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Original Article

Correlation between cytokines and hematological parameters in ovarian cancer

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ABSTRACT

The tumor microenvironment in which ovarian malignant neoplasia develops has been described as enriched with a broad spectrum of proinflammatory cytokines and chemokines that may influence the clinical state and prognosis. The aim of the study was to correlate the dosage of interleukins IL-2, IL-5, IL-6, IL-8, IL-10, and nitric oxide (NO) metabolites in serum, peritoneal and intracystic fluid with hematological parameters of patients with malignant ovarian neoplasia. We evaluated 29 patients diagnosed with primary ovarian malignancy. IL-2, IL-5, IL-6, IL-8 and IL-10 concentrations were quantified by enzyme-linked immunosorbent assay (ELISA). Colorimetric assay was performed for the measurement of nitric oxide (NO) metabolites. Cytokine concentrations were evaluated in serum, peritoneal and intracystic fluids prior to surgical treatment. The results were analyzed by Spearman test. Evaluation of serum showed an inverse correlation of IL-8 and IL-10 with absolute value of lymphocytes, and direct correlation with NLR and PLR; there was also an inverse correlation of iNOS with NLR and direct relationship with red blood cell count (RBC). Evaluation of intracystic fluid showed an inverse correlation of IL-6 and RBC, leukocytes and lymphocytes; direct correlation of IL-8 with platelet-lymphocyte (PLR); inverse correlation of NO metabolites with neutrophil-lymphocyte (NLR) and PLR, and direct correlation with lymphocytes. Evaluation of peritoneal fluid showed an inverse correlation of IL-6 and RBC, absolute lymphocyte value, and hemoglobin. Some cytokines, such as IL-6, IL-8, IL-10 and nitric oxide metabolites, correlate with blood count parameters that influence disease prognosis, such as anemia, absolute lymphocyte value, NLR, and PLR. Thus, these cytokines could be considered new prognostic factors in ovarian cancer, guiding the clinical oncologist for better treatment and follow-up, and being potential targets in the study of new treatments.

KEYWORDS: ovarian cancer, cytokines, correlation, hematological parameters.

INTRODUCTION

Ovarian cancer is the sixth most common cancer and the fifth most common cause of death in women in the United States. About 90% of the cases are epithelial histological type. Currently, it is considered that there is not a single risk factor implicated in the etiology of this type of tumor [1].

Epithelial ovarian cancer is a highly lethal gynecological cancer for which the overall prognosis has remained poor in recent decades, accounting for about 2.5% of all cancers among women, leading to 5% overall cancer deaths in this population [2].

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The tumoral environment in which ovarian carcinoma develops has been described as enriched with a broad spectrum of proinflammatory cytokines and chemokines. In particular, several of these cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, produced by the tumor itself and/or activated immune cells, in addition to stimulating the growth of cancer cells, seem to influence the clinical status and prognosis, reducing the response to chemotherapy and inducing symptoms such as anorexia, altered energy metabolism, anemia, weight loss, depression and fatigue [3].

Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), play an important role in the pathophysiology of anemia in cancer patients. The mechanisms of anemia are related to inflammation and genetic regulatory aspects of erythropoiesis *via* GATA-1 and GATA-2 [4].

New biomarkers for ovarian cancer diagnosis and prognosis are needed. Concentration of some cytokines such as IL-2, IL-5, IL-6, IL-8, IL-10 and nitric oxide (NO) metabolites in serum, intracystic fluid and peritoneal fluid may aid in the diagnosis of ovarian malignancy, and could be prognostic markers of this disease. To this end, our objectives are to correlate the dosage of these cytokines in serum, peritoneal fluid and intracystic fluid with hematological parameters and preoperative tumor markers of patients with malignant ovarian neoplasia.

PATIENTS AND METHODS

Twenty-nine patients diagnosed with primary malignant ovarian cancer treated at the Pelvic Mass Outpatient Clinic of the Department of Gynecology and Obstetrics/Oncology Research Institute (IPON) of the Federal University of Triângulo Mineiro – UFTM were evaluated. The patients underwent surgical treatment according to pre-established criteria [5, 6] from May 2009 to December 2016.

Inclusion criteria were postoperative diagnosis of primary ovarian malignant neoplasia by paraffin anatomopathology, and exclusion criteria were presence of adnexal pedicle torsion, cyst rupture during surgery, secondary ovarian malignancy (metastasis), antineoplastic treatment prior to surgery, relapse, endometrioma, and autoimmune systemic diseases.

The following data from the medical records were recorded in a specific database for the study: age, histological type, histological grade, staging (FIGO), type I and type II carcinogenesis model (in case of epithelial ovarian tumors), laboratory tests and the results of the experiments. Regarding laboratory tests, information on blood count (hemoglobin, absolute value of neutrophils and lymphocytes, platelets) was verified.

Neutrophil/lymphocyte ratio (NLR) and platelet/ lymphocyte ratio (PLR) values were obtained by dividing the absolute number of neutrophils and platelets by the absolute number of lymphocytes. The cutoff value was 2.6 for NRL [7] and 300 for PLR [8].

The study was reviewed and approved by the Research Ethics Committee of the Federal University of Triângulo Mineiro under protocol number 1408. Free written informed consent was obtained from each patient or their family members.

Intracystic fluid collection

All ovarian tumors were punctured immediately after cyst excision to obtain 20 ml of the intracystic content by slow aspiration and then centrifuged at 1500 rpm for 10 minutes. The resulting supernatant was collected and stored in 300 μL aliquots in a freezer at -20 °C until cytokine and NO metabolite dosing [9].

Collection of peritoneal fluid

The peritoneal fluid was collected through peritoneal lavage in surgeries for ovarian tumors. When there was ascites, it was collected. For peritoneal lavage, 100 mL of 0.9% saline was injected into the peritoneal cavity and 20 mL was removed. The peritoneal lavage was centrifuged (2000 rpm, for 10 min) and the supernatant collected was stored (-20 °C) until cytokine dosing.

Enzyme-linked immunosorbent assay (ELISA)

IL-2, IL-5, IL-6, IL-8 and IL-10 concentrations were quantified by enzyme-linked immunosorbent assay (ELISA). For antibody capture, 96-well plates were coated with 50 μL/well of antibody specific for each of the above cytokines, diluted (1 to 3 μg/μL) in binding buffer (Na_2HPO_4) and

incubated for 16-24 hours at a temperature of 4 ºC. The plates were then washed three times with 0.05% PBS/Tween 20 and nonspecific binding was blocked by 1% PBS/BSA (100 µl/well) and incubated for 2 hours at 37 °C. The samples and standards were loaded onto plates (50 µl/well) and incubated for 16-24 hours at a temperature of 4 °C. For the standard curve, previously diluted human recombinant antibodies (PBS/Tween 20) were used. The plates were washed and then biotinylated anti-cytokine antibody (0.5 to 1 mg/ml) diluted in blocking buffer (PBS/1% BSA) was added. After 1 hour incubation at 37 °C the plates were washed with PBS/Tween 20 and 100 µl/well of avidin-peroxidase conjugate, previously diluted in blocking buffer (1:5000), was added. The plate was incubated again for 30 minutes at room temperature. After this time the plates were washed and then the staining reaction was performed by adding 100 µL of the o-phenylenediamine dihydrochloride-OPD substrate. The plates were then incubated at room temperature for a further 15 to 20 minutes. The reaction was then stopped by adding 50 µL of 1 M H_2SO_4, and absorbance measured at 490 nm in an ELISA plate reader. Results were expressed in pictograms of each cytokine dosed per milliliter of the intracystic fluid supernatant [10].

Colorimetric assay for the measurement of nitric oxide (NO) metabolites

The quantification of nitrate in patients' intracystic fluid samples according to their group was determined by enzymatic reduction of nitrite with nitrate reductase, as described by Schmidt *et al*., in 1994 [11]. Samples (40 µL) were incubated with same volume of buffer reductase (0.1 M potassium phosphate, pH 7.5) containing 1 mM nicotinamide phosphate ademine dinucleotide-NADPH, 10 mM flavin adenine didinucleotide-FAD and 4 IU nitrate reductase/mL for 20 hours at 37 ºC. A standard nitrate curve was determined by incubating sodium nitrate (10 to 200 µM) with buffer reductase. The total amount of NO metabolites in these cystic fluid samples was then determined by the Griess reaction-based colorimetric assay [12]. Absorbance was measured at 546 nm. Results were expressed as micromol (µM) of nitrite (NO-2) in intracystic fluid samples.

Statistical analysis

Data were analyzed using GraphPad Prism software 6 and IBM SPSS Statistics 20. According to distribution (D'Agostino & Pearson test), results with non-normal distributions were expressed as medians (minimum and maximum values). Correlations of cytokine IL-2, IL-5, IL-6, IL-8, IL-10 and nitric oxide (NO) metabolite concentrations in serum, peritoneal fluid and intracystic fluid with haematological parameters of patients with ovarian cancer were performed by the Spearman test, with a significance level <0.05.

RESULTS

Twenty-nine patients diagnosed with primary ovarian malignancy were evaluated. The average age was 53.45 ± 16.85 years.

On histological diagnosis of malignant ovarian tumors, we found 6 (20.7%) serous cystadeno-carcinomas, 6 (20.7%) borderline mucinous tumors, 5 (17.2%) granulosa cell tumors, 2 (6.9%) clear cell adenocarcinomas, 2 (6.9%) borderline serous tumors, 2 (6.9%) dysgerminomas, 1 (3.4%) immature teratoma, 1 (3.4%) endometrioid adenocarcinoma, 1 (3.4%) large cell adenocarcinoma, 1 (3.4%) high grade neoplasia, 1 (3.4%) germinative cell tumor, and 1 (3.4%) endodermal sinus tumor.

Staging of ovarian malignant tumors was performed according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO) and we found 12 (41.4%) IA, 2 (6.9%) IB, 6 (20.7%) IC, 1 (3.4%) IIA1, 1 (3.4%) IIIA1, 3 (10.3%) IIIB3, and 4 (13.8%) IIIC.

Table 1 shows the correlations of serum cytokine levels with hematological factors of patients with malignant ovarian neoplasia. There was an inverse correlation of IL-8 with absolute lymphocyte value ($r = -0.499$ and $p = 0.01$), as well as an inverse correlation of IL-10 with absolute lymphocyte value ($r = -0.394$ and $p = 0.046$). There was a direct correlation between IL-8 and IL-10 levels with NLR ($r = 0.519$ and $p = 0.007$; $r = 0.401$ and $p = 0.042$, respectively) and PLR ($r = 0.547$ and $p = 0.004$; $r = 0.478$ and $p = 0.014$, respectively), and inverse correlation of iNOS with NLR ($r = -0.428$ and $p = 0.021$). iNOS was also directly correlated to RBC ($r = 0.883$ and $p = 0.0001$). For the other cytokines no statistical significance was found in this analysis.

Table 1. Correlations of serum cytokine levels with hematological factors of patients with malignant ovarian neoplasia.

		Hemoglobin		**Lymphocytes**		**NRL**		**PLR**		**Red blood cell**		**Hematocrit**		**Leukocyte**	
		r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Serum cytokines	IL-2	-0.052	0.802	-0.037	0.857	0.186	0,363	0.098	0.635	0.007	0.973	-0.091	0.657	0.308	0.126
	IL-5	-0.138	0.503	-0.103	0.615	0.048	0,816	0.049	0.814	-0.133	0.517	-0.136	0.508	0.267	0.188
	IL-6	-0.129	0. 530	-0.220	0.281	0.310	0.123	0.296	0.142	-0.105	0.609	-0.171	0.405	0.339	0.090
	IL-8	-0.285	0.159	**-0.499[a]**	**0.01**	**0.519[a]**	**0,007**	**0.547[a]**	**0.004**	-0.291	0.150	-0.330	0.099	0.110	0.594
	IL-10	-0.342	0.087	**-0.394[a]**	**0.046**	**0.401[a]**	**0.042**	**0.478[a]**	**0.014**	-0.204	0.317	-0.297	0.140	0.162	0.428
	iNOS	0.189	0.355	-0.074	0.720	**-0.428[a]**	**0.021**	-0.028	0.893	**0.883[a]**	**0.0001**	0.250	0.218	0.062	0.751

[a]Correlation is significant at 0.05 level (Spearman's r Test).

Table 2 shows the correlations of cytokine quantification in the intracystic fluid with hematological factors of patients with malignant ovarian neoplasia. There was an inverse correlation of IL-6 with red blood cell value (r = -0.494 and p = 0.019), an inverse correlation of IL-6 with absolute leukocyte value (r = -0.426 and p = 0.048) and an inverse correlation between IL-6 and absolute lymphocyte value (r = -0.606 and p = 0.003). Regarding IL-8, there was a direct correlation with PLR (r = 0.426 and p = 0.043). Regarding iNOS, there was an inverse correlation with both NLR (r = -0.57 and p = 0.04) and PLR (r = -0.454 and p = 0.03), and direct correlation with lymphocytes (r = 0.439 and p = 0.036). For the other cytokines no statistical significance was found in this analysis.

Table 3 shows the correlations of cytokine quantification in peritoneal fluid with hematologic factors of patients with malignant ovarian neoplasia. There was an inverse correlation of IL-6 with red blood cell value (r = -0.433 and p = 0.019), an inverse correlation of IL-6 with absolute lymphocyte value (r = -0.428 and p = 0.021), an inverse correlation between IL-6 and hemoglobin (r = -0.443 and p = 0.016), and direct correlation of IL-6 with hematocrit value (r = -0.440 and p = 0.017). For the other cytokines no statistical significance was found in this analysis.

DISCUSSION

Malignant neoplasm-related inflammation plays a significant role in morbidity and mortality associated with solid tumors such as ovarian cancer, and is a factor associated with debilitating symptoms such as fatigue, thromboembolism, cachexia, and anemia [13].

By correlating the interleukin IL-2, IL-5, IL-6, IL-8, IL-10 dosages, and nitric oxide (NO) metabolites in serum, peritoneal fluid and intracystic fluid with haematological parameters of patients with malignant ovarian neoplasia, we found that some of them (IL-6, IL-8, IL-10 and nitric oxide metabolites) are statistically correlated with parameters that influence the prognosis of the disease.

Elevated levels of various cytokines have been reported in ovarian cancer [14, 15].

Neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) are new inflammatory biomarkers used as prognostic factors in various diseases, including ovarian cancer [16-18].

Scientific evidence suggests that neutrophils and platelets are associated with pro-tumor activities, such as increased angiogenesis that contributes to tumor cell proliferation and promotes metastatic potential [19, 20].

IL-8 is a proangiogenic cytokine and its secretion is related to proliferation, adhesion and tumor invasion in ovarian cancer [21]. In our study, in serum, there was an inverse correlation of IL-8 with absolute lymphocyte value, a direct correlation with NLR e PLR, and in intracystic fluid a direct correlation of IL-8 with PLR, which is consistent with the study of Sanguinete *et al.* (2017) [15]. IL-8 plays an important role in tumor angiogenesis and may accelerate tumor angiogenesis and promote ovarian cancer progression [22, 23].

Lower hemoglobin concentrations in ovarian cancer patients compared to healthy controls have already been seen, as in the study by Qin *et al*. (2017) [24], as inflammatory factors may affect iron metabolism and inhibit erythropoietin expression and maturation of erythrocytes.

In the study by Martins-Filho *et al*. (2017) [14], IL-6 levels were higher in patients with low hemoglobin. Increased plasma levels of IL-6 have been associated with unfavorable prognosis in many cancers. Inflammation has been firmly associated with anemia through IL-6 up-regulation leading to increased production of hepcidin iron regulatory peptide, resulting in iron sequestration by macrophages and decreased iron absorption by the gastrointestinal tract. These observations identified IL-6 as a potential target for the treatment of cancer-related anemia [13]. In our study, in intracystic fluid, there was an inverse correlation of IL-6 with red blood cells (RBC) value, inverse correlation of IL-6 with absolute leukocyte value and inverse correlation between IL-6 and absolute lymphocyte value. In the peritoneal fluid there was an inverse correlation of IL-6 with red blood cell value, inverse correlation of IL-6 with absolute lymphocyte value, inverse correlation between IL-6 and hemoglobin, and direct correlation of IL-6 with hematocrit value.

IL-6 may increase inflammatory proteins causing not only anemia, but also fever, fatigue, lipolysis,

Table 2. Correlations of cytokine quantification in the intracystic fluid with hematological factors of patients with malignant ovarian neoplasia.

		Hemoglobin		**Lymphocytes**		**NRL**		**PLR**		**Red blood cell**		**Hematocrit**		**Leukocyte**	
		r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Cytokines in peritoneal fluid	IL-2	-0.059	0.762	-0.202	0.293	0.100	0.607	-0.012	0.950	-0.121	0.531	-0.113	0.561	0.050	0.799
	IL-5	0.289	0.129	0.104	0.592	0.033	0.867	-0.243	0.203	0.114	0.555	0.249	0.192	0.027	0.889
	IL-6	**-0.443**[a]	**0.016**	**-0428**[a]	**0.021**	0.256	0.180	0.332	0.079	**-0.433**[a]	0.019	**0.440**[a]	**0.017**	0.059	0.763
	IL-8	-0.176	0.360	-0.120	0.534	0.081	0.675	0.214	0.265	-0.017	0.929	-0.096	0.621	-0.082	0.671
	IL-10	-0.144	0.456	0.063	0.744	-0.183	0.341	-0.211	0.271	-0.334	0.077	-0.209	0.276	-0.145	0.452
	iNOS	-0.191	0.322	-0.234	0.222	0.229	0.232	0.175	0.363	-0.114	0.555	-0.117	0.547	0.091	0.638

[a]Correlation is significant at 0.05 level (Spearman's r Test).

Table 3. Correlations of cytokine quantification in the peritoneal fluid with hematological factors of patients with malignant ovarian neoplasia.

		Hemoglobin		**Lymphocytes**		**NRL**		**PLR**		**Red blood cell**		**Hematocrit**		**Leukocyte**	
		r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Cytokines in intracystic fluid	IL-2	-0.383	0.071	-0.068	0.757	0.109	0.621	0.107	0.626	-0.319	0.138	-0.345	0.107	0.355	0.096
	IL-5	0.347	0.105	0.033	0.883	0.014	0.948	-0.174	0.427	0.250	0.251	0.317	0.141	-0.331	0.123
	IL-6	-0.362	0.097	**-0.606**[a]	**0.003**	0.145	0.521	0.295	0.182	**-0.494**[a]	**0.019**	-0.355	0.105	**-0.426**[a]	**0.048**
	IL-8	-0.384	0.070	-0.386	0.069	0.281	0.194	**0.426**[a]	**0.043**	-0.283	0.191	-0.369	0.083	-0.210	0.335
	IL-10	0.181	0.408	-0.214	0.326	0.187	0.393	0.099	0.654	0.029	0.895	0.205	0.349	-0.150	0.494
	iNOS	0.344	0.108	**0.439**[a]	**0.036**	**-0.57**[a]	**0.04**	**-0.454**[a]	**0.03**	0.315	0.143	0.327	0.127	-0.271	0.211

[a]Correlation is significant at 0.05 level (Spearman's r Test).

skeletal muscle catabolism and chemoresistance of tumor cells. Therefore, there is a strong association between elevated serum IL-6 levels and a high degree of tumor progression and a fall in the patient's general condition [25].

Chronic disease anemia refers to impaired red blood cell production associated with chronic inflammatory states, including cancer, chronic infection, or autoimmune diseases. Current research discusses the role of proinflammatory cytokines and iron biology in the pathophysiology of the disease. Among the main contributing factors is the relationship between the action of interleukin-6 and the hepcidin-ferroportin axis, in which IL-6 is a potent inducer of hepcidin, an iron-regulating peptide hormone that contributes to hemoglobin homeostasis [26].

Increased serum IL-6 levels are strongly related to low levels of red blood cells and hemoglobin and, therefore, increased incidence of anemia and its severity [26]. This is in line with the findings from our study, which demonstrate an inverse correlation of IL-6 levels in intracitic fluid, peritoneal lavage, and serum, with red blood cell and hemoglobin values.

IL-10 is related to the escape of neoplastic malignant cells from immune surveillance [27, 28], including epithelial ovarian cancer [29]. Several studies have shown an increase in IL-6 and IL-10 in serum or peritoneal fluid of ovarian cancer patients compared to patients with benign ovarian tumors [30, 31].

Our results demonstrate an inverse correlation of IL-10 and absolute lymphocyte value ($r = -0.394$ and $p = 0.046$) and a direct correlation with NLR e PLR in the serum of patients with malignant ovarian neoplasia.

Nitric oxide (NO) is a multifunctional molecule that plays a multifaceted role in cancer biology through multiple mechanisms [32]. The apparently contradictory roles of NO are attributed to factors such as differences in NOS isoform, expression level, cell line and tumor tissue heterogeneity [33]. While low NO levels may promote tumor progression by inducing cell proliferation, migration, invasion and angiogenesis, on the other hand, high NO levels may induce a cytotoxic effect that leads to tumor regression, tumor death and metastasis inhibition [34].

The drop in immunity is prominent in cancer patients and is probably of multifactorial origin. Factors contributing to the fall in general condition include anemia, weight loss, fever, pain, medication and infection. In cancer patients, many of these factors are influenced by a frequently disrupted balance between endogenous cytokine levels and their natural antagonists. Indeed, cancer cells and the immune system appear to overexpress a range of cytokines in patients with malignant neoplasms. Some of these cytokines act as autocrine or paracrine growth factors for neoplastic tissue and at the same time cause secondary symptoms related to impaired immunity and general condition [35].

Due to all of these mechanisms described, the relationship between increased levels of IL-6, IL-8 and IL-10 and low lymphocyte and leukocyte levels may suggest an alteration in tumor-related immune response. Such inference is corroborated in our study.

CONCLUSION

Some cytokines, such as IL-6, IL-8, IL-10 and nitric oxide metabolites, correlate with blood count parameters that influence disease prognosis, such as anemia, absolute lymphocyte value, NLR, and PLR. Thus, these cytokines could be considered new prognostic factors in ovarian cancer, guiding the clinical oncologist for better treatment and follow-up, and being potential targets in the study of new treatments.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest.

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