Effect of Breast Cancer Adjuvant Therapies on Potential Biomarkers of Pulmonary Inflammation

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Abstract. *Background: The aim of this study was to investigate the effect of breast cancer adjuvant therapies on the levels of circulating surfactant protein-D (SP-D), C-Reactive protein (CRP) and soluble receptor for advanced glycation end-products (sRAGE), as potential biomarkers of subclinical pulmonary inflammation. Materials and Methods: The soluble molecules were serially determined in 38 patients, prior to the initiation of radiation therapy (RT) and during adjuvant treatment, using immunoassays. Results: Significantly higher levels of all three biomarkers were observed in patients prior to the initiation of RT compared to healthy controls (CRP: $p<0.001$, SP-D: $p<0.05$, sRAGE: $p<0.05$). SP-D levels exhibited a gradual increase after RT and during follow-up ($p<0.005$). Patients treated with a combination of RT and hormonal therapy presented a significant, but less pronounced, increase in SP-D and a significant decrease in CRP compared to those who did not receive hormonal therapy ($p=0.0428$ and $p=0.0116$, respectively). Patients treated with a combination of RT and trastuzumab presented a significant increase in SP-D levels ($p=0.0310$). Conclusion: The average rate of change in the levels of circulating SP-D and CRP during postoperative irradiation and adjuvant hormonal therapy suggests that the combined therapeutic regiment may potentially exert important anti-inflammatory effects on the lung. On the contrary, combined administration of RT and trastuzumab is likely to induce or provoke pulmonary inflammation.*

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Breast cancer constitutes a typical example of an inflammation-linked malignancy, since breast tumors are commonly enriched with inflammatory components (1). The process of breast cancer development and progression appears to be influenced not only by the intrinsic properties of the tumor cells, but also by inflammatory mediators within the tumor microenvironment (2-5). As a result, the course of the disease is largely defined by the interplay between tumor cells and a network of factors of inflammation, secreted by inflammatory, tumor and stromal cells.

The inflammatory status has been suggested as an important prognostic factor for breast cancer (6). More specifically, inflammation within the tumor microenvironment has been associated with the development of more aggressive tumors and poor prognosis (7, 8). Moreover, differences in the inflammatory profiles of patients with breast cancer have been associated with tumor characteristics, with potential implications in treatment response (9).

The therapeutic management of breast cancer often induces significant immunomodulation, possibly altering the patient's inflammatory status during treatment. Breast cancer surgery, even though conventionally considered minor, has been associated with the indirect induction of a pro-inflammatory profile (10). Chemotherapy regimens, and in particular taxanes, have been also shown to exert significant immunostimulatory effects against tumor cells, indicating that they are capable of suppressing cancer through multiple mechanisms (11, 12). In addition, postoperative breast irradiation, combined with administration of antineoplastic agents has been associated with the development of pulmonary toxicity (13).

The aim of the present study was to investigate the effect of adjuvant breast cancer therapy, incorporating irradiation with or without administration of hormonal therapy, or trastuzumab, on cancer-related subclinical pulmonary inflammation. Taking into consideration that peripheral blood sampling is a minimally-invasive procedure and a useful tool for the monitoring of the expression of circulating

biomarkers, we quantitatively determined changes in the expression levels of appropriately chosen markers during breast cancer treatment. In that context, a panel of three biomarkers was investigated: surfactant protein-D (SP-D) as a specific biomarker of inflammatory lung disease; C-Reactive protein (CRP) as a sensitive marker of systemic inflammation, and soluble receptor for advanced glycation-end products (sRAGE) as a potential biomarker of lung injury. Expression levels were serially determined during a three-month period starting prior to the initiation of postoperative radiotherapy in an effort to explore their potential associations with applied therapeutic interventions.

Materials and Methods

Participants. A total of 38 female patients with primary breast cancer, aged between 30 and 76 years (median=51.5 years), were recruited from the First Department of Radiation Oncology of St. Savvas Anticancer Hospital of Athens, prior to the initiation of radiation therapy (RT). All patients had previously undergone breast surgery and received irradiation to the whole breast or chest wall and supraclavicular area to a total dose of 50 Gy (2 Gy daily for five weeks), followed by a boost to the tumor bed to a dose of 9 Gy (3 Gy daily). Among patients, 34 women also underwent adjuvant chemotherapy. In addition, 25 and 10 patients received hormonal therapy or treatment with trastuzumab, respectively, during or after the RT course. Patients' demographic and clinicopathological characteristics are presented in Table I. Patients were monitored for a period of three months, starting before the initiation of RT, and were followed-up for an additional two months after completion of RT.

Throughout the study period, patients were evaluated for progression of disease and RT-induced side-effects by medical history, physical examination, blood tests, chest radiograph, electrocardiogram, and cardiac sonogram. In addition, 31 healthy controls, aged between 36 and 73 years (median=54.5 years), were recruited from the Breast Unit of the Hippokratio Hospital of Athens during their annual mammography and clinical breast examination, after exclusion of the presence of breast cancer or other suspicious findings. Participants with metabolic disorders, chronic inflammatory disease, cardiovascular disease or pulmonary dysfunction were excluded from the study. The study protocol was approved by the Hospitals' Ethics Committees. All participants had given their informed consent prior to entering the study.

Table I. *Patients' demographic data, clinicopathological and treatment characteristics*.

Characteristic	N	%
Age (years)		
Median (range)	51.5 (30.0-76.0)	
BMI (kg/m^2)		
Median (range)	27.6 (19.7-40.9)	
Menopausal status		
Pre-	8	21.1
Post-	28	73.7
Smoking status		
Non-smokers	26	68.4
Ex-/current smokers	11	29.0
Tumor location		
Left breast	16	42.1
Right breast	22	57.9
Clinical stage		
0-I	12	31.6
II	15	39.4
III	11	29.0
Histological grade		
I-II	27	71.1
III	10	26.3
ER		
Negative	11	29.0
Positive	26	68.4
PgR		
Negative	12	31.6
Positive	25	65.8
ERBB2		
Negative	25	65.8
Positive	11	29.0
Radiation dose (Gy)		
Median (range)	59.0 (47.0-109.0)	
Surgery		
Lumpectomy/quadrectomy	29	76.3
Mastectomy	9	23.7
Adjuvant chemotherapy	34	89.5
Hormonal therapy	25	65.8
Trastuzumab	10	26.3

ER: Estrogen receptor, PgR: progesterone receptor, ERBB2: epidermal growth factor receptor 2.

Samples. Peripheral venous blood samples were collected from all patients at defined time-points throughout the study period as follows: before initiation of RT, on day 6 of RT, at completion of RT (day 38) and at first follow-up (day 90). Two separate samples were collected from all participants in appropriate vacutainers, for the separation of serum and plasma, respectively, according to standard protocols. Serum and plasma samples were subsequently aliquoted and stored at –80˚C until assayed. All samples were assayed in duplicate.

Determination of serum SP-D levels by enzyme-linked immunosorbent assay (ELISA). Serum SP-D levels were quantified using ELISA (BioVendor GmbH, Heidelberg, Germany) according to the manufacturer's instructions. The assay's limit of detection was 0.2 ng/ml, and the intra-assay and inter-assay precision ranged between 2.2-4.7% and 4.2-9.7%, respectively.

Determination of serum CRP levels by ELISA. Serum CRP levels were quantitatively determined using ELISA (R&D Systems, MN, USA) according to the manufacturer's instructions. The assay's limit of detection was 0.01 ng/ml, and the intra-assay and inter-assay precision ranged between 3.8-8.3% and 6.0-7.0%, respectively.

Determination of plasma sRAGE levels by Luminex xMAP technology. Plasma levels of sRAGE were measured with the Milliplex Human Soluble Cytokine Receptor Panel (Millipore Corp., MA, USA) on a Luminex 100-IS analyzer (Luminex Corp.) according to the manufacturer's protocol. The assay's limit of

Table II. *Comparison between pre-radiotherapy levels of circulating C-Reactive protein (CRP), surfactant protein-D (SP-D) and soluble receptor for advanced glycation end-products (sRAGE) in patients with breast cancer and their corresponding levels in healthy controls.*

Biomarker	Circulating levels (median, range)		*p*-Value
	Patients	Healthy	
CRP (ng/ml)	1442.0 (82.6-9391.2)	719.1 (114.2-2873.4)	0.0008
SP-D (ng/ml)	76.8 (33.7-222.1)	61.3 (21.5-78.0)	0.0240
sRAGE (pg/ml)	97.1 (60.9-426.1)	68.4 (60.9-107.4)	0.0169

Table III. *Changes in levels of circulating C-Reactive protein (CRP), surfactant protein-D (SP-D) and soluble receptor for advanced glycation end-products (sRAGE) in patients with breast cancer during radiation therapy (RT) and at follow-up. p-values represent overall changes throughout the study period (relationship to time).*

Biomarker	Circulating levels (median)				*p*-Value
	Pre-RT	Day 6	Day 38	Day 90	
CRP (ng/ml)	1442.0	999.6	1153.3	1206.5	0.7341
SP-D (ng/ml)	76.8	75.7	96.0	98.4	0.0032
sRAGE (pg/ml)	97.1	128.9	122.7	113.7	0.7359

detection was 4.7 pg/ml, and the intra-assay and inter-assay precision was 5.2% and 8.5%, respectively.

Statistical analysis. Pre-radiotherapy levels of circulating CRP, SP-D and sRAGE in patients with breast cancer were compared to corresponding levels in healthy individuals using an analysis of covariate model with age as a covariate. Changes in CRP, SP-D and sRAGE levels throughout the study period (relationship to time) were explored with a random co-efficient model with patient and patient-by-time terms as random effects. The effect of treatment with hormonal therapy or trastuzumab on the average rate of change in CRP, SP-D and sRAGE levels in patients with breast cancer was also investigated with the same model. In all models, an unstructured variance-covariance matrix was taken. Furthermore, in order to meet the random co-efficient model assumptions, a square root transformation was applied in order to give approximately normal distributions and to produce linear rates of change over time. The statistical analysis was performed with SAS® version 9.2 (SAS Institute, Cary, NC, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Patients were monitored throughout a three-month period, starting prior to the initiation of RT and until two months after the completion of the radiotherapy course. Progression of cancer and development of radiation-induced acute side-effects were not observed for any of the patients after clinical examination, radiological imaging and quantitative assessment of established cancer markers. Moreover, none of the patients developed cardiovascular disease or respiratory problems.

Comparison of levels of circulating SP-D, CRP and sRAGE between patients with breast cancer and healthy individuals. Pre-radiotherapy levels of SP-D, CRP and sRAGE in patients with breast cancer were compared to corresponding levels in healthy individuals (Table II). Significantly higher levels of all three circulating biomarkers were observed in patients prior to the initiation of RT, as compared to healthy controls (CRP: $p<0.001$, SP-D: $p<0.05$, sRAGE: $p<0.05$).

Changes in levels of circulating SP-D, CRP and sRAGE in patients with breast cancer. SP-D, CRP and sRAGE levels were serially measured in patients with breast cancer prior to the initiation of RT (day 0), during RT (day 6), at completion of RT (day 38) and at first follow-up (day 90) (Table III). Changes in levels of CRP and sRAGE during the study period were not significant. However, gradually increasing levels of SP-D were observed after completion of RT and during follow-up. As a result, a significant positive association between the expression of circulating SP-D and time was detected ($p<0.005$).

Changes in levels of circulating SP-D, CRP and sRAGE in patients under treatment with hormonal therapy or trastuzumab. The rate of change in SP-D, CRP and sRAGE levels during the study period was investigated in patients treated with hormonal therapy or trastuzumab, during or after irradiation. Patients under treatment with a combination of RT and hormonal therapy exhibited a significant increase in SP-D levels, which was less pronounced compared to those who did not receive hormonal therapy ($p=0.0428$) (Figure 1A). With respect to CRP levels, a significant decline was observed ($p=0.0116$) (Figure 1B). Regarding patients treated with a combination of RT and trastuzumab, a significant increase in SP-D levels was observed during the study period ($p=0.0310$) (Figure 2), while levels of CRP exhibited no significant changes. The average rate of change in levels of circulating sRAGE exhibited no significant association with treatment with hormonal therapy or trastuzumab.

Discussion

It has long being recognized that RT to the chest may cause pulmonary toxicity, which is further induced by the concomitant or subsequent administration of several antineoplastic agents, including carmustine, paclitaxel, adriamycin and trastuzumab (14-16). Even though the exact mechanisms remain unclear, it has been proposed that

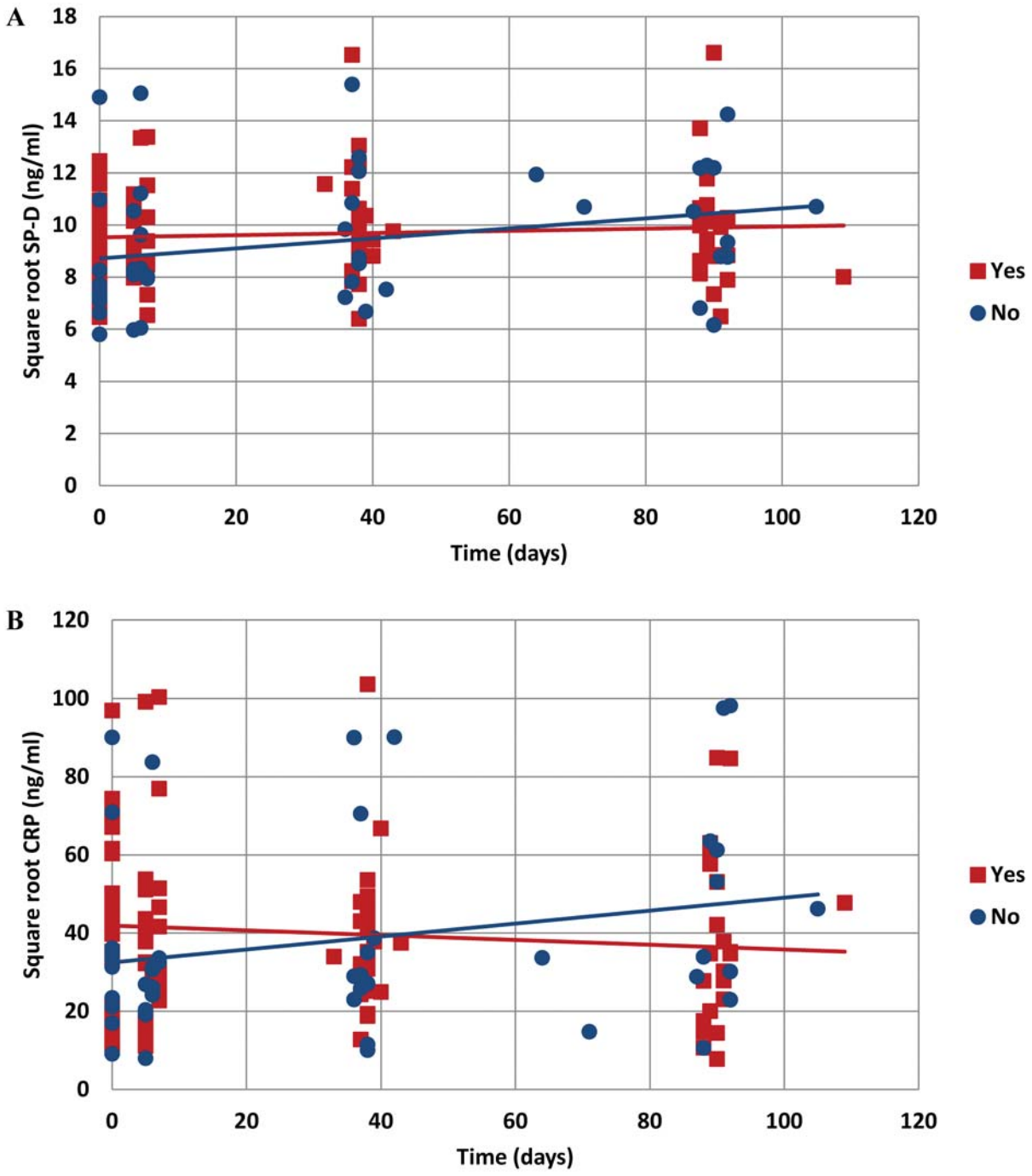

Figure 1. *Linear rate of change in levels of circulating (A) surfactant protein-D (SP-D) and (B) C-Reactive protein (CRP) during therapeutic management of breast cancer patients with radiation therapy (RT) and hormonal therapy, explored with a random co-efficient model using a square root transformation. $p<0.05$ and $p<0.05$, respectively.*

irradiation may cause subclinical injury to the lung parenchyma that becomes reinforced when another pulmonary insult is encountered at a later date. Otherwise, it is likely that irradiation causes injury to type II pneumocytes within the RT field, reducing the ability of the lung to repair itself (13). Antineoplastic agents, on the other hand, may induce pulmonary toxicity effects by direct injury to the pneumocytes or to the alveolar capillary endothelium, followed by the subsequent release of cytokines and the recruitment of inflammatory cells. It has also been suggested that

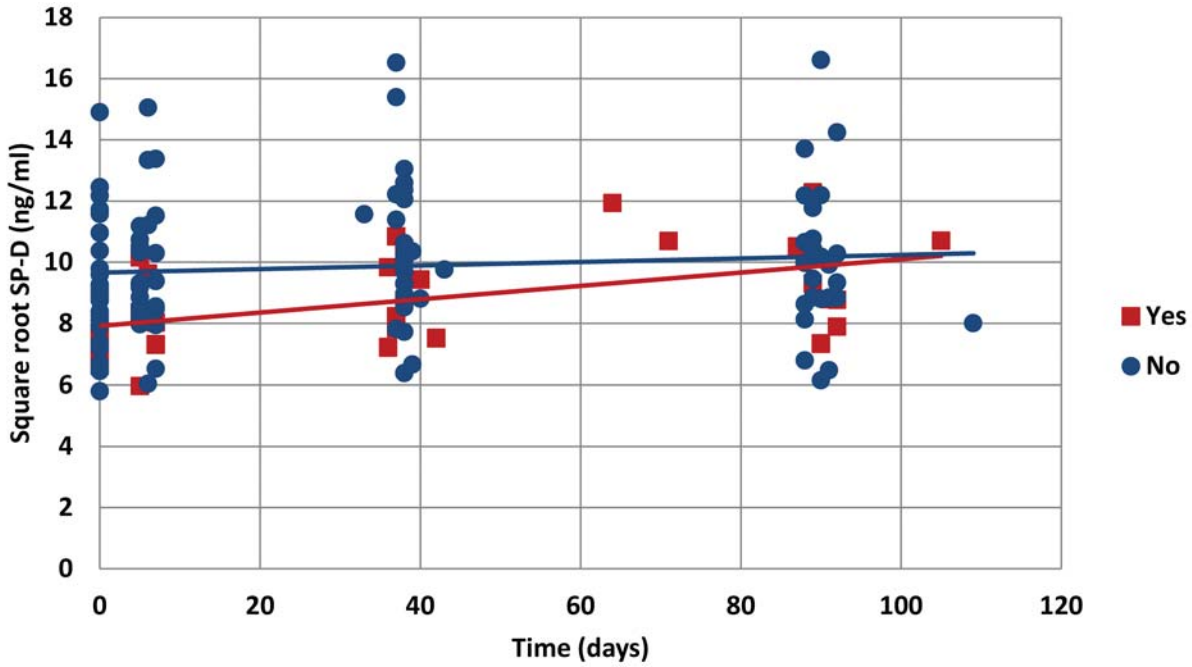

Figure 2. *Linear rate of change in levels of circulating surfactant protein-D (SP-D) during therapeutic management of breast cancer patients with radiation therapy (RT) and trastuzumab, explored with a random co-efficient model using a square root transformation. $p<0.05$.*

antineoplastic agent-induced lung injury may be cell-mediated through activation of lymphocytes and alveolar macrophages. Finally, as epidermal growth factor receptors (EGFR) expressed on type II pneumocytes, are involved in alveolar wall repair mechanisms, EGFR tyrosine kinase inhibitors may potentiate lung injury by impairing the alveolar repair mechanisms (13). Irrespective of the involved mechanisms, therapeutic management of breast cancer seems to be closely-associated with the development of lung inflammation. The aim of the present study was to quantitatively determine the levels of circulating SP-D, CRP and sRAGE in patients with breast cancer under postoperative RT, with or without administration of hormonal therapy or trastuzumab, and to investigate whether the expression of these biomarkers can adequately reflect subclinical pulmonary inflammation.

SP-D. Surfactant protein-D is a glycoprotein, mainly produced by pulmonary alveolar type II cells (17). It is considered an important component of pulmonary surfactant, playing a role in innate immunity and in modulating inflammation in the lung (18, 19). Numerous lung diseases have been associated with elevated circulating SP-D levels (20-25). Moreover, increased SP-D expression has been associated with lung cancer risk (26). Finally, serum SP-D has been suggested as a marker for the early detection of radiation-induced pneumonitis in patients with thoracic malignancies (27, 28).

Our data indicate that SP-D levels were significantly higher in patient with breast cancer prior to the initiation of RT and exhibited a further small but gradual increase after the completion of RT and during follow-up. In agreement with this, previous studies have also shown that SP-D levels increase during RT, even though the effect is more evident in patients who develop radiation-induced pneumonitis (27, 28). In our cohort of patients, the increase in SP-D levels was less pronounced for patients treated with a combination of RT and hormonal therapy. It has been reported that treatment with tamoxifen during post-mastectomy and RT, significantly increases the risk for the development of radiation-induced lung fibrosis (29, 30). The three-month follow-up period of our study limits our ability to observe the development of lung fibrosis, since the latter typically evolves 3-6 months after the completion of RT. However, clinical examination and radiological imaging during the study period have verified that none of our patients experienced RT-induced acute side-effects, which typically progress into fibrosis. Considering that the expression of SP-D is abundant and restricted within the lung, its circulating levels effectively reflect changes in the alveolar compartments and epithelium (18). Thus, we postulate that our observation of a less pronounced increase in SP-D levels in patients under treatment with a combination of radiation and hormonal therapy, is likely to reflect a lower risk of lung toxicity and a protective effect of the combined therapy against pulmonary inflammation.

On the contrary, SP-D levels exhibited a significant increase during the study period in patients treated with a combination of RT and trastuzumab. It has long been recognized that certain antineoplastic agents, including trastuzumab, induce pulmonary toxicity (13). Even though administration of trastuzumab has been associated with a small risk for the development of pneumonitis, its combination with other therapeutic regimens may exert a stronger effect, with a higher frequency of pulmonary toxicity (13). Indeed, adjuvant treatment of patients with breast cancer with chemotherapy and trastuzumab has been associated with the development of several types of pneumonia (31-33). In the absence of clinical and imaging evidence of acute complications, our findings of increasing SP-D levels potentially indicate that the combined administration of RT and trastuzumab in patients with breast cancer is likely to be associated with the development of subclinical pulmonary inflammation.

CRP. C-Reactive protein is a non-specific, acute-phase protein, primarily synthesized in the liver in response to cytokines (34). CRP can be elevated in most forms of tissue damage, infection, inflammation and malignant neoplasia. Elevated CRP levels, reflecting systemic inflammatory response, have been associated with poor survival in several types of cancer (35-39). In breast cancer, CRP may serve as an important predictive and prognostic factor since associations between increased levels and advanced stage, reduced survival and, potentially, breast cancer risk have been observed (40-44).

Our results indicate that levels of CRP were significantly higher in patients with breast cancer prior to initiation of RT and throughout the study period, compared to healthy controls. These findings are in agreement with previous studies reporting elevated CRP during adjuvant breast cancer therapy (45-47). Increased levels of CRP have been also observed during and after RT in patients with various types of cancer (48).

In our study, CRP levels exhibited a significant decrease during the study period for patients treated with hormonal therapy during or after RT. Previous studies have shown that administration of tamoxifen or raloxifene in healthy women is accompanied by significant decreases in circulating CRP levels (49-51). Moreover, patients with breast cancer who received tamoxifen pre-operatively also exhibited a significant dose-dependent decrease in levels of circulating CRP (52). A recent epidemiological analysis from the Health, Eating, Activity and Lifestyle (HEAL) study indicated that tamoxifen administration was associated with reduced CRP levels in a population of 741 patients with breast cancer (53). Finally, according to a study on post-menopausal patients under adjuvant chemoendocrine therapy, CRP levels were significantly increased in patients receiving aromatase inhibitors (AIs) but not in those under tamoxifen, compared to healthy controls (47). Thomson *et al*. reported increased CRP levels in overweight patients with breast cancer under adjuvant hormonal therapy (46). The authors postulate that their observation probably reflects the increasing age and obesity of their patients. However, taking into consideration that 76.2% of the patients received AIs compared to only 23.8% who received tamoxifen, it seems possible that CRP levels were overall increased as an effect of the high percentage of patients under treatment with AIs. Although our data do not allow for the accurate assessment of the differential effect of various hormone therapies on levels of circulating CRP, they provide strong indications that administration of tamoxifen, but not of AIs, is associated with decreased expression of CRP, since the vast majority of our patients (72%) were under tamoxifen treatment.

CRP is a sensitive marker of the systemic inflammatory response, rather than lung inflammation. Moreover, it still remains to be elucidated whether our findings of increased CRP levels in patients with breast cancer are attributable to the disease itself, to the effects of prior and concomitant therapies, or if they reflect an underlying higher baseline inflammatory state in women with breast cancer. Our observation of decreasing CRP levels in patients under RT and hormonal therapy is likely to reflect an anti-inflammatory effect associated with administration of the combined therapy. In support of this, decreases in CRP levels have previously been related to an antiestrogenic effect of tamoxifen on adipocyte cytokine production (50). The potential differential effect of various hormonal therapies for breast cancer on CRP expression remains unclear and is worthy of further investigation.

sRAGE. The receptor for advanced glycation end-products belongs to the immunoglobulin superfamily of cell surface molecules and participates in innate immune function and the inflammatory response (54). RAGE has been implicated in the pathogenesis of many diseases, including inflammatory, vascular and Alzheimer's disease, as well as pulmonary disorders (55-59). *In vivo* research evidence indicates that RAGE plays an important role in cancer, modulating cell proliferation, apoptosis, cell adhesion, migration and invasiveness (60, 61). Many studies on cell lines and malignant tissue specimens support a close association between RAGE expression and invasion and metastasis in various malignancies (62-68). In addition, RAGE has been associated with angiogenesis and has been proposed as an independent prognostic marker in oral squamous cell carcinoma (69, 70).

RAGE synthesis appears to be related to specific tumor types since increased tissue expression has been detected in breast, lung, esophageal, hepatocellular and colorectal cancer, while negative or weak expression has been found in testicular, ovarian and brain carcinomas (71-73). Few studies have so far explored sRAGE expression in human cancer. Jing *et al*. observed decreased tissue and serum RAGE

expression in patients with lung cancer compared to healthy controls, indicating that serum RAGE decreases during lung cancer progression and can effectively reflect the corresponding tissue expression (74). Similarly, decreased serum RAGE levels have been observed in patients with pancreatic cancer (75). Jiao *et al*. recently reported that high pre-diagnostic serum levels of sRAGE are associated with a lower risk of pancreatic and colorectal cancer (76, 77).

In our study, increased sRAGE levels were observed in patients with breast cancer, compared to healthy controls; this finding remained rather unaffected by the administration of adjuvant therapies. In agreement, a recent report on patients with human epidermal growth factor receptor 2 (HER2)-negative invasive breast cancer also found increased tumor tissue expression of RAGE compared to adjacent control samples (78). On the contrary, Tesarova *et al*. reported reduced serum sRAGE levels in patients with breast cancer compared to healthy controls. Nevertheless, higher sRAGE levels were observed in patients with advanced-stage, disease of lower grade, with positive expression of estrogen receptors, intermediate positivity for HER2 and underlying genetic polymorphisms (79). The authors propose that sRAGE is reduced in early breast cancer, and particularly in patients with worse outcome. However, increased levels in more advanced breast cancer may act as a compensatory response to disease progression. Our findings support a role for RAGE in breast cancer, however, it appears that this particular molecule is not associated with pulmonary or systemic complications induced by RT and adjuvant treatments.

In conclusion, we observed significant changes in the levels of circulating SP-D and CRP during postoperative irradiation and adjuvant hormonal therapy in patients with breast cancer, suggesting that this combined therapeutic regiment may exert important anti-inflammatory effects, protecting the lung from injury and inflammation. On the contrary, SP-D levels increased during administration of RT and trastuzumab, indicating that this combination of therapies is likely to induce or provoke pulmonary inflammation. The lack of corresponding changes in CRP levels, in the latter case, could be attributed to the relatively wide distribution of its levels. Pulmonary complications commonly develop during adjuvant breast cancer therapy and their early detection is of uttermost clinical importance. Our findings support the idea that serial monitoring of SP-D, and potentially of CRP, can provide a useful tool for the identification of pulmonary toxicity while it is still in a subclinical phase.

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