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Expression of CCL18 and interleukin-6 in the plasma of breast cancer patients as compared with benign tumor patients and healthy controls

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Abstract

A growing body of laboratory research has shown that pro-inflammatory cytokines can facilitate tumor growth and metastasis. Our goal was to quantify the expression of CCL18 and IL-6 in patients with breast cancer compared with benign breast tumors patients and healthy women, in order to evaluate if these cytokines could serve for breast cancer diagnosis and evaluation. We also correlated the cytokines level of expression with some clinical and pathological characteristics known as prognostic markers for breast cancer. Plasma samples were obtained before treatment from 58 breast cancers, 41 benign breast tumors and 30 healthy women. The quantitative dosage was performed using ELISA. Wilcoxon test was used to compare groups. IL-6 and CCL18 were dramatically upregulated in breast cancers in comparison with healthy controls, but in comparison with benign tumors only CCL18/PARC was overexpressed at borderline significance in cancers (p=0.05). The plasma from benign breast tumor patients exhibited also significant higher levels of the two cytokines than normal controls. The cytokines profile was not linked to patient age, tumor size, histopathological type, lymph node status or histological grade. IL-6 was significantly upregulated in ER-positive and metastasized cancers. CCL18/PARC presented a significantly higher expression in advanced stage and highly proliferative carcinomas. In summary, IL-6 and CCL18 could clearly distinguish between women with breast cancers and healthy controls. High expression of IL-6 seems to confer a poor prognosis for ER-positive cancers. CCL18 was associated with worse prognosis parameters like high Ki67.

Keywords: breast cancer, inflammation, CCL18, IL-6, cytokines.

Introduction

The incidence of breast cancer is increasing almost everywhere. This unfavorable trend is due in part to increases in risk factors (decreased childbearing and breast-feeding, increased exogenous hormone exposure, and detrimental dietary and lifestyle changes, including obesity and less physical activity). Incidence rates for breast cancer vary from 19.3 per 100 000 women in Eastern Africa to 89.7 per 100 000 women in Western Europe, and are high (greater than 80 per 100 000) in developed regions of the world (except Japan) and low (less than 40 per 100 000) in most of the developing regions [1].

On the other hand, mortality is now decreasing in many high-risk countries due to a combination of intensified early detection efforts and the introduction of mammographic screening, resulting in the diagnosis of more small, early stage tumors, and advances in treatment. Although mammogram screening has allowed an undisputable benefit, it has several limitations, for example, it is associated with a significant rate of false-positive results, leading to unnecessary biopsies or surgery [2] or this technology mainly detects slowly proliferating tumors that occur after the age of 50 years and exhibits poor performance to screen aggressive tumors, especially in young women in whom breast density is high. Ultrasonography, combined with a mammogram, also increases the likelihood of breast cancer detection, but it is once again associated with high rate of false positivity [3].

Until now, no serum biomarker has been shown to allow an early diagnosis of breast cancer. Consequently, current ASCO guidelines do not recommend the use of serum biomarker (CA15-3, CEA) for breast cancer screening [4]. These data point out the need to develop new easy-to-do, blood-based tests that would improve breast cancer detection and treatment response surveillance.

A growing body of laboratory research has shown that pro-inflammatory cytokines can facilitate tumor growth and metastasis by altering tumor cell biology and activating stromal cells in the tumor micro-environment, such as vascular endothelial cells, tumor-associated macrophages, and fibroblasts [5–7]. Systemic inflammation may also condition the vasculature in ways that enhance the extravasation, engraftment, and growth of micrometastases or reactivate dormant tumors at distant sites [8–11]. Under Paget's analogy, chronic inflammation may fertilize the soil of systemic tissue

in ways that promote dissemination and growth of metastatic seeds [12].

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by a variety of cell types, including endothelial cells and normal hematopoietic cells [13, 14]. IL-6 is a potent pleiotropic inflammatory cytokine that is considered a key growth-promoting and antiapoptotic factor [15]. The major source of IL-6 within breast tumors is represented by breast tumor-derived fibroblasts, macrophages and tumor infiltrating lymphocytes [16, 17]. However, the nature of IL-6's involvement in cancer has been quite controversial, as dichotomous roles for IL-6 in both tumor-promoting and -suppressive activities have been reported. For example, IL-6 signaling has been linked to both pro- and anti-apoptotic activity in breast cancer cells [18, 19]. Multiple studies have documented high IL-6 levels in the serum of patients with certain carcinomas (i.e., breast, lung, lymphoma) and have correlated high IL-6 levels with a poor clinical prognosis [20]. These data imply an oncogenic role for IL-6; however, lacking is an understanding of the mechanisms governing IL-6 production in tumors and the biological role of this cytokine in tumorigenesis. Some reports provide a molecular rationale for the development of anti-IL-6 therapeutics [21, 22].

PARC (pulmonary and activation-regulated chemokine)/CCL18 (chemokine C–C motif ligand 18) is referred to also as AMAC-1 (alternative activated macrophage associated CC-chemokine), MIP-4 (macrophage inflammatory protein-4), or DC-CK1 (dendritic cell-derived chemokine-1). PARC is chemotactic for both activated CD3(+) T-cells and non-activated CD14 (-) lymphocytes, but not for monocytes or granulocytes [23]. CCL18 is a chemokine predominantly produced by monocyte-derived cells with M2 phenotype [23]. Excessive production of CCL18 in M2 macrophages was demonstrated in various chronic inflammations and fibrotic diseases, including Gaucher's disease and rheumatoid arthritis [24, 25]. In addition, constitutive expression of CCL18 was observed in macrophages infiltrating ovarian cancer, gastric cancer, and glioma [26–28]. However, the role of CCL18 in cancer progression is controversial. CCL18 was reported to participate in immunosuppression of ovarian cancer [28, 29] but was associated with prolonged survival in patients with gastric cancer [27]. Although M2 macrophages are abundant in breast cancer stroma, the role of CCL18 in breast tumor progression remains elusive.

The goal of our study was to quantify the expression of CCL18 and IL-6 in patients with breast cancer, in order to evaluate if these cytokines could serve for breast cancer diagnosis, evaluation, and eventually, if they could represent potential targets for breast cancer treatment. In this regard, we performed quantitative dosage of these selected cytokines in the plasma of breast cancer patients before treatment, compared with both benign breast tumors patients and healthy volunteer women. We also correlated the cytokines level of expression with some clinical and pathological characteristics known as prognostic markers for breast cancer.

Patients and Methods

Patients and tumor characteristics

We evaluated 58 specimens of breast cancers and 42 benign breast tumors from patients who underwent surgery at the University Clinic of Surgical Oncology Timişoara, during 2009–2010. We collected also plasma from 30 healthy volunteer women matched by age with the patients, not pregnant and with no history of cancer. The normal volunteers presented neither fever, nor chronic infections disease history. Informed consent was obtained from all the patients before surgery and from healthy controls, and the study was approved by the Ethical Committee of our University. Table 1 summarizes the characteristics of breast cancer patients included in our study.

Table 1 – ***Characteristics of breast cancer patients included in the study***

Characteristic	Breast cancers n=58 (100%)	
	n	***Percent***
Age (range between 35–90, Mean: 60.42, Median: 60) [years]		
≤50	10	16.67
>50	48	83.33
Tumor size [cm]		
<5	39	66.67
≥5	19	33.33
Nodal status		
Positive	34	57.84
Negative	24	42.16
Histology		
Invasive ductal	42	71.57
*Other types**	16	28.43
Histological grade (G)		
G1	4	5.88
G2–G3	54	94.12
Stage		
Early (I, II, IIIA)	39	66.67
Advanced (IIIB, IV)	19	33.33
Estrogen receptor status		
Positive	45	77.45
Negative	13	22.55
Progesterone receptor status		
Positive	35	59.80
Negative	23	40.20
HER2/neu status		
Negative (0, +1)	51	88.24
Positive (+2, +3)	7	11.76
Ki67 [%]		
<20	23	38.24
≥20	35	61.76

* Mixed (ductal and lobular) (10), Lobular (4), Atypical medullary (2).

Sample preparation

For the assessment of cytokines concentration in the blood, plasma samples were obtained from 58 breast cancer patients, from 41 patients with benign breast tumors before any treatment and from 30 healthy volunteer women. Peripheral venous blood (3 mL) was collected using EDTA as anticoagulant between the hours of 8 and 9 a.m. Within 30 minutes after

collection, samples were centrifuged for 5 minutes at 3500×g; plasma was immediately separated, aliquoted and stored in a -80^{0}C freezer until further analyses. Freeze-thaw cycles were avoided.

Assessment of the concentrations of the selected cytokines in the plasma of breast cancer patients' *vs.* benign tumor patients and healthy volunteers

We used the sensitive and specific ELISA (Enzyme-Linked Immunosorbent Assay) method to assess the levels of IL-6/PARC (Raybiotech, Inc, USA) in the plasma samples. The RayBio® Human PARC or IL-6 ELISA kits are *in vitro* enzyme-linked immunosorbent assays for the quantitative measurement of human PARC or IL-6 in serum, plasma, cell culture supernatants and urine. These assays employ human specific antibodies coated on a 96-well plate. The analyses were performed by following exactly the manufacturer protocol. Briefly, standards and samples (100 µL each) were pipetted into the wells and then the plates were incubated overnight at -4^{0}C. PARC/IL-6 antigens present in the sample were bound to the wells by the immobilized antibodies. The wells were washed and biotinylated antihuman antibodies (100 µL) were added. After washing away unbound biotinylated antibodies, HRP-conjugated Streptavidin (100 µL) was pipetted into the wells. The wells were washed again; a substrate solution (100 µL) was added and the color developed was relative to the amount of primary antibody bounded. The Stop Solution added (50 µL) changed the color from blue to yellow, and the intensity of the color was measured at 450 nm within 30 minutes using an automatic ELISA microplate reader Stat Fax 2100 (Awareness Technology Inc, Palm City, USA). The sensitivity of the assays reported by the manufacturer is less than 2 pg/mL, with an intra-assay and inter-assay reproducibility of CV<10%, respectively CV<12%.

Statistical analysis

Descriptive statistics, including median, mean and standard deviation were computed for each of the cytokines using SPSS software version 15.0 (Chicago, IL, USA). Because the cytokine values were skewed and had large standard deviations relative to their means, the two-sample, rank sum Wilcoxon (Mann–Whitney) test was used to determine differences in the median values and to compare groups. The threshold for significance was set at $p<0.05$.

Results

Cytokines plasma concentration in cancers *vs.* healthy controls and benign tumors

In order to evaluate the expression of the two selected cytokines, we analyzed plasma samples from a total of 58 primary breast carcinoma, 41 benign breast tumor patients and 30 healthy women. All measurements were performed in duplicate.

Each investigated cytokine was dramatically up-regulated in breast cancers in comparison with the healthy controls, with $p=0.02$ for IL-6 and respectively, $p<0.0001$ for CCL18/PARC (Figures 1 and 2). For cancers, IL-6 was ranged between 0–112.7, with a median of 11.2 pg/mL and respectively, for healthy controls, IL-6 was ranged between 0–54.6, with a median of 5.1 pg/mL. CCL18/PARC was ranged 2.1–332.9, with a median of 34.9 ng/mL for cancers, respectively, for healthy controls, CCL18/PARC was ranged between 9.6–35.1, with a median of 17.4 ng/mL.

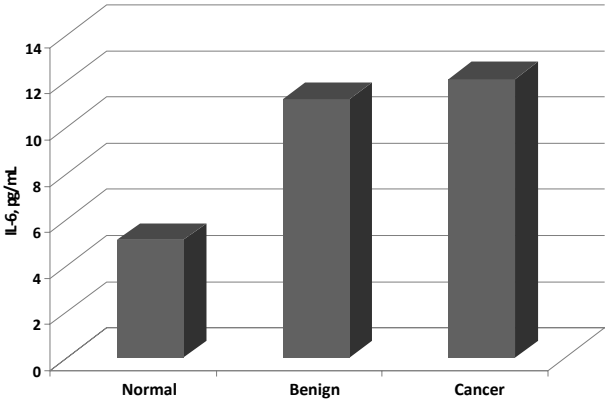

Figure 1 – ***Expression of interleukin-6 in breast cancers vs. benign tumors and healthy controls.***

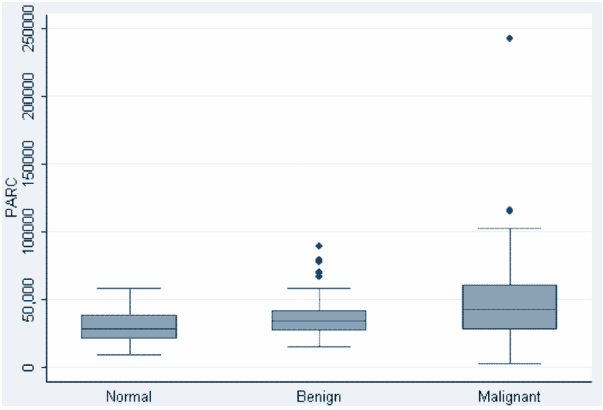

Figure 2 – ***Expression of CCL18/PARC in breast cancers vs. benign tumors and healthy controls.***

The plasma from benign breast tumor patients also exhibited higher levels of the two cytokines than normal plasma; the differences between benign tumors and controls were statistically significant, with $p=0.02$ (range for benign tumors between 0–57.8, median 12.1 pg/mL) for IL-6 and respectively $p=0.0008$ (range for benign tumors between 19.4–705.8, median 587.5 pg/mL) for CCL18/PARC.

When we compared cancers with benign tumors, we observed that the only CCL18/PARC was upregulated at borderline significance in cancers compared with benign tumors ($p=0.05$). Regarding IL-6, we did not obtain a statistically significant difference when we compared benign with malignant breast tumors (Figures 1 and 2).

Correlations between cytokines and the clinicopathological characteristics

A percent of 23%, 40% and 12% of the breast cancer patients were ER-negative, PR-negative, and HER2neu positive (2+ and 3+), respectively. We evaluated whether there was any correlation between the two cytokines

levels and the expression of ER, PR and HER2/neu. We observed that only IL-6 was significantly overexpressed in ER-positive tumors (p=0.03) as compared with ER-negative ones (Figure 3).

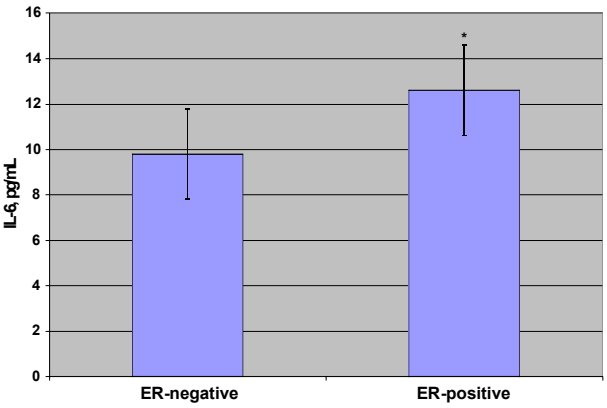

Figure 3 – ***Expression of IL-6 in ER-positive vs. ER-negative cancers.***

We observed no statistically significant correlations between IL-6 and PR or HER2/neu expression, although IL-6 was higher expressed in PR-negative and HER2/neu positive cancers. No significant correlations were observed between CCL18 expression and the expressions of ER, PR and HER2 (Table 2).

Table 2 – ***Correlations between IL-6 and respectively CCL18/PARC and the clinicopathological parameters of breast cancers***

Parameter		IL-6 median (range) [pg/mL]	p	CCL18 median (range) [ng/mL]	p
ER	*Neg*	9.79 (0–112.7)	0.03	35.3 (25.8–35.8)	NS
	Pos	12.59 (0–123.7)		33.9 (2.1–33.2)	
PR	*Neg*	13.5 (0–12.3)	NS	33.8 (2.1–35.8)	NS
	Pos	7.9 (0–112.7)		35.2 (11.6–33.3)	
HER2	*Neg*	9.7 (0–112.7)	NS	35.1 (2.1–55.8)	NS
	Pos	21.4 (13.5–23.8)		30.6 (25.6–33.3)	
Ki67 [%]	*Low ≤20*	7.9 (0–12.7)	NS	32.3 (2.1–33.3)	0.04
	High >20	18.4 (0–49.2)		35.4 (23.5–35.8)	
G	*G1*	6.1 (0–38.2)	NS	34.4 (2.1–33.3)	NS
	G2–G3	13.5 (0–112.7)		35.3 (28.2–35.7)	
N	*Neg*	7.9 (0–112.7)	NS	34.1 (2.1–55.8)	NS
	Pos	14.7 (0–49.2)		35.3 (24.3–333.9)	
T	*<5*	7.9 (0–112.75)	NS	35.1 (24.3–33.3)	NS
	≥5	15.2 (0–123.7)		34.1 (2.1–35.8)	
Stage	*Early (0–II)*	11.2 (0–112.7)	NS	33.8 (2.1–35.8)	0.03
	Advanced (III–IV)	13.6 (0–123.7)		35.2 (24.3–55.8)	
Metastases	*Neg*	11.2 (0–112.6)	0.02	34.8 (11.6–33.2)	NS
	Pos	198.1 (5.6–390.6)		35.3 (2.2–35.5)	
Histological type	*Ductal*	13.5 (0–86.7)	NS	34.9 (2.3–55.8)	NS
	Other	9.7 (0–59.2)		35.3 (23.2–35.8)	
Age [years]	*≤50*	6.1 (0–12.4)	NS	13.3 (0–49.2)	NS
	>50	13.5 (0–112.7)		9.8 (0–112.7)	

Neg – negative; Pos – positive; NS – not significant.

We further evaluated whether chemokine expression is linked to other clinicopathological parameters such as: tumor size, histopathological type, lymph node metastasis status, stage, histological grade (G), proliferation (Ki67) or the patients age.

The cytokine profiles were not linked to patient age, tumor size, histopathological type, lymph node status or histological grade (Table 2). We noticed that IL-6 was significantly upregulated in metastasized tumors (p=0.02) (Figure 4) but not further significant correlations with these investigated parameters were obtained. Regarding CCL18/PARC, we observed a statistically significant correlation with the proliferation rate as determined by Ki67 (p=0.04) (Figure 5) and a significantly higher expression of CCL18 in advanced stage carcinomas (p=0.03) (Figure 6).

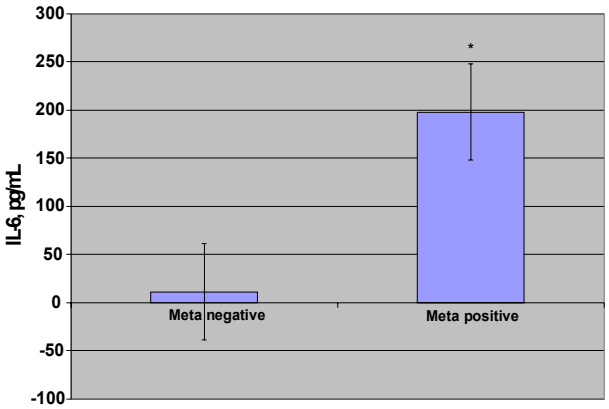

Figure 4 – ***Overexpression of IL-6 in metastasized breast cancers.***

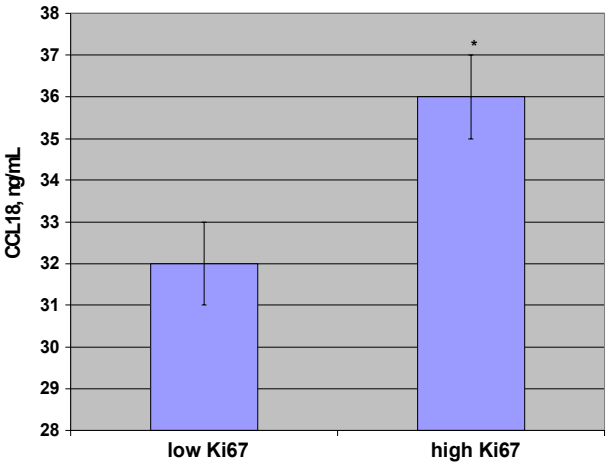

Figure 5 – ***Overexpression of CCL18 in highly proliferative (Ki67≥20) breast cancers.***

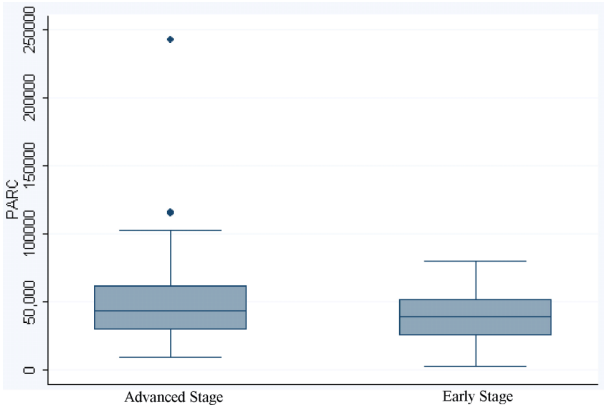

Figure 6 – ***Overexpression of CCL18 in advanced stage breast cancers.***

Discussion

Cancer is a hyperproliferative disorder that involves morphological cellular transformation, dysregulation of apoptosis, uncontrolled cellular proliferation, invasion, angiogenesis, and metastasis [30]. Clinical and epidemiologic studies have suggested a strong association between chronic infection, inflammation, and cancer [31, 32].

In a multisite study of 734 women treated successfully for early stage breast cancer, high levels of circulating acute phase proteins approximately three years after treatment were associated with a two-fold elevation in the risk of subsequent disease recurrence and mortality. Risk ratios were similar across primary tumor types (including stage and estrogen-receptor and progesterone-receptor status) and independent of potential confounders such as age, estrogen level, and adiposity [33]. These results are consistent with previous studies linking circulating inflammatory markers to progression of metastatic breast cancer [34, 35]. The findings of Pierce BL *et al.* [33] suggest that serum inflammatory markers might provide early information about disease recurrence risk in patients with no history of metastatic disease and no current evidence of cancer and could provide a new strategy for assessing the risk of breast cancer recurrence. Much research has suggested that the prognostic value of circulating acute phase proteins stems from their role as stable markers of cumulative exposure to pro-inflammatory cytokines, principally interleukin-6 (IL-6) [21, 36, 37].

IL-6 is a multifunctional cytokine that was originally characterized as a regulator of immune and inflammatory responses; however, elevated expression of IL-6 has been detected in multiple epithelial tumors [20]. High IL-6 levels have been linked to a poor prognosis in patients with advanced breast cancer, the pathogenesis of inflammatory breast cancer, and resistance to chemotherapy [38]. Cytokines, such as IL-6 and tumor necrosis factor (TNF)-α, have an important role in regulating estrogen synthesis in peripheral tissues, including normal and malignant breast tissues. The activities of the aromatase, estradiol 17β-hydroxy-steroid dehydrogenase and estrone sulfatase are all increased by IL-6 and TNF-α [17]. Tamoxifen reduces APP levels [17, 22] raising the possibility that some protective effects of endocrine therapy might stem from their anti-inflammatory actions. In accordance with previous studies [38, 39], in the present study, IL-6 was significantly upregulated in breast cancers as compared with normal controls, but also in ER-positive and metastasized cancers. It was suggested that high levels of the adrenal androgen, DHEA are associated with a Th1 response while a predominantly glucocorticoid environment promotes a Th2-type response [40, 41]. Production of DHEA starts to decline at about the age of 25 years and thus in older subjects there is a switch from a Th1- to Th2-type of environment [40]. Although IL-6 is secreted by Th2 cells, in our study we did not notice any correlations between these cytokine levels and the age of the patients.

Through the analysis of cytokine profile of breast tumor-associated macrophages, the study of Chen J *et al.* [42] shows that CCL18 is abundantly expressed and promotes migration and invasion of breast cancer cells by triggering integrin clustering and enhancing their adherence to extracellular matrix; the CCL18 level in blood or cancer stroma was found to be associated with metastasis of patients with breast cancer; PITPNM3 was identified as a functional receptor for CCL18 that activates intracellular calcium signaling [42]. In accordance with this paper, in our work, CCL18/PARC was significantly more abundant in advanced stage carcinomas and high proliferative tumors determined by Ki67, but there was no difference according to estrogen receptor status and it was unrelated to the tumor size, data that imply that CCL8 correlates with the invasiveness but not the growth kinetics of breast tumor cells.

Conclusions

Both investigated cytokines (IL-6 and CCL18) were dramatically upregulated in breast cancers in comparison with the healthy controls, but when we compared cancers with benign tumors, we observed that only CCL18/PARC was upregulated at borderline significance in cancers compared with benign tumors (p=0.05). Regarding IL-6, we did not obtain a statistically significant difference when we compared benign with malignant breast tumors. The plasma from benign breast tumor patients exhibited also statistically significant higher levels of the two cytokines than normal controls. The cytokines profile was not linked to patient age, tumor size, histopathological type, lymph node status or histological grade. IL-6 was significantly upregulated in ER-positive and metastasized cancers whereas for CCL18/PARC, we observed a significantly higher expression in advanced stage and highly proliferative carcinomas. In summary, these both cytokines are deregulated in breast cancer and could clearly distinguish between women with breast cancers and healthy controls. The high expression of IL-6 seems to confer a poor prognosis for ER-positive cancers and CCL18 was associated in our study with worse prognosis parameters like high Ki67.

Acknowledgments

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