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AVALIAÇÃO DE BIOMARCADORES INFLAMATÓRIOS MOLECULARES EM PACIENTES COM CÂNCER DE MAMA

Tese apresentada ao Programa de Pós-Graduação em Ginecologia, Obstetrícia e Mastologia da Faculdade de Medicina de Botucatu-UNESP, para obtenção do título de Doutora.

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1. Mama - Câncer - Tratamento. 2. Marcadores biológicos de tumor. 3. Citocinas. 4. Citometria de fluxo.

Palavras-chave: Biomarcadores; Câncer de mama; Citocinas; Citometria de fluxo; Micropartículas.

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Sumário

Lista de Abreviaturas e Siglas

°C	grau Celsius
BC	*Breast Cancer*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CBA	*Cytometric Bead Array*
CCL	*(C-C motif) ligand*
CD	*Cluster of differentiation*
CEP	Comitê de Ética em Pesquisa
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CT	*Chemotherapy*
CXCL	*(C-X-C motif) ligand*
DNA	ácido desoxirribonucléico
EDTA	ácido etileno-diamino tetracético
ER	*Estrogen Receptor*
FIOCRUZ	Fundação Oswaldo Cruz
FITC	*fluorescei isothiocyanate*
FL	*Fluorescence*
FSC	*Forward Scatter*
g	unidade de gravidade
HER-2	*Human epidermal growth factor receptor 2*
IFN	Interferon
IL	Interleucina
MAb	*Monoclonal antibody*
min	*minute*
mL	Microlitro
MPs	Micropartículas
mRNA	*RNA mensageiro*
ng	nanograma
p53	proteína 53
PBS	Tampão Fosfato Salino
PE	*Phycoerythrin*

PerCP	*Peridin Chlorophyll Protein*
pg	picograma
Post-CT	*Post-chemotherapy*
PR	*Progesterone Receptor*
Pre-CT	Pre-chemotherapy
PROs	Produtos Reatores do Oxigênio
RNA	ácido ribonucléico
SSC	*Side scatter*
TCLE	Termo de concentimento livre e esclarecido
TNF	Fator de Necrose Tumoral
µm	Micrometro
χ^2	*chi-squared*

Resumo

Resumo:

A alta prevalência do câncer de mama é um fator que nos instiga a investigar novos biomarcadores para a doença. Proteínas séricas ou plasmáticas já são utilizadas rotineiramente no rastreamento de algumas neoplasias. As micropartículas (MPs) são fragmentos da membrana plasmática liberadas por diversos tipos celulares e estão associadas com a resposta inflamatória. Estudos recentes mostram que a presença de MPs e citocinas/quimiocinas circulantes possuem uma relevante associação clínica com o câncer de mama. O objetivo deste estudo foi medir os níveis desses biomarcadores inflamatórios (MPs, citocinas e quimiocinas) no soro de mulheres com câncer de mama pré e pós-quimioterapia comparando com o grupo controle; assim como associar esses dados com diversos parâmetros clínicos e hemograma. Foi coletado sangue periférico de mulheres sem evidências de doenças (n=20) e com câncer de mama (n=38). Foi utilizada a citometria de fluxo para dosagens dos níveis séricos de citocinas (IL-1, IL-2, IL-4, IL-6 IL-10, IL-12, IL- 17A, TNF, IFN-gama), quimiocinas (CXCL-8, CXCL-9, CXCL-10, CCL-2, CCL-5) e micropartículas provenientes de diversas células (neutrófilos, leucócitos, monócitos, eritrócitos, endotélio, plaquetas, linfócitos). As diferenças entre os grupos foram avaliadas pelo teste de Mann-Whitney ou Kruskal- Walis. As diferenças com valor de $p<0,05$ foram consideradas significativas. Não houveram diferenças significativas nos níveis de micropartículas, citocinas e quimiocinas estudadas entre os grupos controle e câncer de mama. Entretanto houve uma diminuição dos níveis de micropartículas derivadas de plaquetas nas pacientes pós-quimioterapia e um aumento de níveis séricos de IL-6, CCL-5 e CXCL-10 pós-quimioterapia. Em associação com os dados clínicos foi demostrado que baixos níveis de micropartículas derivadas de monócitos e altos níveis de CCL-2 e CXCL10 estão associados a tumores mais avançados e metástase. Com esses dados podemos concluir que o câncer de mama possui uma resposta inflamatória mais localizada e induz a uma modulação da resposta imune sistêmica e que o tratamento quimioterápico é capaz de alterar essa configuração.

Palavras-chave: Câncer de mama; Biomarcadores, Micropartículas (MPs), Citocinas, Quimiocinas, Citometria de fluxo e Quimioterapia.

Summary

1. Introdução

1. INTRODUÇÃO

1.1. Câncer de Mama

O câncer de mama é o tipo mais comum entre as mulheres e o segundo tipo de câncer mais frequente no mundo. A cada ano, cerca de 20% a 29% dos casos novos de câncer em mulheres são de mama [1]. São esperados para o Brasil em 2014 57.120 novos casos desta patologia [2].

Os fatores de risco mais importantes para o câncer de mama estão relacionados a eventos hormonais e reprodutivos, excetuando-se a presença de câncer de mama em um parente de primeiro grau. São considerados fatores de risco para o câncer de mama características ou comportamentos que resultam em exposição prolongada a estrógenos, tais como menarca precoce, nuliparidade, idade da primeira gestação a termo acima dos 30 anos, anticoncepcionais orais, terapia de reposição hormonal e menopausa tardia [2, 3]. Mulheres que apresentam mutação nos genes BRCA1, BRCA2 e p53 possuem risco aumentado de desenvolvimento dessa doença, mostrando que fatores genéticos também possuem uma grande associação a um maior risco de desenvolvimento do câncer de mama [2].

Apesar do bom prognóstico se diagnosticado e tratado oportunamente, as taxas de mortalidade por câncer de mama continuam elevadas no Brasil, provavelmente devido ao diagnóstico tardio em estadios avançados da doença [2]. Em países desenvolvidos a sobrevida média após cinco anos de

diagnóstico e tratamento é de 85%, enquanto em países em desenvolvimento está em torno de 60% [2]. A diferença de sobrevida nesses países está provavelmente relacionada ao diagnóstico precoce e maior acesso ao tratamento [4].

A quimioterapia neoadjuvante em câncer de mama é utilizada com a finalidade de reduzir o volume tumoral e levar a uma cirurgia futura menos agressiva [5]. A regressão da doença leva a uma cirurgia mais conservadora e a maiores taxas de cura e sobrevida [6, 7].

A alta prevalência do câncer de mama é um fator que nos instiga a investigar novos biomarcadores para um melhor rastreamento, diagnóstico e posterior prognóstico da doença. Proteínas séricas ou plasmáticas já são utilizadas rotineiramente no rastreamento de algumas neoplasias, como no câncer de próstata e ovário e algumas biomoléculas séricas já vem sendo estudadas quanto à sua eficácia na detecção do câncer de mama [8].

1.2. Micropartículas e Câncer

As micropartículas (MPs) são fragmentos da membrana plasmática de algumas células, formadas por diversos tipos celulares em um processo de vesiculação. Essas são definidas principalmente pelo seu tamanho (menor que 1 micrômetro) e pela presença de fosfatidilserina na sua superfície externa [9].

As MPs possuem na sua constituição as proteínas de membrana da sua célula de origem [10]. Pela presença dessas proteínas é possível identificar a origem das MPs. As mais comuns e numerosas são derivadas de plaquetas [11], mas estas podem ser originadas de qualquer tipo celular como eritrócitos, monócitos, neutrófilos, linfócitos células endoteliais e até mesmo células cancerosas [12-15]

MPs carregam no seu interior uma vasta gama de biomoléculas, tais como: quimiocinas, citocinas, enzimas, factores de crescimento, proteínas de sinalização, lipídios e ácidos nucleicos (microRNAs, mRNAs, e até mesmo DNAs) [15, 16]. MPs estão presentes no sangue periférico de indivíduos saudáveis e podem ser formadas em condições fisiológicas ou quando a homeostase do tecido é perturbada. [17]. Tem sido observado um aumento significativo dessas moléculas em condições patológicas, tais como: doenças autoimunes, diabetes, doenças cardiovasculares, doenças renais, doenças inflamatórias, doenças infecciosas e diversos tipos de câncers [15, 17]. Investigações recentes apontaram uma possível relevância clínica na presença de MPs séricas em diferentes tipos de doenças malignas, incluindo o câncer de mama. No entanto, para utilização dessas moléculas como biomarcadores tumorais ainda são necessários maiores estudos [18].

As MPs têm um importante papel na inflamação, coagulação e homeostase vascular, possuindo várias funções fisiológicas, incluindo o transporte de componentes da membrana da sua célula de origem para outras células, ativando direta ou indiretamente a inflamação e a coagulação. [17,

19]. As micropartículas possuem um papel central na iniciação da coagulação e formação de trombos [20]. A exposição celular a citocinas ou quimioterapia resulta na secreção dessas MPs [21].

O tratamento quimioterápico leva a uma ativação de plaquetas e consequente geração de micropartículas [22]. MPs de pacientes com câncer de mama estão relacionadas a um maior risco de trombose, invasão tumoral e angiogênese e o tratamento quimioterápico perturba o equilíbrio hemostático dessas MPs [22, 23]. A resposta à quimioterapia é heterogênea e dinâmica, envolve uma combinação de mecanismos moleculares independentes que são regulados durante a progressão e tratamento de tumores [24]. Uma melhor compreensão da ação de mediadores moleculares inflamatórios na resposta ao tratamento quimioterápico ajuda a compreender a variabilidade terapêutica observada em oncologia clínica [25].

Recentemente, tem sido sugerida uma relevância clínica para as MPs circulantes em diferentes tipos de doenças malignas, incluindo o câncer de mama [18]. Estudos mostram que a presença de micropartículas está relacionada com câncer de mama avançado, invasão tumoral e metástase [15, 26, 27]. Dada à natureza sistêmica das MPs, estas poderiam ser usadas como fatores de diagnóstico, prognóstico e predição de resposta terapeutica podendo contribuir para estratégias de tratamento individualizado em pacientes com câncer[25].

1.3. Inflamação e Câncer

Observada no século XIX por Rudolf Virchow, a presença de leucócitos ao redor de tumores foi a primeira sinalização de uma possível conexão entre inflamação e câncer. Na última década, foram obtidas evidências elucidando o papel crítico da inflamação no processo de carcinogênese [28, 29].

A inflamação exerce impacto em várias etapas da carcinogênese, desde a iniciação tumoral até a instalação de doença metastática. Diversas evidências conectam câncer e inflamação: doenças inflamatórias crônicas estão associadas a risco aumentado de câncer; células e moléculas inflamatórias estão presentes no microambiente tumoral; ausência de mediadores inflamatórios inibe a progressão tumoral e metástase; e uso prolongado de anti-inflamatórios reduz o risco de mortalidade por câncer [30].

O conceito de que apenas mutações são necessárias para o desenvolvimento de um tumor é incompleto, células do sistema imune inato e adaptativo também são necessárias para os tumores adquirirem características teciduais malignas. Células do sistema imunológico afetam células malignas por meio de produção de citocinas, quimiocinas, fatores de crescimento, prostaglandinas, PROs e nitrogênio [31, 32].

O microambiente tumoral contém células do sistema imune associadas a células neoplásicas. Estas células diversas comunicam-se por meio da produção de citocinas ou quimiocinas, que são capazes de controlar e moldar o crescimento tumoral. A interação dessas células e moléculas variadas no microambiente tumoral define a via predominante – a via promotora ou a

imunidade anti-tumoral. Em tumores estabelecidos, a via dominante é a inflamação pró-tumoral [28].

Portanto, citocinas e quimiocinas possuem um papel bem estabelecido na resposta imune ao câncer e carcinogênese. Estas moléculas estão envolvidas com a iniciação tumoral, progressão e metástase.[33]. O câncer de mama é considerado como sendo fracamente imunogênico e pouco reconhecido pelo sistema imune. Acredita-se que a resposta imunológica a esse tipo de tumor está associada à ação de citocinas moduladoras presentes no microambiente tumoral [34, 35].

Estudos recentes sugerem que o estabelecimento de um perfil de resposta imunológica e inflamatória no câncer de mama pode fornecer informações úteis para o prognóstico e tratamento da paciente [25]. Em pacientes com câncer de mama, a expressão de níveis séricos de várias citocinas parece estar associada a um subgrupo de alto risco de pacientes com menores taxas de sobrevida em comparação com os pacientes que apresentam baixos níveis de citocinas, podendo então ser utilizadas como biomarcadores de fator prognóstico da doença [36, 37].

1.4. Justificativa

Medir a concentração sérica de micropartículas, citocinas e quimiocinas é uma maneira pouco invasiva e indireta de se avaliar a atividade tumoral e sua interação com a microcirculação e resposta inflamatória em pacientes com câncer de mama. Evidências mostram que esses biomarcadores estão associados com uma progressão e metástase no câncer de mama e poucos estudos mostram esse perfil de resposta inflamatória em pacientes pré e pós-quimioterapia. O estudo desses marcadores da inflamação pode propiciar maior compreendimento sobre o comportamento biológico do câncer de mama, assim como sinalizar para futuros marcadores de atividade de doença e resposta ao tratamento.

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2. Objetivos

2. OBJETIVOS

2.1. Objetivo Geral

- Avaliar os níveis de micropartículas e citocinas/quimiocinas séricas em pacientes com câncer de mama.

2.2. Objetivos Específicos

- Comparar os níveis séricos de micropartículas e citocinas/quimiocinas entre mulheres com câncer de mama e mulheres saudáveis (grupo controle).
- Avaliar a associação dos níveis séricos de micropartículas e citocinas/quimiocinas com fatores prognósticos.
- Avaliar a associação dos níveis séricos de micropartículas com o hemograma.
- Avaliar o comportamento das micropartículas e citocinas/ quimiocinas séricas em mulheres com câncer de mama submetidas à quimioterapia.

3. Artigo D

Evaluation of plasma levels of microparticles and cytokines/chemokines in women with breast cancer compared to healthy women

Artigo I

Evaluation of plasma levels of microparticles and cytokines/chemokines in women with breast cancer compared to healthy women

Article Type: Original article

Keywords: breast cancer, microparticles, cytokines, chemokines, network.

Abstract:

The high prevalence of breast cancer is a factor that stimulates the investigation of new biomarkers for the disease. The microparticles (MPs) are released by many cell types and are associated with the inflammatory response. Recent studies show that the presence of circulating MPs and cytokines/chemokines have a relevant clinical association with breast cancer. The aim of this study was to compare the diferent levels of these inflammatory biomarkers (MPs, cytokines and chemokines) in the serum of women with breast cancer with the control group, and to correlate these data with various prognostic factors and hemogram. Peripheral blood of women with no evidence of disease (n = 20) and breast cancer (n = 38) was collected. Flow cytometry was used for determination of serum levels of cytokines (IL-1 , IL-2 , IL-4 , IL-6, IL-10 , IL-12 , IL -17A , TNF , IFN –gamma), chemokines (CXCL8 , CXCL -9 , CXCL 10 , CCL-2, CCL-5) and microparticles from different cells (neutrophils , leukocytes, monocytes, erythrocytes, endothelium , platelets, lymphocytes). A hierarchical network was developed to simulate the interaction between the serum immune microenvironment in healthy and disease. Differences between groups were evaluated by Mann- Whitney or Kruskal-Wallis test. Differences with $p < 0.05$ were considered significant. There were no significant differences in the levels of microparticles, cytokines and chemokines studied between control and breast cancer groups. Hemogram had no association with the levels of circulating microparticles. In association with the prognostic factors data has shown that lower levels of monocyte-derived microparticles and higher levels of CCL -2 and CXCL10 are associated with advanced disease and metastasis.The networks showed presence of more nodes and stronger edges on the breast cancer group compared with the control group as well as in the advanced disease compared with early disease. In conclusion, our data suggests no differences in the levels of MPs and inflammatory cytokines comparing breast cancer patients and healthy women, but breast cancer seems to have a more localized inflammatory response and induces a modulation of the immune system response. The network findings reinforce the importance of the study of biomarkers in association rather individually.

Keywords: breast cancer, microparticles, cytokines, chemokines, network.

1. Introduction

Breast cancer (BC) remains the most common cancer and the leading cause of cancer related death in women worldwide [1]. Although there was some risk reduction with prevention, death rates from BC remain high, especially in developing countries, probably because it is still diagnosed in advanced stages [2]. Therefore, early detection to improve BC outcome and survival remains a crucial issue for its control. Despite of nowadays reliable tools used for diagnosis and prognosis of BC, more specific diagnostic, prognostic and response to treatment biomarkers are important features in the current management and for better understanding of the molecular mechanism of BC development and metastasis.

Microparticles (MPs) are heterogeneous population of small fragments (0.05–1 μm) released from cell membrane during cell activation and apoptosis. Every type of eukaryotic cells are able to release MPs. It is clear its association with thrombosis, inflammation, as well as to mediate cell–cell communication, and currently MPs are considered potent tools in the cellular communication network [3, 4]. Microparticles play a central role in coagulation initiation and thrombus formation [5]. Cell exposure to cytokines or cytotoxic chemotherapy results in the secretion of these microvesicles [6].

MPs contain a wide range of biomolecules such as chemokines, cytokines, enzymes, growth factors, signaling proteins, lipids, and nucleic acids,

(e.g. microRNA, mRNA, and even DNA) [7, 8]. They are present in the peripheral blood of healthy individuals induced by both homeostatic activation and apoptosis, but, a significant increase in pathological conditions, such as autoimmune diseases, diabetes, cardiovascular diseases, cancer, and infectious diseases has been observed [3, 8]. Recent investigations pointed out a possible clinical relevance of circulating cell-derived MP´s in different kinds of malignant diseases, including BC. However, if circulating MPs can be used as a tumor marker is still unknown [9].

Recent studies suggest that the immune response profile and inflammatory signature in breast cancer may provide useful information on patient prognosis and treatment [10]. In patients with BC, the coexpression of serum levels of several cytokines seems to be associated with a high-risk subgroup of patients with significantly shorter survival and higher risk of death compared with patients who present low levels of cytokines. Data shows that cytokines could be used clinically as a useful tumor marker for the extension and the outcome of the disease [11, 12].

In the present study, we compared the levels of MPs and cytokines/chemokines on the serum of healthy women and women with breast cancer, and the association of these biomarkers with prognostic factors and hemogram. A hierarchical network was developed to simulate the interaction between the serum immune microenvironment in healthy and disease.

2. Patients, Material and methods

2.1 Patients

This study was approved by the Ethics Committee (COEP)at Universidade Federal de Minas Gerais (UFMG), CAAE # 01536112.3.0000.5149, and informed consent was obtained from all participants.

The study enrolled 58 women: 20 healthy women, and 38 women with breast cancer. These women were enrolled from Hospital Vera Cruz, Hospital da Baleia and Hospital das Clínicas at UFMG, Belo Horizonte, Minas Gerais State, Brazil. The patients answered a questionnaire encompassing clinical and epidemiological variables, and other clinical data were obtained from their medical records.

Women with no clinical evidence of any disease were included in the control group. Patients with diagnosis of breast cancer were included in the study groups. Exclusion criteria common to the two groups were chronic hypertension, hemostatic abnormalities, diabetes, obesity, and cardiovascular, autoimmune, renal, hepatic diseases or patients who had any immune system disease or had chronic use of nonsteroidal anti-inflammatories, corticosteroids or immunossupressors in the previous three months.

2.2 Blood samples and hemogram

Blood samples were drawn in sodium citrate (0.129 mol/l) in a 9:1 volume ratio for the microparticles analysis, and in EDTA-K (1.8 mg/mL) for flow cytometric cytokine measurements. The samples were centrifuged at 2500×g for 15 min to obtain plasma. Samples were aliquoted, and stored at -80 °C until analysis. Fresh samples were also drawn in EDTA for hemogram analysis.

2.3 Determination of MPs plasma levels

MPs were prepared as described elsewhere [13]. Briefly, samples were centrifuged at 13,000×g for 3 min to obtain platelet-free plasma, which was then diluted 1:3 in citrated phosphate buffered saline (PBS) containing heparin and centrifuged at 14,000×g for 90 min at 15 °C. The subsequent MP pellet was resuspended in 1× annexin V binding buffer (Sigma-Aldrich, MO).

MPs isolated from plasma were gated on the basis of their forward (FSC) and side (SSC) scatter distribution in density plots of synthetic 0.7–0.9 μm SPHEROTM Amino Fluorescent Particles (Spherotech Inc., Libertyville, IL, USA). Taking into account the presence of phosphatidylserine residues on the MP surfaces, events present in the gate were assessed for their positive staining for annexin V (Sigma-Aldrich) — a classical marker for microparticles — using fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against annexin V. Labeling with cell-specific monoclonal antibodies was corrected for isotype-matched control antibodies.

FITC-labeled immunoglobulin G1 (IgG1) and PE-labeled IgG1 isotype controls, monoclonal antibodies directed against neutrophils (CD66-PE), endothelial cells (CD51-PE), monocytes (CD14–PERCP), platelets (CD41–PERCP), leukocytes (CD45–APC), and erythrocytes (CD235a–PECy5) were purchased from BD Biosciences® (CA, USA). Monoclonal antibody directed against T lymphocytes (CD3-PE) was purchased from Beckman Coulter Immunotech (Marseille, France). The results are expressed as percentage of Annexin V^+-Cell $marker^+$ MPs

2.4 Detection of plasmatic cytokine/chemokine levels by cytometric bead array immunoassay (CBA)

Cytokine/chemokine plasma levels were determined using commercially available kits for Cytometric Beads Array– CBA (BD Biosciences Pharmingen, USA), including the Human Inflammatory Cytokines Kit to quantify IL-1β, IL-6, IL-10, TNF, and IL-12p70 along with the Human Th1/Th2/Th17 Kit to quantify Interleukin IL-2, IL-4,, IFN-γ, and IL-17A, and the Human Chemokine Kit to quantify CXCL-8, CXCL-9, CXCL-10, CCL-5, and CCL-2.

The CBA immunoassay uses 7.5 µm polystyrene microbeads, assembled in distinct fluorescent sets, unique on their type 4 fluorescence intensity (FL-4). Each microbead is coupled to monoclonal antibody (MAb) against a given cytokine/chemokine. Following incubation with the test sample, the bead-captured cytokines were detected by a direct immunoassay using a ‘‘detection cocktail’’ of distinct MAbs phycoerythrin-PE-labeled (FL-2).

The method was carried out as recommended by the manufacturer, modified as follows: briefly, 25 µl of undiluted plasma samples or standards (previously diluted) were added to 15 µl of bead-mix and incubated for 90 min at room temperature in the dark. The cytokine standard curves were run daily for each assay. After incubation, the samples and standards were washed with 500 µl of wash buffer and centrifuged at 600g for 7 min at room temperature. Subsequently, 20 µl of detection cocktail were added to each tube, and the bead-mix re-incubated for 90 min at room temperature in the dark. Following incubation, the samples and standards were washed again with 500 µl of wash buffer and centrifuged at 600g for 7 min at room temperature to remove unbound detector reagent. After washing, 250 µl of wash buffer was added to each tube.

Data acquisition and analysis was performed in dual-laser FACScalibur™flow cytometer (BDBiosciences Pharmingen, San Jose, CA, USA), using the BDBioscience CBA software. Although the fluorescent labeled particles in the BD CBA immunoassay are designed to be excited by the 488 nm and 532 nm lasers on other BD flow cytometers, they can also be excited by the red diode laser 633 nm on dual-laser BD FACSCalibur instruments. The detection of beads emission at FL-4 channel simplifies the instrument set-up procedure and reduces the need for fluorescence compensation. Thus, a total of 1,800 beads/tube were acquired after proper set-up of a flow cytometer. Results were expressed as pg/mL for each analyte.

2.5 Biomarker Network Analysis

Biomarker networks were assembled to assess the association between levels of MPs, cytokines/chemokines, and prognostic factors in serum patients. Spearman's correlation test was performed to assess the association between levels of these biomarkers. The positive and negative correlations were significant when the $p<0.05$. To better represent the interactivity of the molecules tested, the open source software, Cytoscape (version 2.8), was used for composing networks of biomolecules interactions [14] Connecting edges display underscore negative, (_ _), moderate (—) and strong (▬) as proposed by Taylor, 1990. [15]

2.6 Statistical analysis

Data were analyzed using Graphpad Instat 4.0 and SPSS 15.0 statistical software. Differences in the means of the frequencies between groups were analyzed using two-tailed student's t test or Mann-Whitney when data did not fit a Gaussian distribution. The log-transformation of data was applied for situations where variances normality assumptions failed followed by linear regression to investigate the association between the clinical parameters and MPs levels. Pairwise correlations were evaluated with Pearson's correlation coefficient r. Multiple linear regression models with stepwise backward deletion were built to describe independent associations between covariates and the presence of biomarkers. In all case, a p value < 0.05 was considered to be statistically significant.

3. Results

As shown in Table 1, patients included in this study had no significant differences between the two groups regarding age, parity, breast-feeding, menopause status, previous use of oral contraceptive or hormone therapy, family history of breast cancer and smoking habits.

In the group of patients with breast cancer, 1 (2.6%) had stage I, 17 (44.7%) had stage II, 10 (26.3%) had stage III. and 10 (26.3%) had stage IV. Regarding histological grade, 5 patients (13.2%) had grade I, 17 (44.7%) had grade II, and 16 (42.1%) had grade III. Ten of the patients (26.3%) had metastasis, 21 (55.3%) had positive estrogen receptor, 21(55.3%) had positive progesterone receptor, and 15(39.5%) had positive Her-2. (Table 1)

Figure 1 summarizes the cellular origin and number of circulating MPs studied. No significant differences between control group and breast cancer group were found for erythrocyte-, lymphocyte- neutrophil-, leucocyte-, monocyte-, endothelial- and platelet-derived MPs. Although lower levels of monocyte-derived MPs are associated with advanced breast cancer and metastasis. Hemogram had no association with any type of MPs.

Also no differences between the two groups were observed for the cytokines - IL-1-□, IL-2, IL-4, IL-6,, IL-10, IL-12, IL-17, TNF, IFN-□, and the chemokines CXCL-8, CXCL-9, CXCL-10, CCL-2 and CCL-5 - evaluated,

however, when associated with prognostic factors had interesting outcomes. In patients with negative Her-2, levels of CXCL-10 were higher. However, higher levels of IL-17 had positive correlation with RE. Elevated levels of CCL-2 and CXCL-10 seem to be associated with advanced breast cancer.

MPs, cytokines/chemokines, and prognosis factors had important correlations in the hierarquical network. (Figures 2 and 3)

4. Discussion:

The great advantage of cytokines/chemokines and microparticles analysis by flow cytometry is the simultaneous measurement of multiple biomarkers in a single sample [8, 16]. This methodology made possible in this study to measure the levels of seven different MPs, nine different cytokines, and five distinct chemokines.

Our data is the first to measure seven different cell-derived MPs. So far, no data have been reported on circulating, lymphocyte- and erythrocyte-derived MPs in breast cancer patients. In this study, the number of neutrophils-, endothelial cells-, monocytes-, platelets-, leukocytes-, erythrocytes- and lymphocytes-derived MPs in patients with breast cancer had no significant differences to the number of MPs in the control group (Figure 1). Other studies with breast cancer women showed the same findings regarding total number of MPs, platelet-, endothelial cell- and neutrophil-derived MPs [9, 17], although, leukocyte-derived MPs were higher in the breast cancer patients compared to the controls [17]. These data discordance could be justified by the fact that the MPs investigated are not specific for breast cancer, they are also released in several diseases as well as in physiological conditions [8].

Our findings show that lower levels of monocyte-derived MPs are correlated with advanced breast cancer and metastasis. Although, different studies have been reported on higher levels of circulating MPs in breast cancer

patients associated with tumor invasiveness [8, 18]. The patients with advanced breast cancer had higher values of endothelial cell- and leukocyte-derived MPs compared to the patients with earlier disease and healthy controls. Furthermore, was detected higher levels of platelets-derived MPs on patients with bigger size of the tumor [17].

In the majority of studies using patients with benign breast tumor as a control group, platelet-derived–MPs were elevated in breast cancer patients as compared to patients with benign breast tumor [18]. Also, patients with smaller tumor size had significantly higher concentrations of neutrophil-derived MPs than benign tumor. However, total MPs levels did not differ significantly between patients with higher as compared with lower tumor stage [19].

Considering nodal status and MPs numbers, higher MPs total and neutrophil-derived MPs levels were found in patients with lymph node metastases compared to the benign tumor group [19]. Other study also shows that the total numbers of circulating MPs and platelet-derived MPs were highest in breast cancer patients with larger tumor size and distant metastases [18]. No data have been reported on monocyte-derived MPs in association with cancer spreading, but our findings showed a negative correlation between levels of this type of MPs and metastasis. MPs released from cancer cells have been implicated to contribute to tumor growth and metastasis. High levels of circulating MPs may reflect cancer activity in patients [19].

Our data showed few correlations between levels of cytokines and prognostic factors: IL-17 seems to have a positive correlation with ER, and

CXCL-10 had a negative correlation with Her-2. Most findings show higher levels of cytokines/chemokines correlated with the presence of these prognosis biomarkers [20, 21]. Positive correlations with disease progression were detected with levels of CCL-2 and CXCL-10. Other studies show that high levels of CCL-2 and CCL-5 correlate with advanced breast carcinoma, and these findings may contribute to breast cancer progression. The role of CCL-2 as a monocyte-attracting chemokine and its significant association with tumor progression may explain the simultaneous positive correlation found in this study between monocyte-derived MPs and CCL2 with advanced disease and metastasis [22-24].

Cytokines and chemokines have a well-established role in cancer immunity and carcinogenesis. These molecules are involved with tumor initiation, growth and metastasis [25]. However, breast cancer is considered to be weakly immunogenic, and poorly recognized by the immune system. This immunological response is believed to be associated with the action of modulatory cytokines in the tumor microenvironment [21, 26].

This prospective case–control study is the first report addressing the association of the MPs with cytokines/chemokines and the development of a network of these biomarkers in breast cancer. Despite the relatively small size of the study population, our data showed considerable network connections between MPs, cytokines/chemokines, and prognostic factors.

It was used the hierarchical network to simulate the microenvironment of the studied groups. Figure 2 demonstrates two different networks, one of the

control group, and another of the breast cancer group. Comparing the two nets, we can see a different profile between groups. The control group net presents two isolated clusters. There were lesser and weaker connections between the biomarkers, and also the majority of cytokines have a negative or no association with the MPs. Analyzing the breast cancer group there were more and stronger connections between the biomarkers. Most cytokines/chemokines are present in the same cluster of MPs. We can see two nodes that play main roles at the net because of their stronger and numerous associations with lymphocyte-, and leucocyte-derived MPs.

When divided the group of cancer in early disease (stages I and II), and advanced disease (stages III and IV), there was a great difference between networks (Figure 3). In early disease, we can see a balance between positive and negative associations, and presence of just one cytokine. In contrast, what we evaluated at the advanced disease network, there were stronger and exclusively positives edges, whereas in the presence of more cytokines/chemokines, the node balance is lost.

Despite the fact that our data showed no significant difference in the levels of biomarkers between groups, the networks showed presence of more nodes and stronger edges on the breast cancer group compared with the control group as well as in the advanced disease compared with early disease. These findings reinforce the importance of the study of biomarkers as a global network rather individually.

The present study has limitations due to the small number of women evaluated. The detection of cancer serum biomarkers is a big challenge in biomedical research. Our data suggest no differences in the levels of MPs and inflammatory cytokines/chemokines comparing breast cancer patients and healthy women. Our main contribution was to define a global biomarker network in breast cancer. Future investigations could be focused on the possible role of MPs as a prognostic factor in breast cancer.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgments

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Tables and Figures:

Table 1: General characteristics of the patients

	Control (n=20)	Breast Cancer (n=38)	P value
Age (years)	55.8 ± 2	54.2 ± 1.4	0.497
Parity (births)	2.6 ± 0.7	2 ± 0.3	0.396
Breast-feeding			0.570
No	8 (40%)	12 (31.6%)	
Yes	12 (60%)	26 (68.4%)	
Post menopausal			0.509
No	3 (15%)	10 (26.3%)	
Yes	17 (85%)	28 (73.7%)	
Oral contraceptives			0.782
No	9 (45%)	15 (39.5%)	
Yes	11 (55%)	23 (60.5%)	
Hormone therapy			0.141
No	19 (95%)	29 (76.3%)	
Yes	1 (5%)	9(23.7%)	
Family history of breast cancer			0.385
No	15 (75%)	23 (60.5%)	
Yes	5 (25%)	15 (39.5%)	
Smoking habit			0.155
No	16 (80%)	23 (60.5%)	
Yes	4 (20%)	15 (39.5%)	
Stage			
I		1 (2.6%)	
II		17 (44.7%)	
III		10 (26.3%)	
IV		10 (26.3%)	
Histological Grade			
I		5 (13.2%)	
II		17 (44.7%)	
III		16 42.1%)	
Metastasis			
No		28 (73.7%)	
Yes		10 (26.3%)	
ER			
Negative		17 (44.7%)	
Positive		21 (55.3%)	
PR			
Negative		17 (44.7%)	
Positive		21 (55.3%)	
Her-2			
Negative		23 (60.5%)	
Posisitve		15 (39.5%)	

Abbreviations: ER (estrogen receptor), PR (progesteron receptor) Her-2 (Human epidermal growth factor receptor 2) - Data are presented as mean ± SEM. Differences between groups were evaluated using t student or chi-square test.

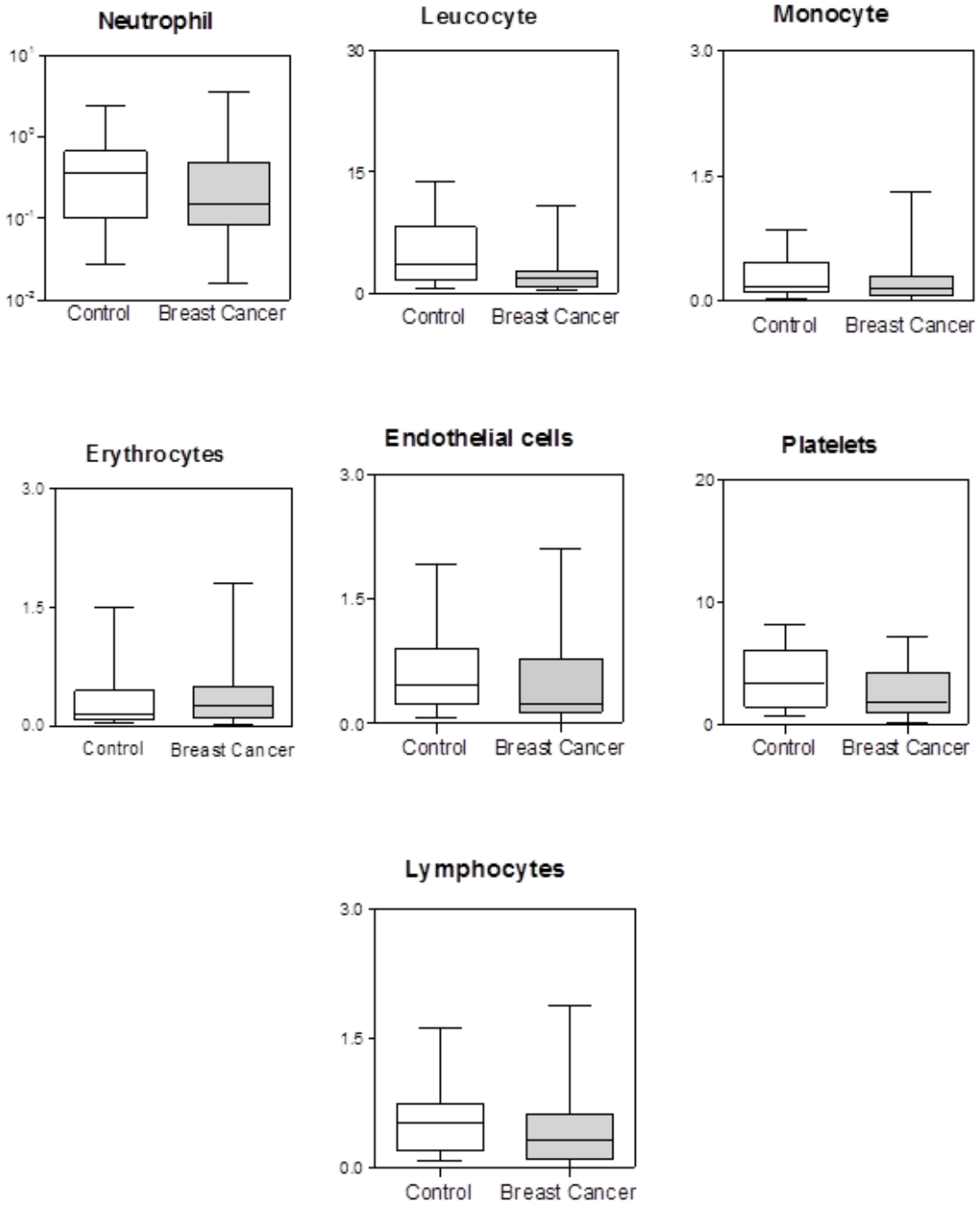


Figure 1: Cellular origin and levels of MPs. Cellular origin and levels of circulating microparticles in healthy patients (Control) and Breast Cancer patients. There was no significant difference between groups. The results are expressed as a interquartile range. Statistical differences between Control and Breast Cancer were considered significant when $p < 0.05$.

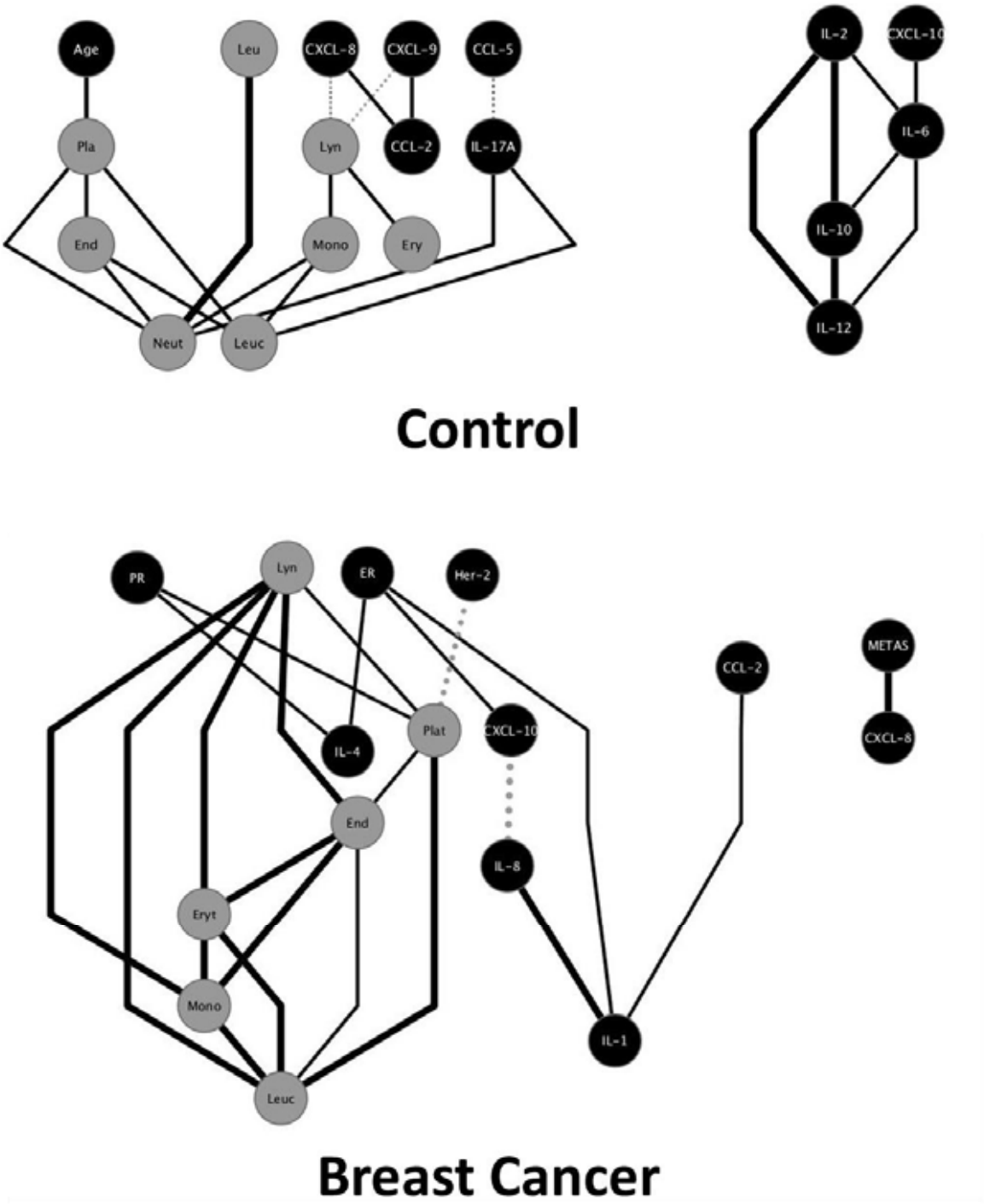
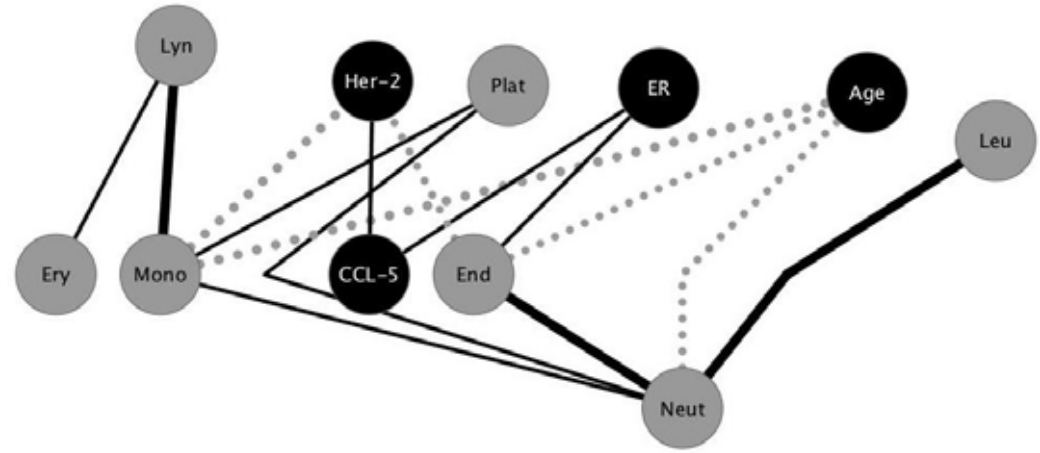
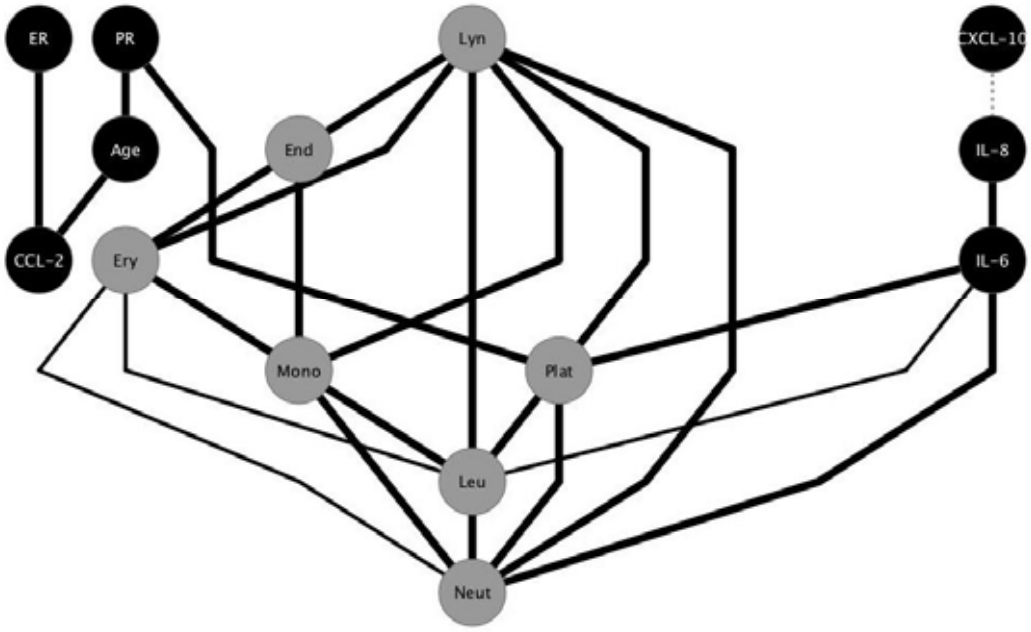


Figure 2: Network analysis. Biomarkers networks in control or Breast cancer groups. Chemokine, cytokine and MPs nodes were assembled as well as the biomarkers correlation indexes amongst groups (negative ⎯ ⎯ ; moderate ⎯⎯, and strong ▬ positive correlation).



Breast Cancer Stage (I, II)



Breast Cancer Stage (III, IV)

Figure 3: Network analysis. Biomarkers networks in Breast cancer, Stages (I, II) or Stages (III, IV) groups. Chemokine, cytokine and MPs nodes were assembled as well as the biomarkers correlation indexes amongst groups (negative – – ; moderate —, and strong ▬ positive correlation).

4. Artigo DD

Measurement of circulating microparticles and cytokine/chemokine levels in women with breast cancer submitted to chemotherapy

Artigo II

Measurement of circulating microparticles and cytokine/chemokine levels in women with breast cancer submitted to chemotherapy

Article Type: Original article

Keywords: breast cancer, chemotherapy, microparticles, cytokines, chemokines, network.

Abstract:

Chemotherapy leads to platelet activation and generation of microparticles (MPs). The combination of microparticles and cytokine/chemokine levels could be used to predict chemotherapy response in breast cancer patients. The aim of this study was to measure the levels of inflammatory biomarkers (MPs , cytokines and chemokines) in the serum of women with breast cancer pre and post-chemotherapy. Peripheral blood of women with breast cancer (n = 18) was collected pre-chemotherapy and post-chemotherapy. Flow cytometry was used for determination of serum levels of cytokines (IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, TNF, IFN –gamma), chemokines (CXCL-8, CXCL-9, CXCL-10, CCL-2 and CCL-5) and microparticles from different cells (neutrophils, leukocytes, monocytes, erythrocytes, endothelium, platelets and lymphocytes). A hierarchical network was developed to simulate the interaction between the serum immune microenvironment pre-chemotherapy and post-chemotherapy. Differences between groups were evaluated by Mann- Whitney or Kruskal-Wallis test. Differences with $p < 0.05$ were considered significant. Our data shows decreased levels of platelet -derived microparticles in post-chemotherapy patients, and increased serum levels of IL-6, CCL and CXCL -5 -10 in post-chemotherapy. In the correlation network we could identify presence of more biomarkers interactions in the pre-chemotherapy net. With these data we can conclude that chemotherapy is able to change the inflammatory response profile in patients with breast cancer.

Keywords: breast cancer, chemotherapy, microparticles, cytokines, chemokines, network.

Introduction:

Chemotherapy, radiotherapy and surgical resection are the current approach in cancer treatment. Neoadjuvant chemotherapy in breast cancer is used for the purpose of reducing tumor size leading to a less aggressive surgery [1]. The regression of the disease leads to a more conservative surgery, higher cure rates and survival [2, 3].

Cytotoxic chemotherapy kills tumor cells and the resulting apoptotic cell death was previously considered an immunological null event. Chemotherapy was thought to have a neutral or even immunosuppressive effect on the host immune system by inducing neutropenia, lymphopenia, thrombocytopenia, and anemia [4].

Chemotherapy leads to platelet activation and generation of microparticles (MPs) associated with increased risk of thrombosis [5]. In addition, chemotherapy leads to rebound angiogenesis facilitating tumor re-growth. It was demonstrated that post-chemotherapy tumor-MPs are involved in cancer thrombogenicity, tumor invasion and angiogenesis. MPs of patients with breast cancer are thrombogenic, and chemotherapy administration disturb the hemostatic balance on MPs [5, 6].

Given the systemic nature of MPs, there is therapeutic significance as they could potentially be used diagnostically to predict therapeutic response and guide individualized treatment strategies in cancer patients. The combination of

microparticles and cytokine/chemokine levels could be used to create an immune signature in breast cancer patients pre-chemotherapy and post-chemotherapy [7].

The overall response to chemotherapy is heterogeneous and dynamic, involving a combination of independent molecular mechanisms that are functionally regulated during tumor progression and treatment [8]. An understanding of the molecular mediators involved in drug responsiveness will help to understand the basis of therapeutic variability observed in clinical oncology [7].

In the present study, we observed the behavior of the serum levels of MPs, cytokines, and chemokines in women with breast cancer pre-chemotherapy and post-chemotherapy, and the interactions between these biomarkers in a network analysis approach.

2. Patients, Material and methods

2.1 Patients

This study was approved by the Ethics Committee (COEP) at Universidade Federal de Minas Gerais (UFMG), CAAE #: 01536112.3.0000.5149, and informed consent was obtained from all participants.

The study included 18 women with diagnosis of breast cancer with proposal of chemotherapy. These women were enrolled from Hospital Vera Cruz, Hospital da Baleia, and Hospital das Clínicas, UFMG, Belo Horizonte, Brazil. The patients answered a survey comprising clinical and epidemiological variables, and other clinical data were obtained from their medical records.

Exclusion criteria were chronic hypertension, hemostatic abnormalities, diabetes, obesity, and cardiovascular, autoimmune, renal, hepatic diseases or patients who had any immune system disease or had chronic use of nonsteroidal anti-inflammatories, corticosteroids or immunossupressors in the previous three months.

2.2 Blood samples

Peripheral blood was collected from patients before their first cycle of chemotherapy, and 21 days after. Blood samples were drawn in sodium citrate (0.129 mol/l) in a 9:1 volume ratio for the microparticles analysis, and in EDTA-K (1.8 mg/mL) for flow cytometric cytokine/chemokine measurements. The samples were centrifuged at 2500×g for 15 min to obtain plasma. Samples were aliquoted, and stored at -80°C until analysis.

2.3 Determination of MP plasma levels

MPs were prepared as described elsewhere [9]. Briefly, samples were centrifuged at 13,000×g for 3 min to obtain platelet-free plasma, which was then diluted 1:3 in citrated phosphate buffered saline (PBS) containing heparin and centrifuged at 14,000×g for 90 min at 15 °C. The subsequent MP pellet was resuspended in 1× annexin V binding buffer (Sigma-Aldrich, MO).

MPs isolated from plasma were gated on the basis of their forward (FSC) and side (SSC) scatter distribution in density plots of synthetic 0.7–0.9 μm SPHEROTM Amino Fluorescent Particles (Spherotech Inc., Libertyville, IL, USA). Taking into account the presence of phosphatidylserine residues on the MP surfaces, events present in the gate were assessed for their positive staining for annexin V (Sigma-Aldrich) — a classical marker for microparticles — using fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies

against annexin V. Labeling with cell-specific monoclonal antibodies was corrected for isotype-matched control antibodies.

FITC-labeled immunoglobulin G1 (IgG1) and PE-labeled IgG1 isotype controls, monoclonal antibodies directed against neutrophils (CD66-PE), endothelial cells (CD51-PE), monocytes (CD14–PERCP), platelets (CD41–PERCP), leukocytes (CD45–APC), and erythrocytes (CD235a–PECy5), were purchased from BD Biosciences® (CA, USA). Monoclonal antibody directed against T lymphocytes (CD3-PE) was purchased from Beckman Coulter Immunotech (Marseille, France).

2.4 Detection of plasmatic cytokine/chemokine levels by cytometric bead array immunoassay (CBA)

Cytokine/chemokine plasma levels were determined using commercially available kits for Cytometric Beads Array – CBA (BD Biosciences Pharmingen, USA), including the Human Inflammatory Cytokines Kit to quantify IL-1β, IL-6, IL-10, TNF, and IL-12p70 along with the Human Th1/Th2/Th17 Kit to quantify Interleukin IL-2, IL-4,, IFN-γ, and IL-17A, and the Human Chemokine Kit to quantify CXCL-8, CXCL-9, CXCL-10, CCL-5, and CCL-2.

The CBA immunoassay uses 7.5 µm polystyrene microbeads, assembled in distinct fluorescent sets, unique on their type 4 fluorescence intensity (FL-4). Each microbead is coupled to monoclonal antibody (MAb) against a given cytokine/ chemokine. Following incubation with the test sample, the bead-

captured cytokines were detected by direct immuno assay using a ‘‘detection cocktail’’ of distinct MAbs labeled with type 2 fluorescence, phycoerythrin- PE (FL-2).

The method was carried out as recommended by the manufacturer, modified as follows: briefly, 25 µl of undiluted plasma samples or standards (previously diluted) were added to 15 µl of bead-mix and incubated for 90 min at room temperature in the dark. The cytokine/chemokine standard curves were run daily for each assay. After incubation, the samples and standards were washed with 500 µl of wash buffer and centrifuged at 600g for 7 min at room temperature. Subsequently, 20 µl of detection cocktail were added to each tube and the bead-mix re-incubated for 90 min at room temperature in the dark. Following incubation, the samples and standards were washed again with 500 µl of wash buffer and centrifuged at 600g for 7 min at room temperature to remove unbound detector reagent. After washing, 250 µl of wash buffer was added to each tube.

Data acquisition and analysis was performed in dual-laser FACScalibur™flow cytometer (BDBiosciences Pharmingen, San Jose, CA, USA), using the BDBioscience CBA software. A total of 1800 beads/tube were acquired after proper set-up of a flow cytometer. Results were expressed as pg/mLfor each analyte.

2.5 Biomarker Network Analysis

Biomarker networks were assembled to assess the association between levels of MPs and cytokines/chemokines, and prognostic factors. Spearman's correlation test was performed to assess the association between levels of these biomarkers. The positive and negative correlations were significant when the $p<0.05$. To better represent the interactivity of the molecules tested, the open source software, Cytoscape (version 2.8), was used for composing networks of biomolecules interactions [10] Connecting edges display underscore negative, (_ _), moderate (—) and strong (▬) as proposed by Taylor, 1990. [11]

2.6 Statistical analysis

Data were analyzed using Graphpad Instat 4.0 and SPSS 15.0 statistical software. Differences in the means of the frequencies between groups were analyzed using two-tailed student's t test or Mann-Whitney when data did not fit a Gaussian distribution. The log-transformation of data was applied for situations where variances normality assumptions failed followed by linear regression to investigate the association between the clinical parameters and MPs levels. Pairwise correlations were evaluated with Pearson's correlation coefficient r. Multiple linear regression models with stepwise backward deletion were built to describe independent associations between covariates and the presence of biomarkers. In all cases, a p value < 0.05 was considered to be statistically significant.

3. Results

Patients included in this study had a mean age of 54 (±2.9) years, ranging from 39 to 69 years. Parity had a mean of 2.2(±2.9) births. Eleven (61.1%) patients had breast-feeding history, and seven (39.9%) family history of breast cancer. Eleven (61.1%) patients were in post-menopause, six (33.3) had previous use of hormone therapy, and nine (50%) previous use of oral contraceptives. Eight (44.4%) patients have had or have smoking habits.

Regarding prognostic factors, in the group of patients with breast cancer, 1 (5.6%) had stage I, 8 (44.4%) stage II, 5 (27.8%) stage III, and 4 (22.2%) stage IV. Regarding histological grade, 3 patients (16.7%) had grade I, 8 (44.4%) grade II, and 7 (38.9%) grade III. Four patients (22.2%) had metastasis, 9 (50%) had positive estrogen receptor, 10 (55.6%) positive progesterone receptor, and 8 (44.4%) positive Her-2.

Figure 1 summarizes the cellular origin and number of circulating MPs studied. A lower number of platelet-derived MPs were observed in post-chemotherapy measures. However, no significant differences between groups were found for erythrocyte-, lymphocyte- neutrophil-, leucocyte-, monocyte- and endothelial-derived MPs. Regarding the cytokines/chemokines assay, higher levels of IL-6, CXCL-10 and CCL-5 were find in the post-chemotherapy measurements as shown in Figure 2.

In the correlation network, a different profile of the biomarkers for the pre and post-chemotherapy groups was detected (Figure 3).

Discussion:

Blood samples were collected from patients with breast cancer pre-chemotherapy (pre-CT) and post-chemotherapy (post-CT). Our data is the first to measure 7 different cell-derived MPs (erythrocyte-, lymphocyte- neutrophil-, leucocyte-, monocyte-, endothelial- and platelet), and 14 different cytokine/chemokine (IL-1-□, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, TNF, IFN-□, CXCL-8, CXCL-9, CXCL-10, CCL-2, and CCL-5) in breast cancer patients pre-CT and post-CT.

Despite the relatively small size of the study population, our data showed considerable differences in circulating cell-derived MPs and cytokines levels post-CT.

MPs originated from platelets are the most numerous and seems to have a key role in thrombosis, inflammation, angiogenesis, vascular dysfunction, and can affect cancer patient prognosis [6]. Chemotherapy induces death of tumor cells and disruption of tumor blood vessels what results in increased liberation of MPs [6]. Differing from other studies, in our study, there was a significant decreased in platelets-derived MPs post-CT (Figure 1). Another study in patients with acute myeloid leukemia presented similar results, after chemotherapy, with lower levels of MPs [12]. Rapidly growing cells tend to secrete more MPs than cells with lower proliferation rate, what could be one of the explanation for the decreased level of MPs post-CT [13].

Post-CT measures showed higher levels of IL-6, CXCL-10, and CCL-5 (Figure 2). In different types of cancer the response to chemotherapy is increased in tumors[8]. The pro-inflammatory/modulatory profiles have important role in chemotherapy response in breast cancer [7].

In Figure 3, it was used the hierarchical network to simulate the serum patients microenvironment pre-CT and post-CT. Comparing the two nets, we can see a different profile between groups. The pre-CT net presents more connections, where we can identify presence of more correlations between cytokines/chemokines. Analyzing the post-CT net, we can clearly see a great difference in the microenvironment. There were fewer molecules involved, and they were divided into two clusters. The presence of cytokines/chemokines was greatly reduced, however, the MPS were still present. It seems that cytokines/chemokines have a faster response to chemotherapy than MPs.

In summary, chemotherapy disturbs the balance on MPs and cytokines/chemokines patterns in patients with breast cancer. This research identifies systemic immune interactions in response to chemotherapy and has potential for translation into clinical by identifying a response profile of breast cancer patients to chemotherapy.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgments

This study was supported by Fundação Oswaldo Cruz (FIOCRUZ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). The authors also thank the program for technological development in tools for health-PDTIS-FIOCRUZ for the use of its facilities. OAMF and ATC thank CNPq for fellowships (PQ).

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Figures:

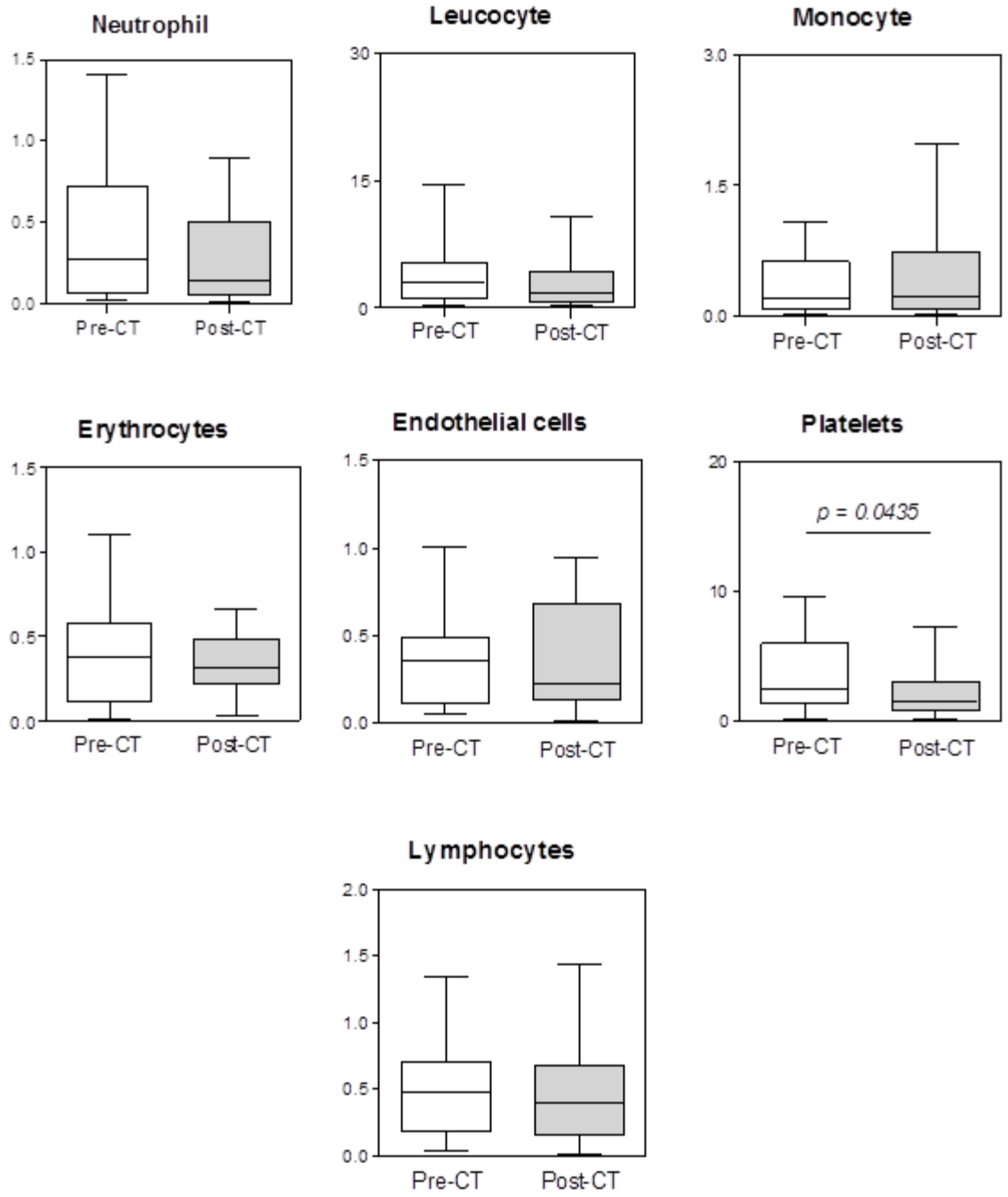

Figure 1: Cellular origin and levels of MPs. Cellular origin and levels of circulating microparticles pre-chemotherapy (pre-CT) and post-chemotherapy (post-CT). Platelet-derived MPs were lower in post-CT measurements. The results are presented as blox-plots, where the minimum, median, 75% and maximum values are represented. Statistical differences were considered significant when $p < 0.05$.

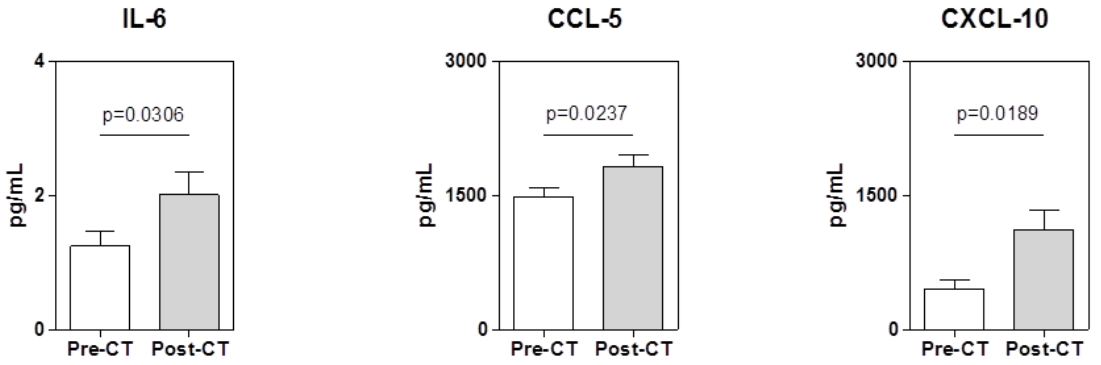

Figure 2: Levels of circulating cytokines. Levels of circulating cytokines/chemokines pre-chemotherapy (pre-CT) and post-chemotherapy (post-CT). IL-6, CCL-5 and CXCL-10 had higher levels in post-CT measurements. The results are presented as blox-plots, where the minimum, median, 75% and maximum values are represented. Statistical differences were considered significant when $p < 0.05$.

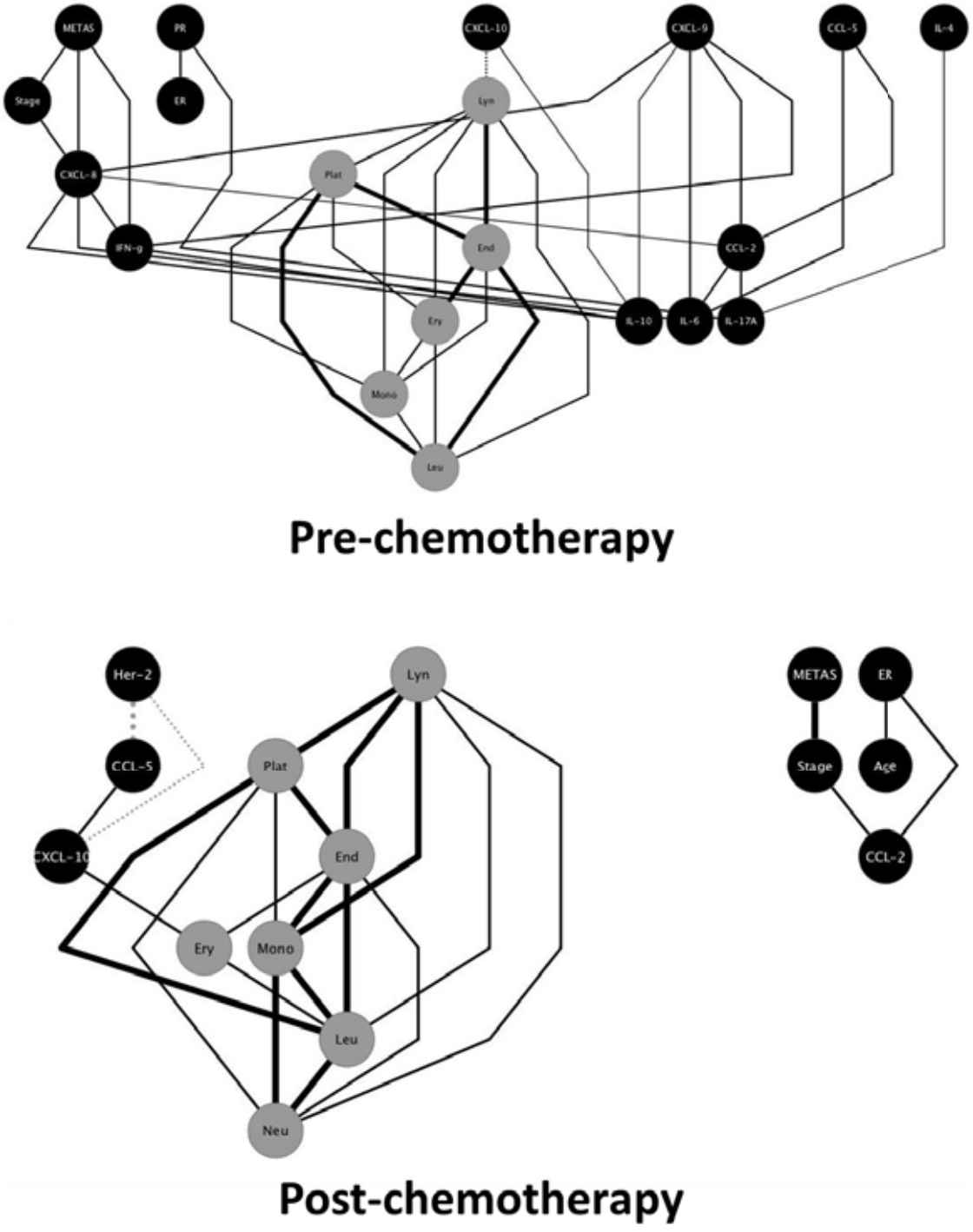

Figure 3: Network analysis. Biomarkers networks in Pre-CT or Post-CT groups. Chemokine, cytokine and MPs nodes were assembled as well as the biomarkers correlation indexes amongst groups (negative – – ; moderate —, and strong ▬ positive correlation).

5. Considerações Finais

5. Considerações finais:

Os resultados obtidos nesse trabalho nos permite concluir que:

- ✓ Não houve diferença significativa nos níveis de micropartículas, citocinas e quimiocinas entre o grupo controle e pacientes com câncer de mama.
- ✓ Baixos níveis de micropartículas derivadas de monócitos estão correlacionados com tumores em estadio avançado e metástase.
- ✓ Altos níveis de CCL-2 e CXCL10 estão correlacionados com tumores em estadio avançado e metástase.
- ✓ Não houve associação entre os níveis de micropartículas e o hemograma das pacientes.
- ✓ Houve uma diminuição dos níveis de micropartículas derivadas de plaquetas pós-quimioterapia
- ✓ Houve um aumento nos níveis séricos de IL-6, CCL-5 e CXCL-10 pós-quimioterapia.

Este trabalho nos mostra que apesar de não haver diferenças nos níveis de citocinas, quimiocinas e micropartículas entre pacientes com câncer de mama e pacientes do grupo controle, alguns desses biomarcadores estão associados a tumores em estadios avançados e metástase assim como na resposta ao tratamento quimioterápico.

A detecção de marcadores biológicos associados ao câncer é um grande desafio na pesquisa biomédica. Nossa principal contribuição foi a definição de uma rede sistêmica de biomarcadores no câncer de mama e em resposta à quimioterapia. Esta pesquisa possui potencial para tradução para a prática clínica, identificando um perfil de resposta inflamatória dessas pacientes. Esses achados reforçam a importância do estudo de biomarcadores como uma rede global de correlações e não individualmente.

Anexos

Anexo I – Aprovação pelo Comitê de Ética em Pesquisa

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Projeto: CAAE – 01536112.3.0000.5149

Interessado(a): Prof. Agnaldo Lopes da Silva Filho
Departamento de Ginecologia e Obstetrícia
Faculdade de Medicina - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 30 de maio de 2012, o projeto de pesquisa intitulado **"Avaliação da resposta inflamatória em mulheres com câncer de mama"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2° andar – Sala 2005 – Cep:31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

Anexo II – Termo de Consentimento Livre e Esclarecido.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está sendo convidada para participar, como voluntária, em uma pesquisa. Após ser esclarecida sobre as informações a seguir, no caso de aceitar fazer parte do estudo, assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável.

Em caso de recusa você não será penalizada de forma alguma. Em caso de dúvida relacionada à pesquisa você pode procurar o Prof. Agnaldo Lopes da Silva Filho pelo telefone (31) 92250909. Em caso de dúvida relacionada a aspectos éticos da pesquisa procure o Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais pelo telefone (31) 3409-4592 ou pelo endereço Av. Antônio Carlos, 6627, Unidade Administrativa II, 2° andar, Sala 2005, Campus Pampulha, Belo Horizonte, MG - Brasil (31270-901).

Título do Projeto: Avaliação da resposta inflamatória em mulheres com câncer de mama

Pesquisador Responsável: Dr. Agnaldo L. Silva Filho

Este projeto tem o objetivo de investigar a presença de algumas substâncias no sangue relacionadas à inflamação (especificamente, mieloperoxidase, n-acetilglucosaminidase, interferon-gama e fator de necrose tumoral alfa), que possam nos ajudar a fazer o diagnóstico precoce dos tumores de mama.

A participação no estudo consiste em doar uma amostra de sangue para ser analisada. Essa participação não modifica o tratamento proposto para a sua doença. Não haverá aumento do risco de complicações devido à coleta do sangue. A coleta de sangue será realizada no momento da punção para administração do soro ou durante a realização de exames pré-operatórios. A sua identidade será preservada e o seu direito de não participar no estudo não a prejudicará no seu tratamento.

Após ler e receber explicações sobre a pesquisa, e ter meus direitos de:

1. receber resposta a qualquer pergunta e esclarecimento sobre os procedimentos, riscos, benefícios e outros relacionados à pesquisa;

2. retirar o consentimento a qualquer momento e deixar de participar do estudo;

3. não ser identificado e ser mantido o caráter confidencial das informações relacionadas à privacidade.

Declaro estar ciente do exposto e desejar participar do projeto de pesquisa.

Belo Horizonte, ___ de _____ de 201_.

Nome do sujeito/ ou do responsável:_____

Assinatura:_____

Eu, Agnaldo L. Silva Filho , declaro que forneci todas as informações referentes ao projeto ao participante e/ou responsável.

_____ Data:___/___/___

Telefone : 31 92250909