The Relation between Soluble Apoptotic Proteins and Subclinical Cardiotoxicity in Adjuvant-treated Breast Cancer Patients

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Abstract. Background: Circulating apoptotic proteins are increased in heart failure patients. We evaluated whether circulating soluble apoptosis-related protein levels change after anthracycline-containing chemotherapy and radiotherapy in relation to cardiac dysfunction or the applied treatment. Patients and Methods: Circulating apoptotic proteins were measured with immunoassay in 40 breast cancer patients following surgery (T0), one month (T1) and one year (T2) after epirubicin-based chemotherapy. Standard-dose (n=21) or high-dose (n=19) myeloablative chemotherapy, preceded irradiation and tamoxifen. Circulating apoptotic proteins were compared with previous cardiac evaluations. Results: Soluble tumor necrosis factor receptor 1 (+30%), 2 (+43%) and Fas (+40%) were transiently increased at T1 compared to T0, whereas Fas ligand (-64%) was transiently decreased, especially in the high-dose group. Apoptosis markers were not associated with cardiac dysfunction. Conclusion: Significant, but transient changes in soluble apoptotic protein levels were observed, particularly after high-dose chemotherapy. No relation was found between apoptosis-related proteins and cardiotoxicity.

Late complications of anticancer treatment have become increasingly relevant due to improved survival of cancer patients. Cardiovascular toxicity for instance, is a well-known adverse effect of several chemotherapeutic agents, especially anthracyclines (1). Anthracyclines can cause heart failure, which mostly occurs during the first year after treatment (2), but which can also take years to develop (3). Asymptomatic cardiac dysfunction is considered to precede symptoms of heart failure. We have previously shown that the left ventricular ejection fraction (LVEF) decreases during the first year following epirubicin-containing chemotherapy and chest wall irradiation in patients treated for breast cancer who did not experience symptomatic cardiac dysfunction (4). In addition to anthracyclines, high-dose chemotherapy, particularly cyclophosphamide-containing regimens, can also induce cardiotoxicity (5). Unfortunately, LVEF measurement is relatively insensitive, since it detects only loss of systolic cardiac function. More sensitive methods, enabling earlier detection or even prediction of anthracycline-related cardiotoxicity, may allow clinicians to intervene before heart failure develops.

In patients with heart failure unrelated to anthracyclines, increased cardiomyocyte apoptosis has been reported (6-7). Plasma levels of several soluble (s) members of the tumor necrosis factor (TNF)-superfamily of apoptosis-related proteins (TNFα, TNF Receptor (TNF-R) 1 and 2, Fas and Fas Ligand) are elevated in heart failure patients and correlate with New York Heart Association (NYHA) class (8-9), while increased TNF-related apoptosis-inducing ligand (TRAIL) cDNA expression has been reported in peripheral blood mononuclear cells of heart failure patients (10). Serum levels of the acute phase reactant high-sensitivity C-reactive protein (HS-CRP) are also elevated in heart failure patients and positively associated with the NYHA class (11).

Currently, it is unknown whether circulating sTNF-related apoptotic protein levels change during follow-up after anticancer treatment in relation to cardiac injury. We have reported that plasma sTNF-related apoptotic proteins are increased after a median follow-up of more than 6 years.
in patients who had received adjuvant epirubicin-containing chemotherapy and chest wall irradiation for breast cancer (12). This increase was particularly prominent in patients who underwent high-dose chemotherapy with hematopoietic stem cell rescue. The question arises whether this rise in soluble apoptotic proteins is causative for, or coinciding with (early) cardiotoxicity in this breast cancer population. Previously, we found that subclinical cardiotoxicity occurred during the first year after anthracycline-containing chemotherapy and chest wall irradiation in 40 patients treated with anthracycline-based chemotherapy for breast cancer (4). In the current study, we extended the results of that prospective study by measuring plasma sTNF-related apoptotic protein levels in plasma samples from those patients.

The primary aim of the current prospective study was to evaluate whether circulating sTNF-related apoptotic protein levels change during the first year following the start of anthracycline-containing chemotherapy and radiation therapy in relation to cardiac function. Secondly, we investigated the influence of the applied chemotherapy regimen on the circulating apoptosis-related protein levels.

Patients and Methods

Plasma samples were examined from 40 breast cancer patients who had participated in a cardiac side study of a nation-wide randomised trial (13) comparing the anti-tumor efficacy of standard-dose chemotherapy to high-dose chemotherapy and hematopoietic stem cell rescue. The study was approved by the medical ethical committee and all patients gave their written informed consent. Twenty-one patients received standard-dose chemotherapy and 19 received high-dose chemotherapy followed by high-dose cyclophosphamide (1500 mg/m²), thiotepa (120 mg/m²) and carboplatin (400 mg/m²) daily for 4 days (4x FEC + CTC). Peripheral stem cell reinfusion took place 7 days after the start of chemotherapy and chest wall irradiation for breast cancer (4). Treatment. Patients were treated with a standard-dose of 5 courses of fluorouracil (500 mg/m²), epirubicin (90 mg/m²) and cyclophosphamide (500 mg/m²) (FEC), or 4 cycles of FEC, followed by high-dose cyclophosphamide (1500 mg/m²), thiotepa (120 mg/m²) and carboplatin (400 mg/m²) daily for 4 days (4x FEC + CTC). Peripheral stem cell reinfusion took place 7 days after the start of CTC. Total epirubicin dose was 450 mg/m² for the standard-dose FEC-treated group and 360 mg/m² for the high-dose 4x FEC + CTC-treated group. Tamoxifen (40 mg/day, orally) was started thereafter in both treatment groups. Locoregional irradiation followed upon haematological recovery from the last chemotherapy cycle. Patients received 46-50 Gy in 23-25 fractions on their supraclavicular and axillary lymph nodes. Chest wall irradiation was performed with doses between 40 and 50 Gy in 20-25 fractions. In patients who underwent breast conserving treatment, the breast was treated with two tangential-wedged fields, in which the ipsilateral mammary lymph nodes were included. A boost of 16-20 Gy in 8-10 fractions was applied to the tumor bed. Twenty-five patients received right sided chest wall irradiation and the remaining 15 underwent radiation therapy to the left side of the chest wall.

Cardiac evaluation. This was performed as described previously (4). Briefly, cardiac evaluations had been performed following surgery but before the start of chemotherapy (T0), one month after chemotherapy before chest wall irradiation (T1) and one year after the start of chemotherapy (T2). In addition to history and physical examination with special attention to signs and symptoms related to heart failure, the cardiac evaluations consisted of a standard electro- and echocardio gram, and plasma natriuretic peptide (N-terminal atrial and B-type natriuretic peptide (NT-ANP and BNP)) measurement. LVEF measurements were done at T0 and T2. The normal LVEF lower limit in our institution was 0.50. None of the patients had pre-existent cardiac disease at the time of enrolment and symptomatic heart failure had not occurred during the one-year follow-up period. Two patients had pre-existent hypertension for which they received medical treatment consisting of an angiotensin converting enzyme inhibitor and a calcium blocker, respectively. Two patients did not complete the cardiac evaluation, but until T1. Table I summarises the earlier published results of the cardiac evaluations (4).

Plasma soluble apoptosis markers. Peripheral blood samples were collected in 10 ml disposable tubes containing either 2-natrium-ethylenediamine tetra-acetic acid (EDTA), heparin or no additive. Tubes containing EDTA or heparin as an additive were placed immediately on ice. In tubes containing no additive, blood was allowed to clot at room temperature until centrifugation. Serum and plasma were separated within 30 min of collection by centrifuging at 4 °C and then stored at –80 °C until determination. Circulating levels of sTNFα, sTNF-R1, sTNF-R2, sFas, sFas ligand and sTRAIL were determined using commercially available ELISA kits (Quanti kine; R&D systems, Minneapolis, MN, USA) following the manufacturer’s instructions. TNFα was measured in EDTA plasma, sTNF-R1 and sTNF-R2 in heparin plasma. Serum was used for sFas, sFas ligand and sTRAIL.

Platinum determination. Serum platinum levels were determined in the patients who received carboplatin. This analysis was performed using a highly sensitive assay in which high-pressure decomposition of plasma is followed by adsorptive voltammetric determination of platinum, with a quantification limit of 6 pg/g (14).

HS-CRP determination. Serum HS-CRP was determined as a marker for inflammation and assayed with the BNII Nephelometer (Dade Behring, Brussels, Belgium). The lower detection limit was 0.16 mg/l (normal values 0.16-10 mg/l).

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. Paired analysis was performed with a Wilcoxon signed ranks test. 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 the Spearman rank sum test. All p values were two-sided and p<0.05 was considered statistically significant.

Results

Plasma soluble apoptosis markers. Table II and Figure 1 show plasma apoptosis marker levels at the different time
points for the whole patient group. Between T0 and T2, an increase in median plasma levels of sTNF-R1, sTNF-R2 and sFas was observed at T1, while plasma sFas ligand concentrations were lower. At T2, sFas remained higher compared to pre-treatment levels. After a decrease at T1 compared to T0, plasma sTRAIL raised from T1 to T2, to levels higher than pre-treatment values.

Positive correlations between serum HS-CRP levels at T1 and serum levels of sTNF-R1 (R=0.573, p<0.001) and sTNF-R2 (R=0.362, p=0.035) were found. One year after chemotherapy, a weak positive correlation was observed between serum HS-CRP and sTNF-R2 (R=0.418, p=0.038) and sFas (R=0.450, p=0.024).

**Plasma soluble apoptosis markers, HS-CRP and cardiac dysfunction.** Plasma apoptosis marker levels and serum HS-CRP did not differ between the patients with a decrease in LVEF of more than 0.10 from pre-treatment values or a total LVEF value less than 0.50 one year after the start of chemotherapy. Furthermore, the changes in plasma apoptosis markers and serum HS-CRP levels between the different time points were not associated with a change in LVEF during the study period.

No associations were observed between the plasma apoptosis marker concentrations and echocardiographic functional parameters or the QTc time. At T1, serum HS-CRP levels were positively correlated with the corrected QT time (QTc) (R=0.622, p<0.001).

In the patients with increased plasma natriuretic peptide levels (NT-ANP >500 pmol/l; BNP >10 pmol/l) at T2, circulating apoptosis marker values were not different from patients with natriuretic peptide levels within the normal range.
Limits. No correlations between natriuretic peptide plasma concentrations and plasma apoptosis marker levels or HS-CRP were observed.

Figure 2 represents plasma apoptosis marker levels according to the applied treatment regimen. Before the

Plasma soluble apoptosis marker, HS-CRP and treatment.

Figure 1. Bar plots of 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). Bars represent median values, error bars represent range (except for TNFα; interquartile range). T0: after surgery, before chemotherapy; T1: 1 month after chemotherapy, before radiation therapy; T2: 1 year after the start of chemotherapy.
start of treatment, apoptosis marker levels did not differ between the treatment groups. At T1 however, sTNF-R1, sTNF-R2 and sFas plasma levels were markedly higher in the patients who had received high-dose chemotherapy. Plasma sFas and sTRAIL levels were lower in the high-dose treated group. One year after the start of chemotherapy, only a higher median sTNF-R1 plasma level was detected in the high-dose group.

No differences were observed between patients who had received left-sided compared to right-sided chest wall irradiation with regard to the circulating apoptosis marker concentrations.

Serum HS-CRP levels did not differ between the treatment groups at any of the time points.

**Platinum.** In the patients treated with the high-dose carboplatin-containing regimen, serum platinum was analysed at T2 to evaluate whether persisting circulating platinum could explain for the higher plasma apoptosis marker levels observed in this subgroup of patients. Circulating platinum levels were available for 16 of the 19 patients in the high-dose group and was a median value of 4091 (range 2027-7022) pg/g plasma. Serum platinum correlated positively with plasma sFas levels ($R=0.684$, $p=0.003$) and with sTNF-R1 ($R=0.558$, $p=0.025$) at T2, but were not associated with serum HS-CRP levels.

**Discussion**

The main finding of the present prospective study is a transient change in the levels of several of the soluble TNF-related apoptotic proteins following adjuvant antineoplastic treatment for early stage, high-risk breast cancer, particularly a rise in sFas and a decrease in sFas ligand. The changes in serum soluble apoptosis marker levels were not associated with the development of cardiac dysfunction, since no relation with symptomatic cardiac dysfunction, decreased LVEF or increased natriuretic peptide plasma levels was observed.

Anthracyclines are well known to induce cardiac toxicity in a dose-dependent manner (15). Both chemotherapy regimens applied in this study contained a relatively low dose of epirubicin, either 360 mg/m² or 450 mg/m². This may be an explanation for the fact that none of the patients developed symptoms of cardiac failure during the first year after the start of treatment. However, an asymptomatic decrease in LVEF to a value below 0.50 was observed in 15% of the patients. This indicates that even low doses of anthracyclines have detrimental effects on the heart, which may ultimately lead to the development of heart failure. We observed no differences in plasma apoptosis marker levels between patients with a LVEF decline more than 0.10 or to an absolute value less than 0.50, and patients with a normal LVEF. At one year, apoptosis marker levels were not different in patients with plasma natriuretic peptide concentrations above the normal limit. As a consequence, the value of circulating apoptosis marker measurement for the early detection of cardiac dysfunction appears limited.

It is known, particularly from studies performed in childhood cancer survivors, that anthracycline-induced cardiotoxicity can occur years to decades after treatment (1, 3). Although we observed no associations between circulating apoptosis marker levels and the development of cardiotoxicity during the first year post-treatment, an association between the transient increase in circulating apoptotic proteins and the development of cardiotoxicity in the long term cannot be excluded based on our findings.

In patients with heart failure due to coronary artery disease and non-ischemic causes, increased serum HS-CRP levels have been reported (11). Although HS-CRP serum levels were not associated with other parameters representative of cardiac function, we observed a positive association of serum HS-CRP levels with the QTc at T1.

Currently, only limited data with regard to circulating soluble apoptosis marker levels during and after adjuvant antineoplastic treatment are available. In several types of (active) malignant diseases, plasma levels of TNF-related apoptotic proteins are raised. Compared to healthy controls, breast cancer patients have higher TNFα plasma concentrations before treatment, and a more advanced TNM stage is positively associated with higher TNFα levels (16). A small study among 17 breast cancer patients showed that pre-treatment serum sTNF-R levels were higher in patients than in healthy controls (17). Plasma sFas concentrations were also elevated in 162 primary and 71 recurrent breast cancer patients compared to controls (18). Additionally, plasma sFas levels were higher in breast cancer patients before surgery, than in healthy subjects (19). In our study population, the baseline (T0) measurement was performed after surgery. In general, in order to evaluate the effects of systemic anticancer treatment on circulating levels of the apoptotic proteins, samples should preferably be taken in the adjuvant setting, with baseline levels taken after removal of the tumour and recovery from surgery. In our study, these criteria were applied to all patients included. Therefore, the observed changes in circulating apoptotic protein levels cannot be attributed to the presence of active malignancy. In addition, there were no signs of cancer recurrence at the different times of blood sampling.

Previously, we described that plasma apoptosis markers are elevated in long-term disease-free breast cancer survivors, after a median follow-up duration of more than 6 years after the start of chemotherapy, especially after high-dose chemotherapy (12). Compared to healthy controls, higher plasma TNFα, sFas, sFas ligand and sTRAIL levels were observed in the population of long-term disease-free...
Figure 2. Dot plots of 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) according to chemotherapy regimen. T0: after surgery, before chemotherapy; T1: 1 month after chemotherapy, before radiation therapy; T2: 1 year after the start of chemotherapy. Lines represent median values. *p<0.01.
breast cancer survivors. In particular, patients who had received 4 cycles of FEC followed by myeloablation with high-dose CTC, had higher sFas ligand and sTRAIL plasma concentration at a median follow-up of 6 years, compared to the patients treated with 5 cycles of FEC. Remarkably, in the current study, we found a transient increase in sFas, but a transient decrease in sFas ligand plasma levels, at one month after chemotherapy. Plasma sFas levels tend to increase with advancing age (19). This may partly explain the higher circulating apoptotic protein levels which we observed in our population of long-term disease-free breast cancer survivors (12). Alternatively, the initial change in circulating apoptosis marker levels observed in the current study may be indicative of the generalised injury induced by the antineoplastic treatment, and the high-dose chemotherapy regimen in particular. The return to normal levels of the circulating apoptotic proteins one year after the start of chemotherapy, may point to a recovery of the body after the induced damage. It can be hypothesized that the increase in circulating apoptotic proteins after longer follow-up may be the result of an autonomically progressive process, which is initiated as a response to the initial injury induced by the anticancer treatment. As a result, these patients might be at increased risk of developing late sequelae related to the anticancer treatment. In addition, patients with altered circulating apoptotic protein levels after antineoplastic treatment may be more susceptible to chemotherapy-related complications, for instance when they are re-treated with cytostatic agents.

The origin of the elevated sTNF-related apoptotic proteins remains to be determined. In addition to the inflamed heart (20), several extracardiac sources of circulating apoptotic proteins, such as peripheral skeletal muscles (21) and activation of the immune system have been described as possible sources (22). The increased serum apoptosis marker levels may indicate an augmented apoptotic state. Hypothetically, the increase in plasma apoptosis marker levels may be the result of an increase in membrane-expressed apoptotic proteins. Elevated plasma concentrations of the soluble forms of these proteins may be the result of subsequent cleavage and shedding of membrane-bound proteins. Alternatively, an increase in the rate of proteolytic cleavage, leading to reduced membrane-expressed protein, might also account for the rise in plasma apoptosis marker levels. The circulating apoptosis-related receptors are viewed as anti-apoptotic (23, 24), while the ligands are considered to be pro-apoptotic (25-27). The transient increase in plasma sFas and decrease in plasma sFas ligand might suggest a natural protection or compensation mechanism against the external noxe, induced by the antineoplastic treatment. The exact association between the basal apoptotic rate and the current findings of transient change in plasma apoptosis-related protein levels remains unclear and warrants further investigation.

The high-dose chemotherapy regimen contained carboplatin. Previously, we showed that platinum can reside in the circulation long after treatment and is associated with long-term sequelae of chemotherapy (28, 29). In the current study, we evaluated the influence of circulating platinum retention on the plasma apoptosis marker levels and observed that, one year after the start of chemotherapy, circulating platinum was positively associated with plasma sTNF-R1 and sFas levels. This might be interpreted as a continuous external noxe for the apoptotic rate.

The transient increase in circulating apoptosis markers at one month after chemotherapy was especially pronounced in the patients treated with high-dose chemotherapy followed by hematopoietic stem cell rescue, compared to women treated with standard-dose chemotherapy. Data with regard to serum markers for apoptosis following high-dose chemotherapy during and after adjuvant treatment for setting is scarce. In 22 patients who received autologous peripheral blood stem cell transplantation for malignant non-Hodgkin’s lymphoma or lung cancer, a transient increase in TNFα plasma levels was observed at one week after stem cell transplantation (30).

Several pathophysiological mechanisms may contribute to the difference in apoptosis marker levels in high-dose compared to standard-dose treated patients. For instance, elevated circulating TNFα, sTNF-R, sFas, sFas ligand and sTRAIL levels have been reported in inflammatory conditions (31-33). Additionally, several reports have suggested a pathogenetic role of TNFα and its receptors in, particularly allogeneic, bone marrow transplantation-related complications (34, 35). In this light, the increased circulating TNF-related apoptotic proteins may reflect an acute phase reaction as part of the neutropenic period induced by the high-dose regimen. We observed a positive association between HS-CRP serum levels and serum levels of the sTNF-Rs at T1, and sTNF-R1 and sFas one year after chemotherapy. In our study however, plasma apoptosis markers were measured after the neutropenic period. The higher circulating apoptosis marker levels in patients treated with high-dose chemotherapy could als be indicative of greater tissue and/or cellular injury induced by the myeloablative regimen in comparison to the standard-dose chemotherapy.

In conclusion, we could not detect a relation between serum apoptosis markers and cardiac function in breast cancer patients within the first year of treatment. The multiple comparison evaluation used, which may have increased a type I (α) error, and the relatively small size of the study population, might have played a role in this
finding. The fact that we previously observed elevated apoptosis markers in long-term disease-free breast cancer survivors, especially after high-dose chemotherapy, warrants precaution in ruling out the pathogenic role of apoptotic proteins too early. Hence, it is known that anthracycline cardiotoxicity may become overt even many years after treatment (1, 3).

References


