FACULDADE DE MEDICINA DE BOTUCATU
Universidade Estadual Paulista "Júlio de Mesquita Filho"
Faculdade de Medicina de Botucatu

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AVALIAÇÃO DA RESPOTA IMUNE SISTÊMICA E COMPARTIMENTALIZADA EM PACIENTES PORTADORAS DE CÂNCER DE OVÁRIO

Orientador: Agnaldo Lopes da Silva Filho

Co-orientadora: Andréa Teixeira de Carvalho

Botucatu - SP
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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ginecologia, Obstetrícia e Mastologia, Área de Concentração em Tocoginecologia, da Faculdade de Medicina de Botucatu-UNESP para obtenção do Título de Doutor.

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1. Ovário - Câncer - Tratamento. 2. Câncer - Cirurgia. 3. Resposta imune Citometria de fluxo. 4. Citometria de fluxo. 5. Reação em cadeia de polimerase. 6.Inflamação. 7 Quimioterapia.

Palavras-chave: Câncer epitelial de ovário; Citometria de fluxo; Cytometric Bead Array; RT-qPCR; Resposta inflamatória.

"Quando se olha com o coração é como estar olhando através do microscópio. Enxergamos coisas tão pequenas e simples que com um pouco de amor podem mudar nossas vidas".

PTraiman

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Dedico esta tese...

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Sumário

Lista de Abreviaturas e Siglas

°C	grau Celsius
CA-125	cancer antigen 125
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CBA	cytometric bead array
CCL	(C-C motif) ligand
cDNA	complementary DNA
CEO	Câncer Epitelial de Ovário
CEP	Comitê de Ética em Pesquisa
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
C_t	threshold
CXCL	(C-X-C motif) ligand
DNA	deoxyribonucleic acid
EOC	Epithelial Ovarian Cancer
FIGO	International Federation of Gynecology and Obstetrics
FIOCRUZ	Fundação Oswaldo Cruz
FL	Fluorescence
FSC	Forward Scatter
g	unidade de gravidade
IFNG	gene of interferon gamma
IFNγ	Interferon gamma
IL	Interleukin
min	minute
□L	Microlitro
MoAb	monoclonal antibody
ng	nanograma

NTC	No template control
PCR	Polymerase Chain Reaction
PE	phycoerythrin
qPCR	quantitative real time PCR
r	correlation index
RNA	Ribonucleic acid
SSC	Side Scatter
TP53	tumor protein p53
µm	Micrometro
χ^2	chi-squared

Resumo

Resumo

O câncer epitelial de ovário representa um desafio para Oncologia Ginecológica, devido à sua natureza insidiosa e alta mortalidade. Há também uma compreensão limitada da etiologia da doença ao nível molecular, o que continua a dificultar o desenvolvimento de alvos terapêuticos. Estudos recentes de morfologia, imuno-histoquímica e de genética molecular têm levado ao desenvolvimento de um novo paradigma para a patogênese e a origem do câncer epitelial de ovário, baseado em um modelo dualista de carcinogênese que divide o câncer epitelial de ovário (CEO) em duas grandes categorias chamadas de tipos I e II. O objetivo deste estudo foi avaliar o padrão das citocinas e quimiocinas encontradas no tecido ovariano de mulheres saudáveis e mulheres com CEO tipo I e tipo II. Além disso, descrever a associação desses biomarcadores com os dados clínico-patológicos. Foram analisadas amostras de tecido ovariano e ascite obtidas de mulheres com CEO (n=26) e amostras de tecido ovariano e lavado peritoneal de mulheres sem evidências de malignidade (n=16 - grupo de controle). Nas amostras de tecido, a expressão gênica foi avaliada utilizando a metodologia de PCR quantitativo em tempo real (qPCR) para os genes *IFNG, IL-10, TGFB1, CCL2, CCL3, CCL5, CXCL8, CXCL9 e CXCL10*. A detecção dos níveis de citoquinas/quimioquinas nos fluidos peritoneais foi realizada através do método *Cytometric Bead Array* (CBA) os marcadores IL-12p70, TNF, IL-10, IL-6, IL-1-□, IL-8, IL-17A, IFN-□, IL-4, IL-2, CCL2, CCL5, CXCL-9 e CXCL-10. No grupo das pacientes com CEO, 10 (38,5%) apresentavam estágios I/II e 16 (61,5%) estágios III/IV. Com relação ao tipo de tumor, de acordo com a nova classificação, 8 (30,8%) eram do tipo I e 18 (69,2 %) do tipo II. A citorredução ótima foi obtida em 15 (57,7%) das mulheres com CEO. Mulheres com CEO tipo II apresentaram maiores níveis séricos do marcador CA-125 quando comparado às do tipo I. Não houve óbito no grupo das mulheres com tumor do tipo I, enquanto no grupo das mulheres com tumor tipo II seis (33,3%) foram a óbito. Foi detectado o aumento da expressão gênica no grupo das mulheres com CEO tipo II quando comparado com o tipo I, para os genes *IL-10, CXCL8* e *CXCL9*. Houve uma diminuição na expressão de *TGFB1*, *CCL2* e *CXCL10* no grupo de mulheres com CEO tipo II quando comparado ao tipo I. Dentre os genes estudados, não houve diferença com relação os parâmetros clínicos: citorredução cirúrgica (ótima ou subótima) e o marcador CA-125 (<600 U/mL ou >600 U/mL). Nossos resultados suportam a hipótese de que existe uma interferência intercelular entre as células do CEO e o infiltrado de células do sistema imunológico, sugerindo um microambiente imunológico mais supressivo no CEO tipo I, quando comparado com CEO tipo II.

Palavras-chave: expressão genética, câncer de ovário, tumor, citocinas, quimiocinas

Summary

Summary

The epithelial ovarian cancer represents a challenge to Gynecologic Oncology due to its insidious nature and high mortality. There is also a limited understanding of disease etiology at the molecular level, which continues to hamper targeted therapeutic development. Recent morphological, immunohistochemical, and molecular genetic studies have led to the development of a new paradigm for the pathogenesis and origin of epithelial ovarian cancer based on a dualistic model of carcinogenesis that divides epithelial ovarian cancer into 2 broad categories designated types I and II. The aim of this study was to evaluate the cytokines and chemokines pattern in ovarian tissue from healthy women and women with EOC type I and type II. Also, we described the association of these biomarkers with the clinicopathological data. Samples of ovarian tissue and ascite obtained from women with EOC (n=26), samples of ovarian tissue and peritoneal wash from women with no evidence of malignancy were analyzed (n=16 – control group). In the tissue samples, gene expression were evaluated by quantitative real time PCR (qPCR) *IFNG, IL-10, TGFB1, CCL2, CCL3, CCL5, CXCL8, CXCL9* and *CXCL10*. The detection of cytokine/chemokine levels in peritoneal fluids was measured by cytometric bead array immunoassay (CBA), IL-12p70, TNF, IL-10, IL-6, IL-1-□, IL-8, IL-17A, IFN-□, IL-4, IL-2, CCL2, CCL5, CXCL-9 and CXCL-10. In the group of women with EOC, 10 (38.5 %) had stage I/II and 16 (61.5 %) were stage III/IV . Concerning tumor type , according to the new classification, 8 (30.8 %) were type I and type II were 18 (69.2 %) . Optimal cytoreduction was achieved in 15 (57.7 %) women with EOC. The CA-125 showed higher serum levels in patients with EOC type II compared to type I. There were no deaths in women with type I tumor while 6 (33.3 %) of patients with type II tumor died . Increased expression of IL-10, CXCL8 and CXCL9 genes in the group of women with type II EOC was detected as compared to type I. In contrast, there was a decrease in the gene expression of *TGFB1, CCL2* and *CXCL10* in the group of women with EOC type II when compared to type I. Among the genes studied there was no association between them and clinical parameters: surgical cytoreduction (optimal or suboptimal) and CA-125 serum marker (<600 U/mL or >600U/mL). Our results support the hypothesis that an intercellular crosstalk between the cells of EOC and infiltrating immune cells suggesting a more suppressive immune microenvironment in EOC type I when compared with EOC type II.

Keywords: gene expression, ovarian cancer, tumor, cytokines, chemokines

1. Introdução

1. INTRODUÇÃO

1.1 Câncer Epitelial de ovário

O câncer epitelial do ovário (CEO) representa um desafio à Oncologia Ginecológica devido ao seu caráter insidioso e à sua alta letalidade [1]. Para o ano de 2014, são estimados 21.980 novos casos e 14.270 mortes em mulheres americanas [2]. No Brasil configura-se como o oitavo tumor ginecológico mais comum, porém o de maior letalidade. Estudos mais recentes apontam a incidência de 5.680 novos casos para o ano de 2014 e a ocorrência de 3.027 mortes no ano de 2011 [3]. A sintomatologia inespecífica e a falta de métodos propedêuticos de rastreamento dificultam o diagnóstico precoce dessa neoplasia [4, 5].

Aproximadamente 2/3 dos casos são diagnosticados nos estádios III e IV, com uma sobrevida em cinco anos variando de 10 a 20%, principalmente pela ausência de métodos de rastreamento e diagnóstico inadequados, tendo os exames de imagem como a ultrassonografia, a tomografia computadorizada e a ressonância nuclear magnética, associadas aos marcadores séricos como o CA-125, baixos valores de predição [6]. Embora o CEO possa ocorrer em qualquer idade, é mais comum em mulheres com mais de 50 anos. O tratamento convencional inclui citorredução cirúrgica seguida de quimioterapia, quando indicada [7].

Citam-se como fatores de risco importantes para o desenvolvimento do câncer de ovário, não relacionado a alterações genéticas, a idade, a menarca precoce, a menopausa tardia, a obesidade e a síndrome dos ovários policísticos. Inibidores da ovulação, como as anticoncepcionais têm um efeito protetor [8]. As manifestações clínicas do câncer de ovário são em sua maioria inespecíficas e variam desde desconforto abdominal com mudanças do hábito intestinal, com anorexia e perda ponderal até o encontro de grande massa no abdome e distensão por ascite nos estágios avançados [9].

O CEO é uma doença heterogênea composta por diferentes tipos de tumores com uma grande variedade de características e comportamentos clínico patológicos [10]. Estudos de morfologia, imunohistoquímica e genética molecular têm levado ao desenvolvimento de um novo paradigma para a patogênese e origem do CEO baseado em um modelo dualista de carcinogênese que divide o CEO em duas grandes categorias designadas tipo I e II. Os tumores tipo I são de baixo grau, geralmente não causam dor e são detectados nos estádios iniciais da doença. Os tumores tipo I possuem maior estabilidade genética e raramente apresentam mutações em TP53, sendo caracterizados por mutações específicas, incluindo KRAS, BRAF, ERBB2, CTNNB1, PTEN, PIK3CA, ARID1A e PPP2R1A. Os tumores do tipo II são mais agressivos e de alto grau, sendo geralmente diagnosticados em estadios avançados, estes tumores apresentam alta frequência de mutações em TP53, mas raramente abrigam as mutações encontradas nos tumores tipo I [11-15].

Um dos sintomas clínicos no CEO é o aumento da circunferência abdominal devido à ascite [16-18]. A ascite surge a partir da desregulação da função endotelial, levando ao aumento da permeabilidade dos vasos peritoneais [19]. Em condições fisiológicas, a permeabilidade vascular é mediada estritamente pela abertura e fechamento das junções célula-célula [20-22]. Por este motivo, qualquer alteração na organização destas junções, podem resultar numa desregulação da função vascular levando a condições patológicas devido as modificações da parede dos vasos [23]. Nas pacientes com CEO, a ascite pode ser evidenciada devido ao aumento da permeabilidade vascular.

O câncer de ovário apresenta alto potencial inflamatório, evidenciado pela presença de infiltrados linfocitários (TILs) no seu microambiente, os quais se relacionam diretamente à sobrevida das pacientes. O fluxo e as atividades destes infiltrados linfocitários são mediados por citocinas, sendo estas um reflexo do estado imunológico do hospedeiro, podendo servir como meio de se entender a resposta imune

O CEO é um tumor imunogênico, que apresenta linfócitos infiltrantes de tumores (TIL), que cria um microambiente supressor imunitário para escapar da eliminação do sistema imune.

Epithelial ovarian cancer is an immunogenic tumor, as indicated by the presence of tumor infiltrating lymphocytes (TIL), which creates an immune suppressive microenvironment in order to escape from the immune elimination[24].

The clinical outcome of EOC is strongly dependent on the immune response, which is critically dependent on macrophages, the most prevalent immune cell within the EOC microenvironment [25, 26]. The ability of expansion of ovarian malignant tumors requires the collaboration between malignant and stromal cells via cellular interactions. Therefore, malignant cells and subsidiary stromal cells communicate and exchange information by direct cell-to-cell contacts as well as the release of signaling molecules, such as soluble factors [27, 28].

Cytokines are small polypeptides released from cells to regulate the activities of other cells via interactions with specific cytokine receptors expressed on their surface [29]. At least 16 different cytokines are expressed in normal ovaries, along with many of the corresponding receptors [30]. In ovarian cancer there is increased expression of particular cytokines and/or receptors [31]. These act to enhance key phenotypes associated with tumor progression, such as proliferation, migration and survival, including chemo-resistance [32]. This can be due to direct effects on the cancer cells themselves, or via indirect effects on components of the immune system.

REFERENCIAS BIBLIOGRÁFICAS

1. Silva-Filho, A.L.d., et al., *Cirurgia não ginecológica em pacientes com câncer de ovário.* Revista Brasileira de Ginecologia e Obstetrícia, 2004. 26: p. 411-416.

2. Siegel, R., et al., *Cancer statistics, 2014.* CA Cancer J Clin, 2014. 64(1): p. 9-29.

3. INCA, *Estimativa de câncer no Brasil*, 2013, Ministério da Saúde: BRASIL.

4. Look, K.Y., *Epidemiology, etiology, and screening of ovarian cancer*, in *Ovarian Cancer*, S.C. Rubin and G.P. Sutton, Editors. 2001, Lippincott Williams & Wilkins: Philadelphia. p. 167-180.

5. Reis, F.M., et al., *Biochemical markers of ovarian cancer: diagnostic and prognostic value.* Italian Journal of Gynaecology & Obstetrics, 2004. 16(3): p. 94-99.

6. Brewer, M.A., et al., *Prevention of ovarian cancer: intraepithelial neoplasia.* Clin Cancer Res, 2003. 9(1): p. 20-30.

7. Roett, M.A. and P. Evans, *Ovarian cancer: an overview.* Am Fam Physician, 2009. 80(6): p. 609-16.

8. George, S.H. and P. Shaw, *BRCA and Early Events in the Development of Serous Ovarian Cancer.* Front Oncol, 2014. 4: p. 5.

9. Burges, A. and B. Schmalfeldt, *Ovarian cancer: diagnosis and treatment.* Dtsch Arztebl Int, 2011. 108(38): p. 635-41.

10. Kurman, R.J. and M. Shih Ie, *The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory.* Am J Surg Pathol, 2010. 34(3): p. 433-43.

11. Shih Ie, M. and R.J. Kurman, *Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis.* Am J Pathol, 2004. 164(5): p. 1511-8.

12. Jones, S., et al., *Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma.* Science, 2010. 330(6001): p. 228-31.

13. Wiegand, K.C., et al., *ARID1A mutations in endometriosis-associated ovarian carcinomas.* N Engl J Med, 2010. 363(16): p. 1532-43.

14. Samartzis, E.P., et al., *ARID1A mutations and PI3K/AKT pathway alterations in endometriosis and endometriosis-associated ovarian carcinomas.* Int J Mol Sci, 2013. 14(9): p. 18824-49.

15. Kurman, R.J. and M. Shih Ie, *Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer--shifting the paradigm.* Hum Pathol, 2011. 42(7): p. 918-31.

16. Ayantunde, A.A. and S.L. Parsons, *Pattern and prognostic factors in patients with malignant ascites: a retrospective study.* Ann Oncol, 2007. 18(5): p. 945-9.

17. Ayhan, A., et al., *Is there a correlation between tumor marker panel and tumor size and histopathology in well staged patients with borderline ovarian tumors?* Acta Obstet Gynecol Scand, 2007. 86(4): p. 484-90.

18. Cheng, M.H., et al., *Differential diagnosis of gynecologic organ-related diseases in women presenting with ascites.* Taiwan J Obstet Gynecol, 2008. 47(4): p. 384-90.

19. Garrison, R.N., et al., *Malignant ascites. Clinical and experimental observations.* Ann Surg, 1986. 203(6): p. 644-51.

20. Dejana, E., *Endothelial cell-cell junctions: happy together.* Nat Rev Mol Cell Biol, 2004. 5(4): p. 261-70.

21. Bazzoni, G. and E. Dejana, *Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis.* Physiol Rev, 2004. 84(3): p. 869-901.

22. Walz, A., et al., *Effects of luteinizing hormone and human chorionic gonadotropin on corpus luteum cells in a spheroid cell culture system.* Mol Reprod Dev, 2005. 72(1): p. 98-104.

23. Herr, D., et al., *Human chorionic gonadotropin controls luteal vascular permeability via vascular endothelial growth factor by down-regulation of a cascade of adhesion proteins.* Fertil Steril, 2013. 99(6): p. 1749-58.

24. Yigit, R., et al., *Cytokine analysis as a tool to understand tumour-host interaction in ovarian cancer.* Eur J Cancer, 2011. 47(12): p. 1883-9.

25. Zhang, L., et al., *Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.* N Engl J Med, 2003. 348(3): p. 203-13.

26. Kumar, J. and A.C. Ward, *Role of the interleukin 6 receptor family in epithelial ovarian cancer and its clinical implications.* Biochim Biophys Acta, 2014.

27. Yellapa, A., et al., *Interleukin 16 expression changes in association with ovarian malignant transformation.* Am J Obstet Gynecol, 2013.

28. Dong, Y.L., et al., *CXCR2-Driven Ovarian Cancer Progression Involves Upregulation of Proinflammatory Chemokines by Potentiating NF-kappaB Activation via EGFR-Transactivated Akt Signaling.* PLoS One, 2013. 8(12): p. e83789.

29. Leonard, W.J. and J.X. Lin, *Cytokine receptor signaling pathways.* J Allergy Clin Immunol, 2000. 105(5): p. 877-88.

30. Burke, F., et al., *A cytokine profile of normal and malignant ovary.* Cytokine, 1996. 8(7): p. 578-85.

31. Nash, M.A., et al., *The role of cytokines in both the normal and malignant ovary.* Endocr Relat Cancer, 1999. 6(1): p. 93-107.

32. Shield, K., et al., *Alpha2beta1 integrin affects metastatic potential of ovarian carcinoma spheroids by supporting disaggregation and proteolysis.* J Carcinog, 2007. 6: p. 11.

2. Objetivos

Objetivo geral

Avaliação da resposta imune tumoral compartimentalizada em pacientes com câncer epitelial de ovário (CEO).

Objetivos específicos

1. Comparar a resposta inflamatória tecidual em mulheres com CEO tipo I e tipo II.
2. Comparar a resposta inflamatória no líquido ascítico/lavado peritoneal de pacientes com CEO com o grupo controle.
3. Avaliar a associação da resposta inflamatória tecidual com níveis séricos de CA-125, citorreduçao ótima, estadiamento e mortalidade em mulheres com CEO.
4. Correlacionar o nível de expressão gênica de citocinas no CEO.

3. Artigo

Cytokine and chemokine pattern in epithelial ovarian cancer - type I and type II

CYTOKINE AND CHEMOKINE PATTERN IN EPITHELIAL OVARIAN CANCER - TYPE I AND TYPE II

Article Type: Original article

ABSTRACT

The epithelial ovarian cancer represents a challenge to Gynecologic Oncology due to its insidious nature and high mortality. There is also a limited understanding of disease etiology at the molecular level, which continues to hamper targeted therapeutic development. Recent morphological, immunohistochemical, and molecular genetic studies have led to the development of a new paradigm for the pathogenesis and origin of epithelial ovarian cancer based on a dualistic model of carcinogenesis that divides epithelial ovarian cancer into 2 broad categories designated types I and II. The aim of this study was to evaluate the cytokines and chemokines pattern in ovarian tissue from healthy women and women with EOC type I and type II. Also, we described the association of these biomarkers with the clinicopathological data. Samples of ovarian tissue and ascite obtained from women with EOC (n=26), samples of ovarian tissue and peritoneal wash from women with no evidence of malignancy were analyzed (n=16 – control group). In the tissue samples, gene expression were evaluated by quantitative real time PCR (qPCR) IFNG, IL-10, TGFB1, CCL2, CCL3, CCL5, CXCL8, CXCL9 and CXCL10. The detection of cytokine/chemokine levels in peritoneal fluids was measured by cytometric bead array immunoassay (CBA), IL-12p70, TNF, IL-10, IL-6, IL-1-□, IL-8, IL-17A, IFN-□, IL-4, IL-2, CCL2, CCL5, CXCL-9 and CXCL-10. In the group of women with EOC, 10 (38.5 %) had stage I/II and 16 (61.5 %) were stage III/IV. Concerning tumor type, according to the new classification, 8 (30.8 %) were type I and type II were 18 (69.2 %). Optimal cytoreduction was achieved in 15 (57.7 %) women with EOC. The CA-125 showed higher serum levels in patients with EOC type II compared to type I. There were no deaths in women with type I tumor while 6 (33.3 %) of patients with type II tumor died. Increased expression of IL-10, CXCL8 and CXCL9 genes in the group of women with type II EOC was detected as compared to type I. In contrast, there was a decrease in the gene expression of *TGFB1, CCL2* and *CXCL10* in the group of women with EOC type II when compared to type I. Among the genes studied there was no association between them and clinical parameters: surgical cytoreduction (optimal or suboptimal) and CA-125 serum marker (<600 U/mL or >600U/mL). Our results support the hypothesis that an intercellular crosstalk between the cells of EOC and infiltrating immune cells suggesting a more suppressive immune microenvironment in EOC type I when compared with EOC type II.

Keywords: gene expression, ovarian cancer, tumor, cytokines, chemokines

1 INTRODUCTION

The Epithelial Ovarian Cancer (EOC) is the most lethal gynecological cancer worldwide, with approximately 200,000 new cases diagnosed each year globally, and a mortality rate greater than 60% within five years [1]. Epithelial ovarian cancer (EOC) develops on the surface of the ovary (epithelium) and is known to be the sixth most common type of cancer in women [2]. Almost 75% of the patients are diagnosed in the advanced stages (III and IV), and therefore have a very poor prognosis [2, 3]. Its poor prognosis is partly due to the largely asymptomatic nature of the disease, at the time of diagnosis, the primary tumor is already spread [1]. There is also a limited understanding of disease etiology at the molecular level, which continues to hamper targeted therapeutic development.

Epithelial ovarian cancer is a heterogeneous disease composed of different types of tumors with widely differing clinicopathologic features and behavior [4]. Recent morphological, immunohistochemical, and molecular genetic studies have led to the development of a new paradigm for the pathogenesis and origin of epithelial ovarian cancer based on a dualistic model of carcinogenesis that divides epithelial ovarian cancer into 2 broad categories designated types I and II. Type I tumors comprise low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas, and Brenner tumors. They are generally indolent, present in stage I (tumor confined to the ovary), and are characterized by specific mutations, including KRAS, BRAF, ERBB2, CTNNB1, PTEN, PIK3CA, ARID1A, and PPP2R1A, which target specific cell signaling

pathways [5-8]. Type I tumors rarely harbor TP53 mutations and are relatively stable genetically. Type II tumors comprise high-grade serous, high-grade endometrioid, malignant mixed mesodermal tumors (carcinosarcomas), and undifferentiated carcinomas. They are aggressive, present in advanced stage, and have a very high frequency of TP53 mutations but rarely harbor the mutations detected in type I tumors [9].

One of the most impressing clinical symptoms in EOC is an increase of the abdominal circumference due to ascites [10-12]. Development of ascites is a consequence of dysregulated endothelial function leading to increased vascular permeability of peritoneal vessels [13]. Under physiological conditions, vascular permeability is mediated by strictly regulated opening and closure of cell–cell-junctions [14-16]. For that reason, any disturbance of junctional organization might result in dysregulated vascular function leading to pathological conditions by modifying the regular structure of the vessel wall [17]. Under malign circumstances, the typical example with clinical importance caused by increased vascular permeability is ascites.

Epithelial ovarian cancer is an immunogenic tumor, as indicated by the presence of tumor-infiltrating lymphocytes (TILs), which creates an immune suppressive microenvironment in order to escape from the immune elimination [18]. The clinical outcome of EOC is strongly dependent on the immune response, which is critically dependent on macrophages, the most prevalent immune cell within the EOC microenvironment [19, 20]. The ability of expansion of ovarian malignant tumors requires the collaboration between malignant and stromal cells via cellular interactions. Therefore, malignant cells and subsidiary

stromal cells communicate and exchange information by direct cell-to-cell contacts as well as the release of signaling molecules, such as soluble factors[21, 22].

Cytokines are small polypeptides released from cells to regulate the activities of other cells via interactions with specific cytokine receptors expressed on their surface [23]. At least 16 different cytokines are expressed in normal ovaries, along with many of the corresponding receptors [24]. In ovarian cancer there is increased expression of particular cytokines and/or receptors [25]. These act to enhance key phenotypes associated with tumor progression, such as proliferation, migration and survival, including chemo-resistance [26]. This can be due to direct effects on the cancer cells themselves, or via indirect effects on components of the immune system.

The aim of this study was to evaluate the cytokines and chemokines pattern in ovarian tissue from healthy women and women with EOC type I and type II. Also, we described the association of these biomarkers with the clinicopathological data.

2 PATIENTS, MATERIAL AND METHODS

2.1 Patients and specimens

The study was conducted in agreement with the Helsinki Declaration and Resolution 446/2012 of the National Health Council at the Brazilian Ministry of Health, which regulates research involving human subjects in Brazil. This study was approved by the Ethics Committee of UFMG, Brazil, ETIC #: 326/08, and informed consent was obtained from all participants.

This study included 42 women enrolled from a Brazilian clinical center in the period from June 2010 to October 2013. The patients answered a questionnaire encompassing clinical and epidemiological variables and other clinical data including age, surgical findings, pathological and immunohistochemical characteristics (i.e type I or II tumors), CA-125 levels, and survival were obtained from their medical records. The maximum diameter of the residual tumor after surgery was also retrieved. Optimal debulking surgery was defined as the maximum diameter of residual tumor ≤1 cm. Otherwise, the debulking surgery was considered sub-optimal.

Patients with proposal of laparotomy and diagnosis of epithelial ovarian cancer were included in the study group. The control group was comprised from women who underwent patients, when indicating hysterectomy for benign disease, by the surgeon option (intraoperative findings), or for technical reasons (bleeding vascular pedicle and without possibility of maintaining the

gonad). Overall, 26 patients with ovarian cancer and 16 controls have been analyzed.

Histological grading and disease staging were based on the International Federation of Gynecology and Obstetrics (FIGO) classification [27, 28]. In this study, FIGO stage I/II and FIGO stage III/IV ovarian cancers were considered early and advanced disease, respectively.

Exclusion criteria common to the groups were 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 Evaluation of gene expression by quantitative real time PCR (qPCR)

2.2.1 Extraction of total RNA from tissues

Fragments of ovarian tissue were collected and properly packaged in tubes containing RNA Later solution (Ambion Inc., Austin, TX, USA), stored in RNase-free microtube, and kept in a freezer at -80°C until the extraction of total RNA.

The total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. Total RNA was quantified using the Qubit Fluorometer® (Life Technologies, Carlsbad, CA, USA), and the Quant-iT™ RNA Assay Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer´s protocol, and NanoDrop ND - 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) to

determine the purity samples for the presence of protein (absorbance reading at 260 and 280nm).

The quality of total RNA was determined on a 1 % agarose gel in denaturing sample buffer (7 M urea, 30 % glycerol, 0.25 % bromophenol blue, 0.6 % SDS and 60 mM EDTA).

2.2.2 Synthesis of cDNA

After quality verification, 50 ng of total RNA and random hexamers were used to synthesize cDNA using the Improm II Reverse Transcription Synthesis kit (Promega, Madison, WI, USA) following the manufacturer's protocol. The resulting cDNA was stored at temperature -20°C until qPCR.

2.2.3 Selection of primers

In the present study, a total of 10 genes, including IFNG, CCL2, CCL3, CXCL8, CXCL9, CXCL10, IL10, CCL5, TGFB1 and ACTB (the latter was used as reference gene), were selected for quantitative assessment. qPCR optimization was established following the MIQE guidelines [29].

The primers for the genes evaluated in this study (Table 1) were selected from the literature according to the following validation criteria:

1. G + C content of 50-60 %;

2 . Primer size 18-22 bases ;

3 . Melting temperature (Tm) between 58-60ºC;

4. Amplicon size between 80-200 base pairs (bp);

5 . Sequences that were between exons-introns junctions.

Primer matrix experiments were conducted by selecting, for each gene, the primer concentrations that provide the lowest Ct and the highest ΔRn using a fixed amount of target template. As template, a cDNA sample from control group was used and also a no template control (NTC) was included to assess primer-dimer formation or nonspecific amplification. The specificity of each qPCR product was checked by dissociation curves and gel analysis.

The qPCR reactions consisted of 12.5 □L of Power Syber Green PCR Master (Life Technologies, Carlsbad, CA, USA), 1.0 - 2.0 µL of cDNA and conditions universal cycling: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C or 62°C (IL10, CCL2, CXCL10) or 1 minute in ABI Prism Step One Plus Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). For each specific set of primers, all group were run in two technical replicates The results were analyzed using StepOne software version 2.1 (Applied Biosystems, Carlsbad, CA, USA).

2.2.4 Evaluation of the uniformity of the expression levels of the endogenous control

In this study, the constitutive ACTB gene was previously selected as reference gene, and the uniformity of expression of this gene was determined by a qPCR with cDNA samples from each study group. The reaction and cycling conditions were established on the choice of primers and optimization of the test phase, according to item 2.3.3 of the methodology.

Subsequently, the obtained data were analyzed by comparing the average levels of threshold (C_t) cycle by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The results showed no significant difference ($p>0.05$) among the groups. Thus, the ACTB gene was chosen as reference gene for the qPCR reactions in this study.

2.2.5 Evaluation of the efficiency of amplification primers

The relative quantification procedure was chosen following a validation experiment to compare the PCR efficiency for each target and reference gene by using standard curves and slope evaluation. The standard curves were constructed using serial dilutions of cDNA from control group (1.0 ng/□L - 0.05 ng/□□L). Subsequently, the results were plotted on the X axis which represented the log of the concentration of cDNA and the Y axis, the C_t value for each concentration. Primers were considered adequate for evaluation of gene expression when presented with efficiency above 95 %, determined by the slope of the curve applied to the following formula: Efficiency = [$10^{(-1/\text{slope})}$ - 1] x 100. The baseline and threshold values were adjusted for each test using the StepOne software version 2.1 (Applied Biosystems, Carlsbad, CA, USA).

2.2.6 Detection of gene expression levels of cytokines and chemokines by qPCR

The qPCR reactions consisted of 12.5 □L of Power Syber Green PCR Master (Life Technologies, Carlsbad, CA, USA), 1.0 - 2.0 µL of cDNA and specific concentration for each primer (Table 2), performing in a final volume of

25□L of reaction. The conditions universal cycling were following: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C or 62°C (IL10, CCL2, CXCL10) or 1 minute in ABI Prism StepOne Plus Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Assays were performed in duplicate with the other genes and evaluated with the ACTB gene present on the same plate. Furthermore, two internal controls were included to assess primer-dimer formation or nonspecific amplification (no template control - NTC). The baseline and threshold values were adjusted for each test using the StepOne software version 2.1 (Applied Biosystems, Carlsbad, CA, USA). The results were expressed by method $2^{-\Delta\Delta Ct} = 2^{-(Ct_t \text{ target gene} - Ct_t \text{ ACTB gene) target sample} - (Ct_t \text{ target gene} - Ct_t \text{ ACTB gene) control sample}}$ [30].

2.3 Detection of cytokine/chemokine levels in peritoneal fluids by cytometric bead array immunoassay (CBA)

The secreted cytokine/chemokine analysis by flow cytometry is a methodology in which the simultaneous measurement of multiple biomarkers in a single sample is performed. For biomarkers determination in ascite or peritoneteal wash, peritoneal fluids of patients with EOC were collected during laparotomy, centrifuged, and stored at -80°C until laboratory analysis. The CBA immunoassay kit (Becton Dickinson Biosciences Pharmingen, San Diego, CA, USA) was used for quantitative analysis of biomarkers levels. The CBA kit uses 7.5 μm polystyrene microbeads, consisting of distinct populations, unique on their type 3 fluorescence intensity (FL-3) each coupled to monoclonal

antibody (MoAb) against one of each biomarker to capture IL-1-β, IL-6, IL-10, TNF, and IL-12p70 (Human Inflammatory Cytokines Kit); IL-2, IL-4, IFN-γ, and IL-17A (Human Th1/Th2/Th17 Kit), and CXCL8, CXCL9, CXCL10, CCL5, CCL2 (Human Chemokine Kit). The captured cytokines/chemokines were then detected via direct immunoassay using a cocktail of different MoAbs coupled to phycoerythrin (PE) (FL-2). Briefly, 25µl of plasma (diluted 1:5 in diluent G, supplied by CBA kit) or standard (previously diluted in diluent G, as recommended by the manufacturer) were added to 15µl of mixture beads and incubated for 90 min at room temperature in the dark. The biomarkers standard calibrator mixture was used for each assay. After incubation, the samples and standards were washed with 500 µl of wash buffer (supplied with CBA kit) and centrifuged at 600 g for 7 min at room temperature. Subsequently, 20µl of detection cocktail consisting of PE-conjugated MoAbs were added to each tube and the mixture 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 600 g for 7 min at room temperature to remove unbound detector reagent. After washing, 250µl of wash buffer was added to each tube prior to data acquisition using a fluorescence activated cell sorter (FACS) Calibur® flow cytometer (Becton Dickinson). Although the fluorescently labelled particles in the BD CBA immunoassay are designed to be excited by the 488 nm laser common to all BD flow cytometers, they can also be excited by the red diode laser on dual-laser BD FACSCalibur instruments. The detection of particle emission on the FL-4 channel simplifies the instrument set-up procedure and reduces the need for fluorescence compensation. Thus, a

total of 1,800 events/gate were acquired after proper set-up of a flow cytometer to measure forward (FSC) and side (SSC) light scatters, and dual-colour (FL-4 and FL-2) flow cytometric acquisition, using a dual-laser BD CBA template. Data analysis was performed using BD Bioscience CBA software.

2.4 Biomarker Network Analysis

Biomarker networks were assembled to assess the association between the gene expression levels of cytokines and chemokines in tissues of EOC and EOC for type I and type II groups. Spearman's correlation test was performed to assess the association between gene expression levels of biomarker. 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 [31]. Connecting edges display underscore negative - $r<0$ (_ _), moderate - $0.36>r<0.67$ (—), and strong - $r>0.68$ (▬) as proposed by Taylor, 1990 [32].

2.5 Statistical analysis

Statistical analyses were performed using the Graphpad Prism 5.00 software (San Diego, USA). Shapiro–Wilk tests were used to verify if the variables were normally distributed. Data not normally distributed were compared using the Kruskal–Wallis test followed by the Dunns post-hoc test.

Comparison between 2 groups was done using the Mann–Whitney U test with Bonferroni's correction (non-normal data). Normal data are presented as mean and standard deviation, while non-normal variables are presented as median and interquartile range (25th–75th percentiles). Correlations were analyzed using the Pearson or Spearman 2-sided test and Pearson χ^2 test to frequency differences.

3 RESULTS

Characteristics of the study population are summarized in Table 2. There were no differences between the groups regarding age and menopause status (p= 0.824 and p= 0.055; respectively). However there was a significant difference of parity (p= 0.016) between groups.

In the group of patients with EOC, 8 (30.8%) had type I and 18 (69.2%) had type II ovarian cancer. Patients with EOC type II had more tumor stage III and IV (p= 0.001), higher levels of CA-125 (p= 0.024), higher mortality (p= 0.023) and less optimal cytoreduction (p= 0.004). Optimal cytoreduction occurred in 15 (57.7%) patients with ovarian cancer. All women with type I tumors had optimal cytoreduction. Death was not observed in patients with type I, whereas in type II, 6 cases (33.3%) were registered.

Figure 1 summarizes the gene expression levels of cytokines/chemokines detected in the ovarian tumor type I and type II. Higher levels of *TGFB1, CCL-2*, and *CXCL-10* were detected in EOC type I group compared with EOC type II group (p=0.0127, p=0.0372 and p=0.0167, respectively). In contrast, levels of *IL-10*, *CXCL-8*, and *CXCL-9* were higher in the EOC type II group compared with the EOC type I group (p=0.0312, p=0.0402 and p=0.0480, respectively). No significant differences between the two groups were observed for *IFNG*, *CCL-3*, and *CCL-5* gene expression.

There was no significant difference regarding the association of CA-125 and cytoreduction with the gene expression of cytokines/chemokines studied (Figure 2 and Figure 3).

In this study, the levels of cytokines and chemokines were also analyzed in ascite or peritoneal fluid by CBA. Using this methodology, we are able to detect some cytokines/chemokines Figure 4 demonstrated in a spike plots the comparison between levels of INF-□ in ascite (A) with gene expression of *IFNG* in the ovarian tissue from womens with EOC. This biomarker was chosen because was the analyte found more frequently in the samples.

Gene expression levels of cytokines and chemokines presented important correlations in the circle network of EOC type I and type II (Figure 5). While in EOC type 1 the correlation between *TGFB1*, *CCL*-2 and *CXCL-10* are negative in the tumor type 2 they are strong and positive, although they also involve the participation of *CCL-2*, interactions with this chemokines occur between IL-10 and CXCL-9. There is also a positive correlation between two

chemokines responsible for the recruitment of cells with pro-inflammatory potential (*CCL-5* and *CCL-3*). That interaction may contribute to clinical outcome of the disease more severe.

4 DISCUSSION

The present study evaluated the gene expression of cytokines that could potentially be used as tumor markers of prognostic for ovarian cancer. Our study investigated the gene expression profile in 2 different types of ovarian cancer tissue, and also, the presence of levels of cytokines/chemokines was assessed in ascite or peritoneal fluid. The analysis of immune response may contribute to a better understanding of the conflicting results reported in the literature in the association between cytokine/chemokine levels and EOC.

Recent study shows no significant differences in the level of the IFN-gamma between patients with malignant and benign tumors [33]. In the same study, women with advanced cancer had significantly higher serum levels of IL-6, IL-10 and TGF-□1 than women with early stages or benign tumors. In our study, the data shows no difference between EOC type I and type II tumor for the gene expression levels of *IFNG* in accordance with different biological sites analyzed by other authors. Moreover, in EOC type II tumor higher levels of *IL-10* gene expression and lower levels of *TGFB1* were found when compared to EOC type I tumor.

The biomarkers *IL10* and *CXCL8* had higher levels in patients with advanced cancer (EOC type II). This group of biomarkers showed highest percentage of suboptimal cytoreduction, however, the group of patients with less advanced cancer (EOC type I) had the marker *TGFB1* in higher levels in the group of patients who had the highest percentage of optimal cytoreduction. Our data in tumor tissue are similar to other studies that evaluated serum samples profile, moreover, women with very advanced cancer in which the optimal cytoreduction was disabled had the highest serum levels of IL-10, TGF-□1, and CXCL-8 [33].

The Th1 response is activated by IFN-gamma, whereas Th2 response is activated by IL-10. Polarized macrophages differ in terms of the production of cytokines and in their receptor expression. The tumor inflammatory microenvironment facilitates breakage of the basal membrane in the process of cancer invasion of the surrounding tissue and metastases. IL-10 has been shown to inhibit inflammatory response and crosstalk between innate and adaptive immune responses in human cancers, thereby favoring unrestrained tumor growth [34, 35].

The present study showed that a positive association exists between factors associated with poor prognosis and higher levels of IL-10. This finding is supported by many studies. For instance, it is has been proposed that IL10 modulates immunosuppressive response by itself or in conjunction with other cytokines, including TGF-□. Also, IL10 is known to play a role in downregulating the expression of Th1 cytokines, augmenting the immunosuppressive activity.

The IL-10 suppress the T-cell immune response against cancer cells buy downregulating the expression of MHC class I and class II [36].

The CXCL-8, formerly known as the pro-inflammatory cytokine interleukin (IL)-8, is expressed in various cancer cells [37]. CXCL-8 overexpression has been shown in multiple malignancies, and is frequently associated with poor clinical outcome [38]. It has been widely reported that increased CXCL-8 expression in ovarian cyst fluid, ascites, serum, and tumor tissue from ovarian cancer patients was found to be associated with high-grade and advanced-stage cancers, as well as with decreased disease-related patient survival [39-44]. In this study, the higher levels of gene expression of *CXCL8* were detected in EOC type II, and in patient whom did not observe optimal cytoreduction. This data corroborate with the hypothesis that higher levels of CXCL-8 measured in serum and tumor site are related to poor prognosis and survival.

CXCL-9 is an interferon gamma-inducible chemokine, which has been found to be over-expressed in several malignancies [45]. Its elevated activity on tumor cells was originally believed to exert the anti-tumor effects on human cancers, because it can promote the immune activities, such as elevation in cytotoxic T lymphocyte responses and infiltration of $CD4^+$ or $CD8^+$ lymphocytes into tumors, against the tumor cells [45, 46]. In addition, CXCL-9, when coupled with its receptor CXCR3, has anti-angiogenic properties through direct interaction with the endothelium and/or the recruitment of T or NK cells, which can destroy the tumor vasculature [46]. However, recent studies on the direct and pro-tumorigenic activities of CXCL-9 expression on tumor cells are gaining

much recent attention. First, CXCL-9 has been found to be up-regulated at transcripts or overexpressed at tumor cells in several different types of malignancies, and to promote the proliferation of cancer cells [47-51]. Another manner of CXCL-9 overexpression promoting malignancies has been demonstrated that the involvement of CXCL-9 and/or CXCR3 inducing tumor cell metastasis [48, 52]. On the other hand, CXCL-9 and/or CXCR3 antagonism were reported to inhibit the cancer metastasis in melanoma and breast cancers [47, 52, 53]. Furthermore, the elevated CXCL-9 concentrations in blood samples of patients with early breast cancers compared to those of normal volunteers indicated that CXCL-9 could be a screening blood marker for breast cancers [51]. Our recent results support the suggestions about the pro-tumor importance of CXCL-9 in tumorigenesis and that this axis could be a potential therapeutic target for these malignancies.

Interleukin-10 (IL-10), a cytokine with anti-inflammatory and immunomodulatory functions, regulates the biology of B and T cells [54].The consequence of IL-10 presence and the resulting inhibition of the antitumor immune response might be critical in progression of cancers [55]. The complexity of IL-10 activities defines a broad spectrum of the properties of IL-10. The principal function of IL-10 is to control inflammation and instruct adaptive immune responses. IL-10 inhibits the activation and differentiation of antigen-presenting cells, such as dendritic cells and macrophages [54, 56-58]. In EOC type I, the gene expression of *IL-10* is lower than EOC type II, however, there was an increase expression of *CXCL-10* and *TGFB1* in EOC type I, whereas the gene of both CLXC-8 and CXCL-9 were overexpressed in EOC

type II than compared with EOC type I, suggesting a profile less inflammatory in EOC type I.

It was used the circle network to simulate the microenvironment of the studied groups. Figure 5 demonstrate two different networks, one of the EOC type I (A), and another of EOC type II (B). Comparing the two nets, we can see a different profile between groups. The A network presented negative correlations, while B network showed stronger and positive correlations. The A network revealed negative correlations between*TGFB1* with *CXCL10* and *CCL2*, whereas the B network demonstrated strong and positive correlations between *CCL2* with *IL10* and *CXCL9,* and strong positive correlation between *CCL5* with *CCL3*.

Our results support our hypothesis that an intercellular crosstalk between the cells of EOC and infiltrating immune cells suggest an more immune suppressive microenvironment status in EOC type I when compared with EOC type II. The understanding of these molecular networks may be helpful in developing novel therapeutic strategies, which might enable an immunomodulatory intervention in the future. Understanding not only the gene expression of tumor but also the dynamic between a growing tumor and the host immune response may contribute to generate a rich set of opportunities for the specific therapy of cancer. Further studies with a larger number of patients, before and after therapeutic approaches are required to evaluate the importance of these components in the EOC clinical outcome.

Declaration of Conflicting Interests

The authors declare that there are no conflicts of interest.

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REFERENCES

1. Lengyel, E., *Ovarian cancer development and metastasis.* Am J Pathol, 2010. **177**(3): p. 1053-64.

2. Hennessy, B.T., R.L. Coleman, and M. Markman, *Ovarian cancer.* Lancet, 2009. **374**(9698): p. 1371-82.

3. Polverino, G., et al., *Survival and prognostic factors of women with advanced ovarian cancer and complete response after a carboplatin-paclitaxel chemotherapy.* Gynecol Oncol, 2005. **99**(2): p. 343-7.

4. Kurman, R.J. and M. Shih Ie, *The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory.* Am J Surg Pathol, 2010. **34**(3): p. 433-43.

5. Shih Ie, M. and R.J. Kurman, *Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis.* Am J Pathol, 2004. **164**(5): p. 1511-8.

6. Jones, S., et al., *Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma.* Science, 2010. **330**(6001): p. 228-31.

7. Wiegand, K.C., et al., *ARID1A mutations in endometriosis-associated ovarian carcinomas.* N Engl J Med, 2010. **363**(16): p. 1532-43.

8. Samartzis, E.P., et al., *ARID1A mutations and PI3K/AKT pathway alterations in endometriosis and endometriosis-associated ovarian carcinomas.* Int J Mol Sci, 2013. **14**(9): p. 18824-49.

9. Kurman, R.J. and M. Shih Ie, *Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer--shifting the paradigm.* Hum Pathol, 2011. **42**(7): p. 918-31.

10. Ayantunde, A.A. and S.L. Parsons, *Pattern and prognostic factors in patients with malignant ascites: a retrospective study.* Ann Oncol, 2007. **18**(5): p. 945-9.

11. Ayhan, A., et al., *Is there a correlation between tumor marker panel and tumor size and histopathology in well staged patients with borderline ovarian tumors?* Acta Obstet Gynecol Scand, 2007. **86**(4): p. 484-90.

12. Cheng, M.H., et al., *Differential diagnosis of gynecologic organ-related diseases in women presenting with ascites.* Taiwan J Obstet Gynecol, 2008. **47**(4): p. 384-90.

13. Garrison, R.N., et al., *Malignant ascites. Clinical and experimental observations.* Ann Surg, 1986. **203**(6): p. 644-51.

14. Dejana, E., *Endothelial cell-cell junctions: happy together.* Nat Rev Mol Cell Biol, 2004. **5**(4): p. 261-70.

15. Bazzoni, G. and E. Dejana, *Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis.* Physiol Rev, 2004. **84**(3): p. 869-901.

16. Walz, A., et al., *Effects of luteinizing hormone and human chorionic gonadotropin on corpus luteum cells in a spheroid cell culture system.* Mol Reprod Dev, 2005. **72**(1): p. 98-104.

17. Herr, D., et al., *Human chorionic gonadotropin controls luteal vascular permeability via vascular endothelial growth factor by down-regulation of a cascade of adhesion proteins.* Fertil Steril, 2013. **99**(6): p. 1749-58.

18. Yigit, R., et al., *Cytokine analysis as a tool to understand tumour-host interaction in ovarian cancer.* Eur J Cancer, 2011. **47**(12): p. 1883-9.

19. Zhang, L., et al., *Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.* N Engl J Med, 2003. **348**(3): p. 203-13.

20. Kumar, J. and A.C. Ward, *Role of the interleukin 6 receptor family in epithelial ovarian cancer and its clinical implications.* Biochim Biophys Acta, 2014.

21. Yellapa, A., et al., *Interleukin 16 expression changes in association with ovarian malignant transformation.* Am J Obstet Gynecol, 2013.

22. Dong, Y.L., et al., *CXCR2-Driven Ovarian Cancer Progression Involves Upregulation of Proinflammatory Chemokines by Potentiating NF-kappaB Activation via EGFR-Transactivated Akt Signaling.* PLoS One, 2013. **8**(12): p. e83789.

23. Leonard, W.J. and J.X. Lin, *Cytokine receptor signaling pathways.* J Allergy Clin Immunol, 2000. **105**(5): p. 877-88.

24. Burke, F., et al., *A cytokine profile of normal and malignant ovary.* Cytokine, 1996. **8**(7): p. 578-85.

25. Nash, M.A., et al., *The role of cytokines in both the normal and malignant ovary.* Endocr Relat Cancer, 1999. **6**(1): p. 93-107.

26. Shield, K., et al., *Alpha2beta1 integrin affects metastatic potential of ovarian carcinoma spheroids by supporting disaggregation and proteolysis.* J Carcinog, 2007. **6**: p. 11.

27. *Classification and staging of malignant tumours in the female pelvis.* Acta Obstet Gynecol Scand, 1971. **50**(1): p. 1-7.

28. *Changes in definitions of clinical staging for carcinoma of the cervix and ovary: International Federation of Gynecology and Obstetrics.* Am J Obstet Gynecol, 1987. **156**(1): p. 263-4.

29. Bustin, S.A., et al., *The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.* Clin Chem, 2009. **55**(4): p. 611-22.

30. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

31. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks.* Genome Res, 2003. **13**(11): p. 2498-504.

32. Taylor, R., *Interpretation of the Correlation Coefficient: A Basic Review.* Journal of Diagnostic Medical Sonography, 1990. **6**(1): p. 35-39.

33. Nowak, M., et al., *Proinflammatory and immunosuppressive serum, ascites and cyst fluid cytokines in patients with early and advanced ovarian cancer and benign ovarian tumors.* Neuro Endocrinol Lett, 2010. **31**(3): p. 375-83.

34. Zitvogel, L., A. Tesniere, and G. Kroemer, *Cancer despite immunosurveillance: immunoselection and immunosubversion.* Nat Rev Immunol, 2006. **6**(10): p. 715-27.

35. Wang, T., et al., *Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells.* Nat Med, 2004. **10**(1): p. 48-54.

36. Yue, F.Y., et al., *Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules.* Int J Cancer, 1997. **71**(4): p. 630-7.

37. Ogura, M., et al., *Clinical significance of CXCL-8/CXCR-2 network in esophageal squamous cell carcinoma.* Surgery, 2013. **154**(3): p. 512-20.

38. Xie, K., *Interleukin-8 and human cancer biology.* Cytokine Growth Factor Rev, 2001. **12**(4): p. 375-91.

39. Ivarsson, K., et al., *The chemotactic cytokine interleukin-8--a cyst fluid marker for malignant epithelial ovarian cancer?* Gynecol Oncol, 1998. **71**(3): p. 420-3.

40. Ivarsson, K., et al., *Upregulation of interleukin-8 and polarized epithelial expression of interleukin-8 receptor A in ovarian carcinomas.* Acta Obstet Gynecol Scand, 2000. **79**(9): p. 777-84.

41. Penson, R.T., et al., *Cytokines IL-1beta, IL-2, IL-6, IL-8, MCP-1, GM-CSF and TNFalpha in patients with epithelial ovarian cancer and their relationship to treatment with paclitaxel.* Int J Gynecol Cancer, 2000. **10**(1): p. 33-41.

42. Fasciani, A., et al., *Vascular endothelial growth factor and interleukin-8 in ovarian cystic pathology.* Fertil Steril, 2001. **75**(6): p. 1218-21.

43. Herrera, C.A., et al., *Expression of metastasis-related genes in human epithelial ovarian tumors.* Int J Oncol, 2002. **20**(1): p. 5-13.

44. Kassim, S.K., et al., *Vascular endothelial growth factor and interleukin-8 are associated with poor prognosis in epithelial ovarian cancer patients.* Clin Biochem, 2004. **37**(5): p. 363-9.

45. Ben-Baruch, A., *The multifaceted roles of chemokines in malignancy.* Cancer Metastasis Rev, 2006. **25**(3): p. 357-71.

46. Vicari, A.P. and C. Caux, *Chemokines in cancer.* Cytokine Growth Factor Rev, 2002. **13**(2): p. 143-54.

47. Liu, C., et al., *Chemokine receptor CXCR3 promotes growth of glioma.* Carcinogenesis, 2011. **32**(2): p. 129-37.

48. Ohshima, K., et al., *Expression of chemokine receptor CXCR3 and its ligand, mig, in gastric and thyroid marginal zone lymphomas. Possible migration and autocrine mechanism.* Leuk Lymphoma, 2003. **44**(2): p. 329-36.

49. Sreekanthreddy, P., et al., *Identification of potential serum biomarkers of glioblastoma: serum osteopontin levels correlate with poor prognosis.* Cancer Epidemiol Biomarkers Prev, 2010. **19**(6): p. 1409-22.

50. Suyama, T., et al., *Up-regulation of the interferon gamma (IFN-gamma)-inducible chemokines IFN-inducible T-cell alpha chemoattractant and monokine induced by IFN-gamma and of their receptor CXC receptor 3 in human renal cell carcinoma.* Cancer, 2005. **103**(2): p. 258-67.

51. Ruiz-Garcia, E., et al., *Gene expression profiling identifies Fibronectin 1 and CXCL9 as candidate biomarkers for breast cancer screening.* Br J Cancer, 2010. **102**(3): p. 462-8.

52. Amatschek, S., et al., *CXCL9 induces chemotaxis, chemorepulsion and endothelial barrier disruption through CXCR3-mediated activation of melanoma cells.* Br J Cancer, 2011. **104**(3): p. 469-79.

53. Walser, T.C., et al., *Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer.* Cancer Res, 2006. **66**(15): p. 7701-7.

54. Sinuani, I., et al., *Role of IL-10 in the progression of kidney disease.* World J Transplant, 2013. **3**(4): p. 91-98.

55. Terai, M., et al., *Human interleukin 10 receptor 1/IgG1-Fc fusion proteins: immunoadhesins for human IL-10 with therapeutic potential.* Cancer Immunol Immunother, 2009. **58**(8): p. 1307-17.

56. Mosmann, T.R., *Role of a new cytokine, interleukin-10, in the cross-regulation of T helper cells.* Ann N Y Acad Sci, 1991. **628**: p. 337-44.

57. Hedrich, C.M. and J.H. Bream, *Cell type-specific regulation of IL-10 expression in inflammation and disease.* Immunol Res, 2010. **47**(1-3): p. 185-206.

58. Fiorentino, D.F., et al., *IL-10 inhibits cytokine production by activated macrophages.* J Immunol, 1991. **147**(11): p. 3815-22.

FIGURES LEGENDS

Figure 1: Gene expression profile in EOC tissues according to type of tumor.Relative transcript levels of modulatory (*IL10* and *TGFB1*), and proinflammatory (*IFNG, CCL2, CCL3, CCL5, CXCL8, CXCL9* and *CXCL10*) cytokines and chemokines in inflammatory infiltrate of EOC tumor type I (light gray bar, n = 8) and EOC tumor type II (dark gray bar, n = 18). The results are presented in a column chart format and are expressed as the median $2^{-\Delta\Delta Ct}$ and interquartile range. Statistical differences between type I and type II EOC groups were considered significant when $p < 0.05$

Figure 2. Gene expression profile in EOC tissues according to CA-125 serum level.Relative transcript levels of modulatory (*IL10* and *TGFB1*), and proinflammatory (*IFNG, CCL2, CCL3, CCL5, CXCL8, CXCL9* and *CXCL10*) cytokines and chemokines in group with CA-125 <600U/mL (light gray bar, n=14) and CA-125 >600U/mL (dark gray bar, n = 12). The results are presented in a column chart format and are expressed as the median $2^{-\Delta\Delta Ct}$ and interquartile range. Statistical differences between CA-125 <600U/mL and CA-125 >600U/mL groups were considered significant when $p < 0.05$

Figure 3: Gene expression profile in EOC tissues according to Cytoreduction. Relative transcript levels of modulatory (*IL10* and *TGFB1*), and proinflammatory (*IFNG, CCL2, CCL3, CCL5, CXCL8, CXCL9* and *CXCL10*) cytokines and chemokines in group with cytoreduction optimal (light gray bar, n=15) and cytoreduction suboptimal (dark gray bar, n = 11). The results are presented in a column chart format and are expressed as the median $2^{-\Delta\Delta Ct}$ and interquartile range. Statistical differences between cytoreduction optimal and suboptimal groups were considered significant when $p < 0.05$

Figure 4: Detection of IFN-gamma in ascite and simultaneously gene expression of *IFNG* in tumor tissue.

Figure 5: Biomarkers networks in Epithelial Ovarian Cancer (EOC). Gene expression levels were used to calculate correlations between the cytokine and chemokine biomarkers present in distinct groups, (A) EOC type I ( ) and (B) EOC type II ( ). The strength of the interactions is represented by different line style according to the following ranges, negative (r<0 - – –); moderate (0.36>r<0.67 - ——) and strong (r>0.68 - ▬).

TABLES:

Table 1. Sequences of primers used for quantification of mRNA expression by qPCR

GENES	PRIMER SEQUENCE (5'- 3')	AMPLICON SIZE (bp)	Primer concentration Forward/Reverse (µM)	cDNA volume (µL)	Melting temperature (ºC)	Reaction Efficiency (%)	R2	REFERENCE
ACTB	FW: CCGAGCGCGGCTACAGCTTCA RV:GGAAATCGTGCGTGACATTAAG	59bp	0,6 / 0,6	1,0	60	99,1	0,992	(Musso et al., 1996)
IL10	FW: GTGATGCCCCAAGCTGAGA RV: CACGGCCTTGCTCTTGTTTT	138bp	0,9 / 1,2	1,0	62	99,3	0,962	(Costa-Silva et al., 2013)
TGFB	FW:CAGCAACAATTCCTGGCGATA RV: AAGGCGAAAGCCCTCAATTT	119bp	2,0 / 2,0	1,0	60	77,4	0,998	(Costa-Silva et al., 2013)
IFNG	FW: TTCAGCTCTGCATCGTTTTG RV: TCCGCTACATCTGAATGACCT	112bp	1,0 / 1,0	2,0	60	93,0	0,940	(Dhanasekaran et al., 2010)
CCL2	FW: AGTCTCTGCCGCCCTTCT RV: GTGACTGGGGCATTGATTG	93bp	1,2 / 1,2	1,0	62	93,3	0,941	(Lin and Chuang, 2010)
CCL3	FW: GCAACCAGTTCTCTGCATCA RV: TGGCTGCTCGTCTCAAAGTA	140bp	1,0 / 1,0	2,0	60	84,6	0,986	(Zhang et al., 2010)
CCL5	FW:CACGCCTCGCTGTCATCCTCA RV:TTGGCGGTTCTTTCGGGTGAC	197bp	0,1 / 0,1	2,0	60	80,1	0,939	(Costa-Silva et al., 2013)
CXCL8	FW:GTCTGCTAGCCAGGATCCACAA RV:GAGAAACCAAGGCACAGTGGAA	51 pb	1,0 / 1,0	1,0	60	95,3	0,982	(Toki et al., 2009)
CXCL9	FW: CCAATACAGGAGTGACTTG RV: GGATTGTAGGTGGATAGTC	172 pb	0,5 / 0,5	2,0	60	85,8	0,952	(Fernandez, 2008)
CXCL10	FW:GCCAATTTTGTCCACGTGTTG RV:AGCCTCTGTGTGGTCCATCCT	193bp	0,6 / 0,6	1,0	62	87,4	0,948	(Toki et al., 2009)

Table 2. Characteristics of the Patients With EOC (Study Group) and Gynecological Patients With No Ovarian Disease (Control Group)

		EOC, n=26		
	Normal Ovary, n= 16	Type1, n=8	Type 2, n=18	p
Age	42.7 ± 7.9	63 ± 19.9	62 ± 11.3	0.824
Parity	2.2 ± 1.7	1.7 ± 1.3	3.8 ± 1.9	0.016
CA-125 (U/mL)				0.024
<600		6 (75%)	5 (27.8%)	
>600		2 (25%)	13 (72.2%)	
Stage				0.001
I-II		7 (87.5%)	3 (16.7%)	
III-IV		1 (12.5%)	15 (83.3%)	
Cytoreduction				0.004
Optimal (<1 cm)		8 (100%)	7 (38.9%)	
Suboptimal (>1 cm)		0	11 (61.1%)	
Menopause				0.055
No	9 (56.2%)	3 (37.5%)	3 (16.7%)	
Yes	7 (43.8%)	5 (62.5%)	15 (83.3%)	
Death				0.023
No		8 (100%)	12 (66.7%)	
Yes		0	6 (33.3%)	

Abbreviations: CA-125, serum marker for epithelial ovarian tumors; EOC, epithelial ovarian cancer. ANOVA e chisquare test.

FIGURES:

FIGURE 1

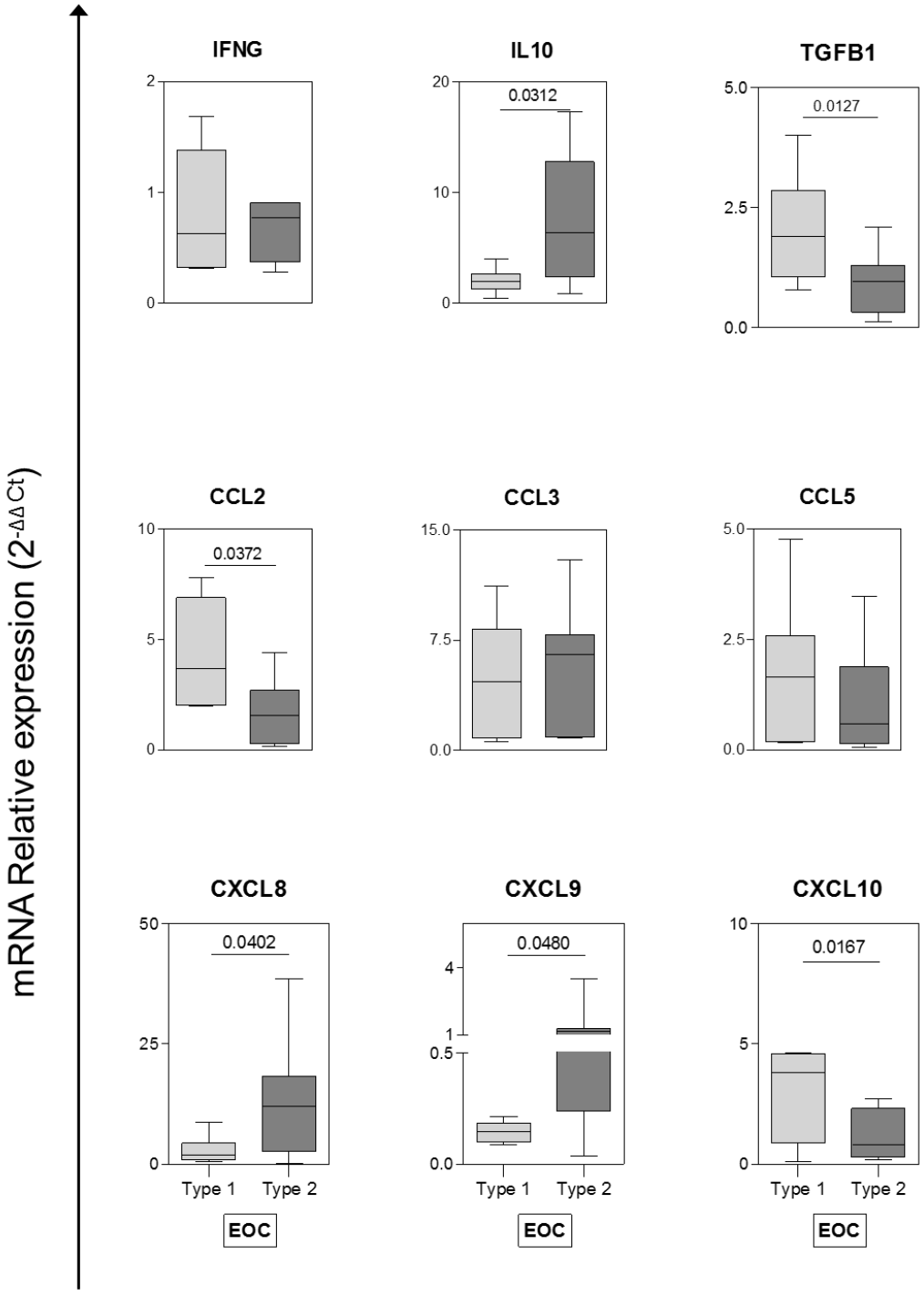


FIGURE 2

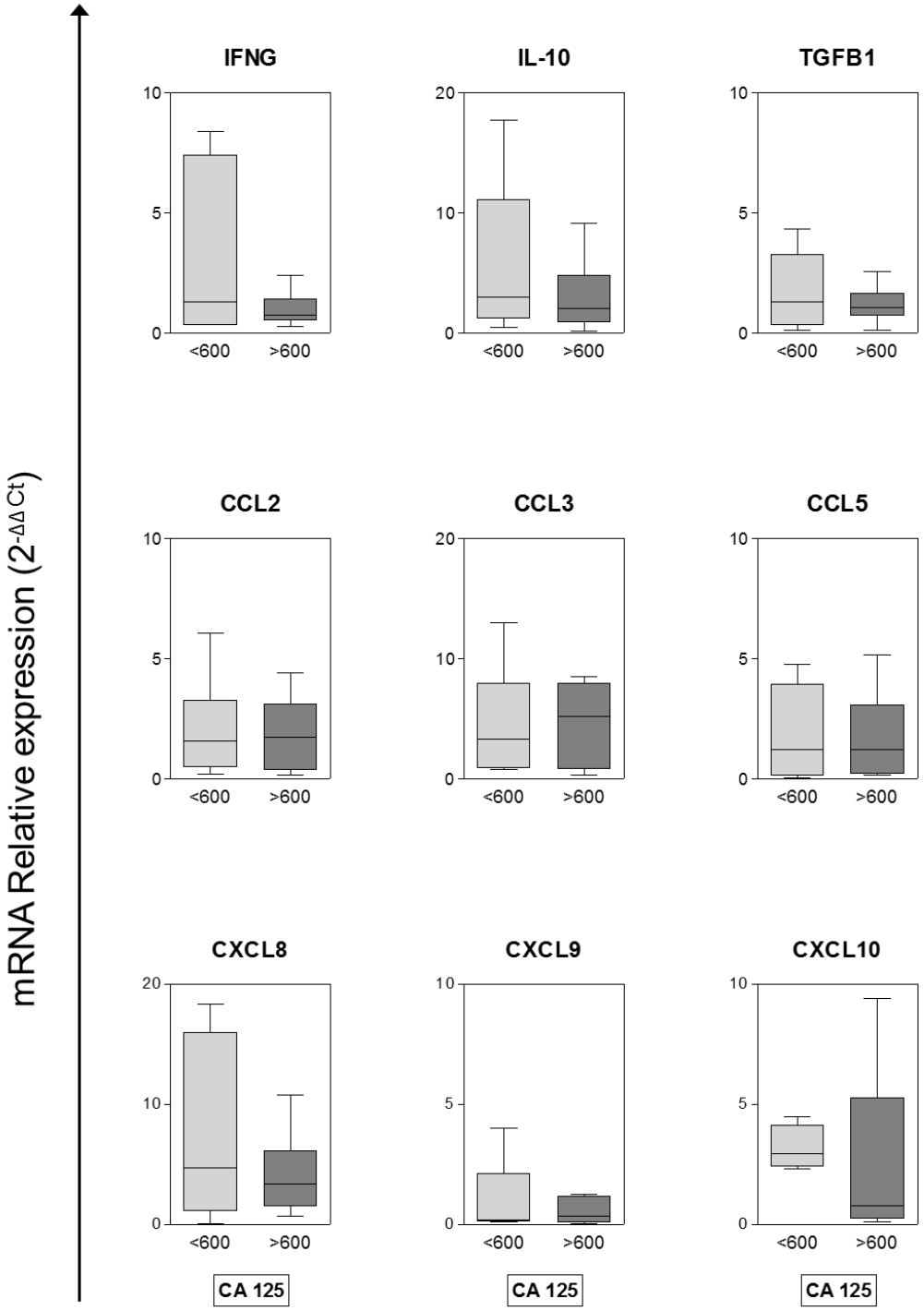

mRNA Relative expression (2-ΔΔCt)
IFNG
IL-10
TGFB1
CCL2
CCL3
CCL5
CXCL8
CXCL9
CXCL10
<600
>600
CA 125

FIGURE 3

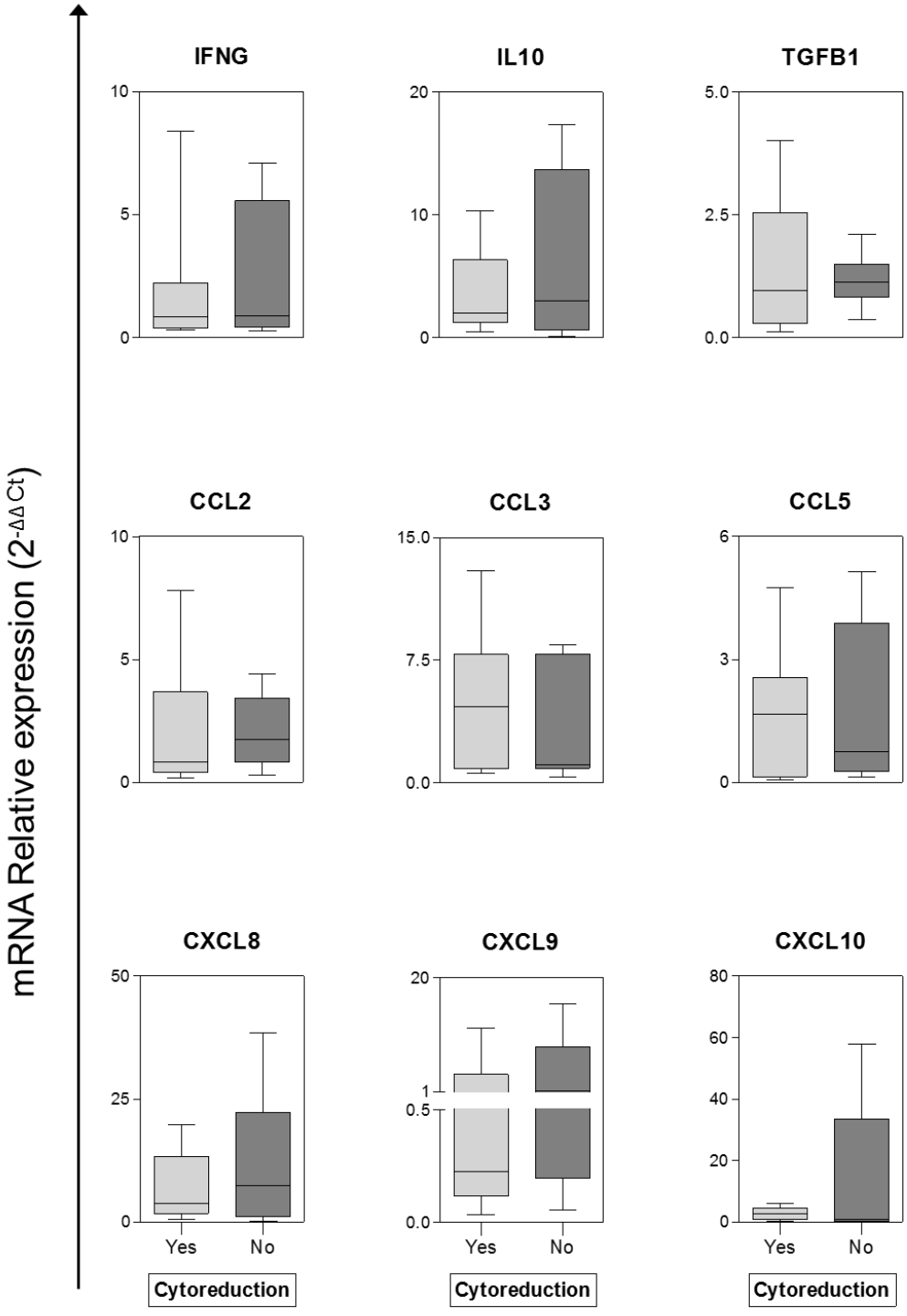

mRNA Relative expression ($2^{-\Delta\Delta Ct}$)
IFNG
IL10
TGFB1
CCL2
CCL3
CCL5
CXCL8
CXCL9
CXCL10
Yes
No
Cytoreduction

Figure 4

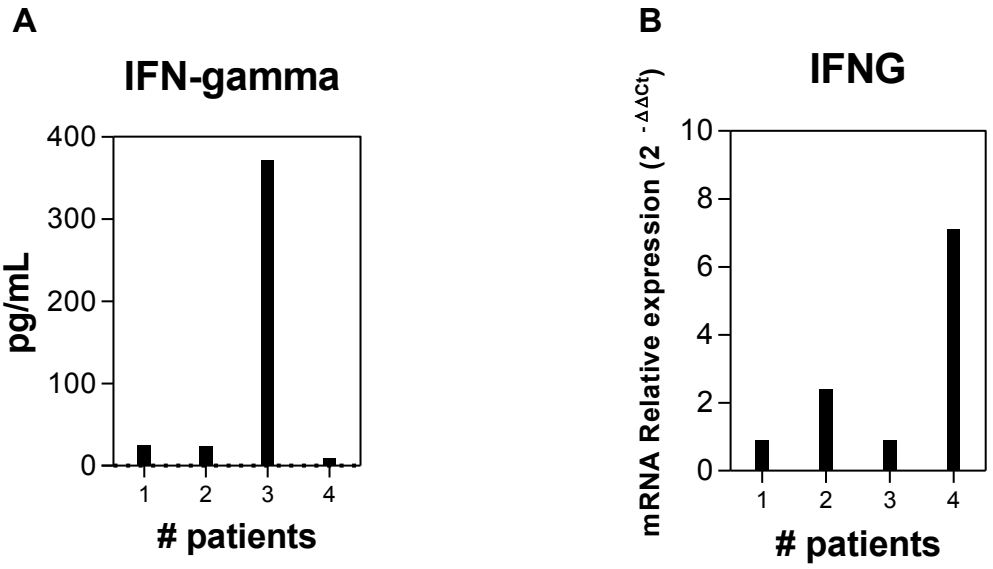
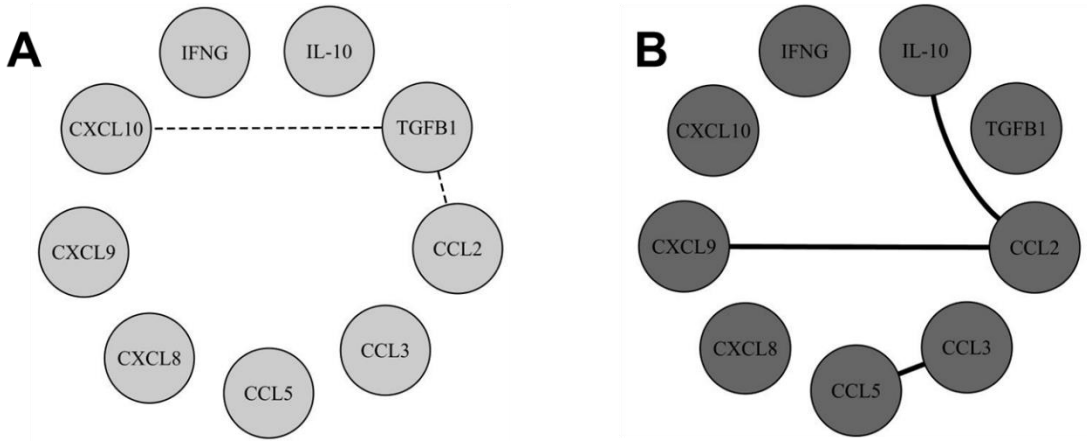

A
IFN-gamma
pg/mL
400
300
200
100
0
1
2
3
4
patients
B
IFNG
mRNA Relative expression (2 - ΔΔCt)
10
8
6
4
2
0
1
2
3
4
patients

Figure 5


A
IFNG
IL-10
CXCL10
TGFB1
CXCL9
CCL2
CXCL8
CCL3
CCL5
B
IFNG
IL-10
CXCL10
TGFB1
CXCL9
CCL2
CXCL8
CCL3
CCL5

4. Considerações Finais

CONSIDERAÇÕES FINAIS

O estudo da resposta imune sistêmica e compartimentalizada em pacientes portadoras de câncer de ovário associados aos genes *IFNG, CCL2, CCL3, CXCL8, CXCL9, CXCL10, IL10, CCL5, TGFB1* e citocinas e quimiocinas IL-1-β, IL-6, IL-10, TNF, IL-12p70; IL-2, IL-4, IFN-γ, IL-17A, CXCL8, CXCL9, CXCL10, CCL5 e CCL2 permite concluir que:

- ✓ Foi detectado o aumento da expressão gênica no grupo das mulheres com CEO tipo II quando comparado com o tipo I, para os genes *IL-10, CXCL8* e *CXCL9*. Houve uma diminuição na expressão de *TGFB1*, *CCL2* e *CXCL10* no grupo de mulheres com CEO tipo II quando comparado ao tipo I. Os resultados suportam a hipótese de que existe uma interferência intercelular entre as células do CEO e o infiltrado de células do sistema imunológico, sugerindo um microambiente imunológico mais supressivo no CEO tipo I, quando comparado com CEO tipo II.
- ✓ Com a metodologia de análise proposta, não foi possível detectar níveis mínimos necessários de citocinas e quimiocinas no líquido ascítico ou lavado peritoneal em um número suficiente de casos para realização da análise estatística.
- ✓ Não houve diferença significativa com relação aos níveis de expressão gênica associados aos níveis séricos de CA-125, citorredução ótima, estadiamento e mortalidade em mulheres com CEO tipo I quando comparadas com tipo II.

- ✓ Enquanto no tumor do tipo I as correlações existentes entre *TGFB1*, *CCL-2* e *CXCL-10* são negativas, no tumor do tipo 2 elas são fortes e positivas e embora envolvam também a participação de CCL-2, as interações com essa quimiocinas ocorrem com *IL-10* e *CXCL-9*. Há ainda uma correlação positiva entre duas quimiocinas responsáveis pelo recrutamento de células com potencial pró-inflamatório (*CCL-5* e *CCL-3*). Essa interação pode estar contribuindo para um desfecho clínico mais grave da patologia. De forma geral, podemos dizer que as redes do tumor do tipo I e tipo II reforçam a existência de duas entidades clínicas distintas.

Anexos

ANEXO I. Parecer do Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais

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

Parecer nº. ETIC 326/08

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

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 17 de setembro de 2008, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"Avaliação da resposta imune em pacientes com câncer ginecológico"** 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 - 2º andar – Sala 2005 – Cep: 31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.uling.br

Anexo II. Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

AVALIAÇÃO DA RESPOSTA IMUNE EM PACIENTES COM CÂNCER GINECOLÓGICO

A senhora está sendo convidada a participar de um projeto de pesquisa que visa investigar a presença de algumas substâncias no sangue, ascite (líquido localizado no abdome) e no útero ou ovário que possam nos ajudar a fazer o diagnóstico precoce das doenças desse órgão.

A participação no estudo consiste em doar uma amostra de sangue, ascite e um fragmento da peça cirúrgica (útero ou ovário) para serem analisados. Essa participação não modifica o tratamento proposto para a sua doença.

A sua identidade será preservada e o seu direito de não participar no estudo não a prejudicará no seu tratamento.

1. DESCRIÇÃO DAS COMPLICAÇÕES DOS MÉTODOS: não haverá aumento do risco de complicações devido à retirada de uma biópsia do útero ou ovário. A coleta de sangue será realizada no momento da punção para administração do soro. As complicações serão as mesmas relacionadas ao procedimento cirúrgico (retirada do útero e/ou ovário).

2. DESCRIÇÃO DA ANESTESIA: poderá haver necessidade de me submeter à anestesia para a realização da cirurgia proposta. O tipo de anestesia vai depender do procedimento realizado, podendo ser bloqueio (anestesia peridural ou raque) ou geral.

3. DESTINO DA PEÇA OPERATÓRIA: a peça cirúrgica será encaminhada à Anatomia Patológica para ser examinada.

4. Recebi todas as informações que desejava conhecer e a possibilidade de fazer perguntas e questionar dúvidas.

5. Também entendi que, a qualquer momento e sem necessidade de dar nenhuma explicação poderei suspender o consentimento que agora presto.

Investigador: Prof. Agnaldo L. Silva Filho

Endereço: Avenida Professor Alfredo Balena 110/4^{0}andar. Santa Efigênia. Belo Horizonte. Minas Gerais. CEP: 30 130 100. Tel: (31) 32489764 / 92250909

Dra. Andréa Teixeira de Carvalho: Tel (31) 33497764

COEP/UFMG:

Endereço: Av. Pres. Antônio Carlos, 6627 - Unid. Adm. II - 2º andar - Sl 2005 - Pampulha - Belo Horizonte/MG - 31.270-090 Tel: (31) 34094592

Nome: Registro:

Endereço:

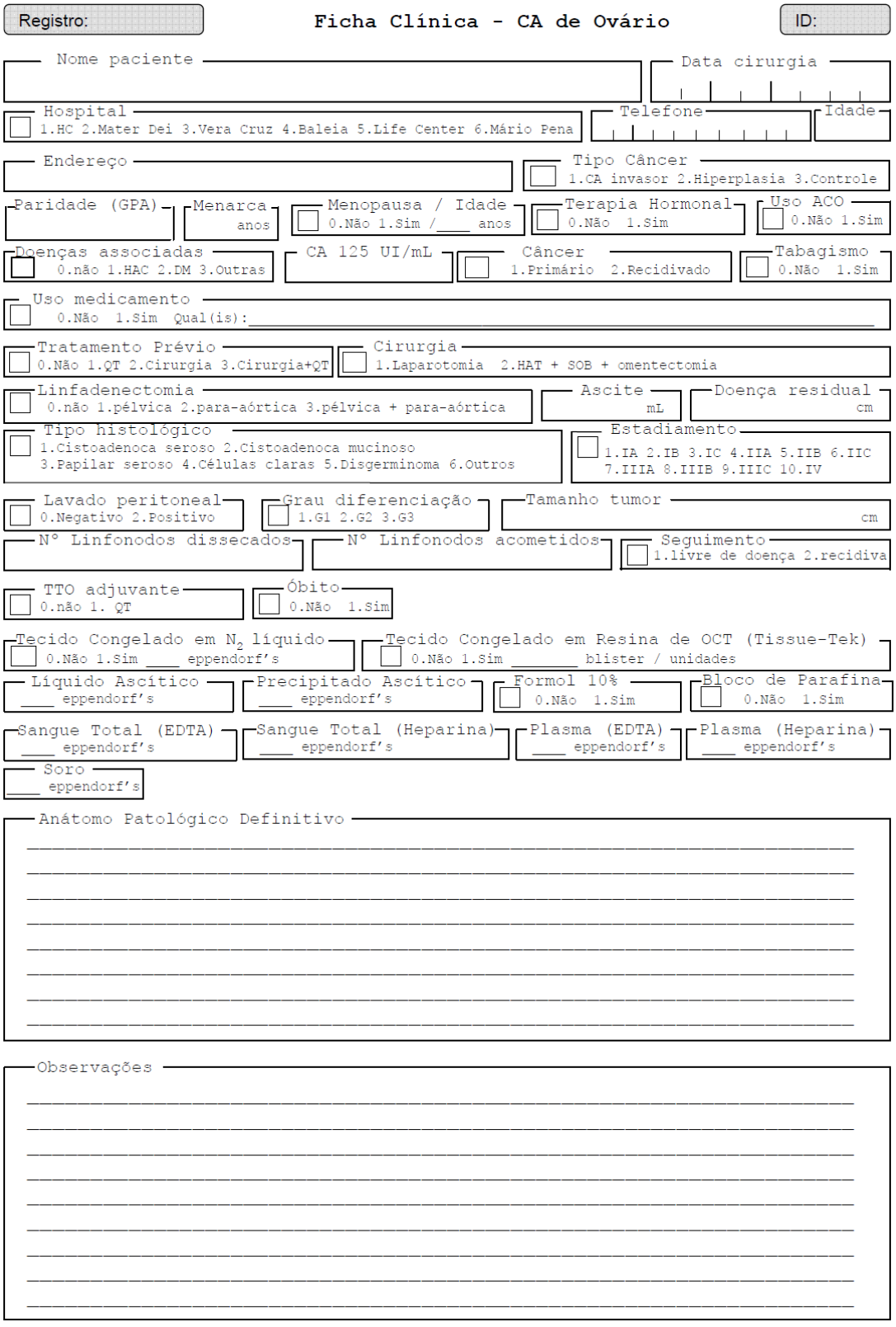
Telefone: Carteira de identidade:

De pleno acordo

Cidade: Data:

Assinatura do médico	Assinatura da paciente
Testemunha	Testemunha

Anexo 3. Ficha clínica



Registro:

Ficha Clínica - CA de Ovário

ID:

Nome paciente

Data cirurgia

Hospital
☐ 1.HC 2.Mater Dei 3.Vera Cruz 4.Baleia 5.Life Center 6.Mário Pena

Telefone

Idade

Endereço

Tipo Câncer
☐ 1.CA invasor 2.Hiperplasia 3.Controle

Paridade (GPA)

Menarca
anos

Menopausa / Idade
☐ 0.Não 1.Sim /____ anos

Terapia Hormonal
☐ 0.Não 1.Sim

Uso ACO
☐ 0.Não 1.Sim

Doenças associadas
☐ 0.não 1.HAC 2.DM 3.Outras

CA 125 UI/mL

Câncer
☐ 1.Primário 2.Recidivado

Tabagismo
☐ 0.Não 1.Sim

Uso medicamento
☐ 0.Não 1.Sim Qual(is):_____

Tratamento Prévio
☐ 0.Não 1.QT 2.Cirurgia 3.Cirurgia+QT

Cirurgia
☐ 1.Laparotomia 2.HAT + SOB + omentectomia

Linfadenectomia
☐ 0.não 1.pélvica 2.para-aórtica 3.pélvica + para-aórtica

Ascite
mL

Doença residual
cm

Tipo histológico
☐ 1.Cistoadenoca seroso 2.Cistoadenoca mucinoso
3.Papilar seroso 4.Células claras 5.Disgerminoma 6.Outros

Estadiamento
☐ 1.IA 2.IB 3.IC 4.IIA 5.IIB 6.IIC
7.IIIA 8.IIIB 9.IIIC 10.IV

Lavado peritoneal
☐ 0.Negativo 2.Positivo

Grau diferenciação
☐ 1.G1 2.G2 3.G3

Tamanho tumor
cm

N° Linfonodos dissecados

N° Linfonodos acometidos

Seguimento
☐ 1.livre de doença 2.recidiva

TTO adjuvante
☐ 0.não 1. QT

Óbito
☐ 0.Não 1.Sim

Tecido Congelado em N_2 líquido
☐ 0.Não 1.Sim ____ eppendorf's

Tecido Congelado em Resina de OCT (Tissue-Tek)
☐ 0.Não 1.Sim _____ blister / unidades

Líquido Ascítico
____ eppendorf's

Precipitado Ascítico
____ eppendorf's

Formol 10%
☐ 0.Não 1.Sim

Bloco de Parafina
☐ 0.Não 1.Sim

Sangue Total (EDTA)
____ eppendorf's

Sangue Total (Heparina)
____ eppendorf's

Plasma (EDTA)
____ eppendorf's

Plasma (Heparina)
____ eppendorf's

Soro
____ eppendorf's

Anátomo Patológico Definitivo

Observações