364-7370.
44. Meinardi MT, Gietema JA, van Veldhuisen DJ, et al. Long-term chemotherapy-related cardiovascular morbidity. Cancer Treat Rev 2000; 26: 429-447.
45. Tiersten A, Wo J, Jacobson C, et al. Cardiac toxicity observed in association with high-dose cyclophosphamide-based chemotherapy for metastatic breast cancer. Breast 2004; 13: 341-346.
46. Miura M, Friedlander RM, Yuan J. Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway. Proc Natl Acad Sci U S A 1995; 92: 8318-8322.
47. Pitti RM, Marsters SA, Ruppert S, et al. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 1996; 271: 12687-12690.
48. Cascino I, Fiucci G, Papoff G, Ruberti G. Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. J Immunol 1995; 154: 2706-2713.
49. Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. J Exp Med 1992; 175: 323-329.
50. Bower JE, Ganz PA, Aziz N, Fahey JL. Fatigue and proinflammatory cytokine activity in breast cancer survivors. Psychosom Med 2002; 64: 604-611.