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# Immune Response Evaluation Through Determination of Type 1, Type 2, and Type 17 Patterns in Patients With Epithelial Ovarian Cancer

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## Abstract

Innate and adaptive immune cells secrete different cytokines, which participate through distinct mechanisms in cell-mediated immunity and humoral immune responses. The aim of this study was to evaluate the immune response through analysis of type 1 (Th1), Th2, and Th17 cells in patients with epithelial ovarian cancer (EOC). Our study included 44 patients with EOC (study group) and 32 gynecological patients with no ovarian disease (control group). Fragments of ovarian tissue and blood samples were collected in both groups and aliquots of intracystic fluid and peritoneal fluid were recovered from the EOC patient group. Interleukin (IL)-2/IL-4/IL-6/IL-10/IL-17/tumor necrosis factor (TNF)- $\alpha$ /interferon (IFN)- $\gamma$  levels were measured by cytometric bead array. Statistical analysis included chi-squared, Student *t*, Mann-Whitney, Kruskal-Wallis tests, and Cox regression model. Patients with EOC were associated with higher levels of TNF- $\alpha$ /IL-4/IL-6/IL-10 compared to the control group. Both IL-10 and TNF- $\alpha$  concentrations were higher in patients with stage III/IV EOC and also associated with higher levels of cancer antigen 125. Higher Th1-mediated immune response was observed when the cytoreduction was considered optimal. However, patients with EOC with unsatisfactory cytoreductive surgery and undifferentiated tumors were associated with higher concentrations of Th2 cytokines in the 4 sites studied. Higher IL-6/IL-10 and lower IFN- $\gamma$  concentrations were also associated with a lower overall survival rate in patients with EOC. The EOC group presented a predominantly Th2 response and an immunosuppressant standard and had association between IL-6/IL-10/IFN- $\gamma$  and prognosis.

## Keywords

epithelial ovarian cancer, adaptive immune response, flow cytometry, cytokines, type 1, type 2, type 17.

## Introduction

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.<sup>1</sup> Almost 75% of the patients are diagnosed in the advanced stages (III and IV)<sup>1,2</sup> and therefore have a very poor prognosis. 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.<sup>3</sup> Immune response is characterized by the division of CD4+ T-helper (Th) cells into 2 functional subsets, type 1 (Th1) cells and Th2 cells.<sup>4</sup> The former secretes interleukin (IL)-2, tumor necrosis factor (TNF)- $\alpha$ , and interferon (IFN)- $\gamma$  and is responsible for promoting cell-mediated immunity to activate macrophages and killer T-cells.<sup>5</sup> The latter secretes IL-4, IL-6, and IL-10<sup>5</sup> and is associated with humoral immune

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responses, which trigger the differentiation of B-cells to antibody-producing cells.<sup>5</sup>

Unlike normal cells, many malignant cells constitutively produce small amounts of TNF. There is evidence from animal models that malignant cell-derived TNF enhances the growth and spread of tumors in the skin, ovary, pancreas, pleural cavity, and bowel.<sup>6,7</sup> The IL-10 has the ability to inhibit differentiation, maturation, and activation of immune cells (for instance by inhibiting antigen presentation and IL-12 production) in processes in which dendritic cells (DCs) are involved. Moreover, IL-10 enhances spontaneous apoptosis, thereby interfering with the induction of an antitumor response and protects cancer cells from cytotoxic T lymphocytes by downregulating the human leukocyte antigen class I and II.<sup>8</sup> Interleukin 6 is a proinflammatory cytokine that can cause uncontrolled proliferation in epithelial cancers.<sup>9</sup> Over the past years, increasing evidence has shown that IL-6 is an important factor in promoting the progression of EOC.<sup>10</sup> Interleukin 4 is involved in many immunomodulatory activities such as overlapping of Th2 response and inhibition of Th1 activity, the production of immunoglobulin G (IgG) and IgE, and abnormal expression of MHC II.<sup>11</sup> In vivo and in vitro studies using receptor blockade of IL-4 by monoclonal antibodies, in ovarian cancer models, have shown significant tumor regression.<sup>12,13</sup>

In EOC, the shift in the immune response from Th1 toward Th2, a process mediated by cytokines, is considered to be one of the escape mechanisms to prevent tumor cell destruction.<sup>14</sup> The TILs correlate positively and strongly with patient survival<sup>15</sup> and the influx and activities of TILs are mediated by cytokines. The cytokine profile is, therefore, a reflection of the host immune status and indicates the type of immune response.<sup>16</sup>

The Th17 cells are a newly identified subset of effector CD4+ Th cells. The Th17 cells secrete IL-17 and play an active role in the inflammatory process, in cell-mediated immunity, and in the development of human tumors.<sup>17</sup> Through synergistic action between IL-17 and IFN- $\gamma$ , Th17 cells induce Th1-type cytokines and active effector cells controlling tumor growth. In contrast, inhibition of Th17 cells represents a novel immune evasion mechanism.<sup>18</sup>

Serum cancer antigen 125 (CA-125) is a cell surface glycoprotein used as a biomarker, which has shown to be elevated in the sera of patients with EOC as well as in other malignancies and certain benign gynecologic diseases such as endometriosis. The CA-125 is elevated in approximately 80% of the patients with EOC, but in only 50% of the patients with stage I disease.<sup>19</sup>

The aim of this study was to evaluate the immune response during EOC through analysis of Th1, Th2, and Th17 profile by measuring the cytokine (L-2/IL-4/IL-6/IL-10/IL-17/TNF- $\alpha$ /IFN- $\gamma$ ) levels in the blood, fragments of the ovarian tissue, and in the intracystic and peritoneal fluid of these patients and in gynecological patients with normal healthy ovaries. The CA-125 levels were also assessed in both the study groups.

## Materials and Methods

### Patients

This prospective study was approved and performed in accordance with the guidelines of the Ethical Committee for Research in Human Beings of the Federal University of Minas Gerais. A written informed consent was obtained from all the patients participating in the study. A total of 98 patients admitted to the Department of Obstetrics and Gynecology at Federal University of Minas Gerais between November 2008 and July 2010, with a pelvic mass suspected to be associated with ovarian tumor were examined. Following operative treatment and histopathology examination, 44 patients with stage I, II, III, or IV EOC (study group; mean age:  $58.7 \pm 12.6$  years, range: 33-78 years) and 32 patients with normal healthy ovaries (control group; mean age:  $62.3 \pm 8.17$  years, range: 49-74 years) were included in this study. Exclusion criteria were (i) patients with a previous surgical intervention associated with a malignant neoplasia or transplantation, (ii) autoimmune disease, (iii) diabetes, or (iv) signs of infection. Patients from both the groups had an overall parity ranging from 0 to 6 births (mean  $2.1 \pm 0.6$  births).

Tumor removal was performed by laparotomy and surgical cytoreduction was done in all patients with EOC. Optimal cytoreduction was defined as leaving no residual disease greater than 1 cm of diameter after primary surgery.

The ovarian cancer was classified after pathologic examination (stage I, II, III, or IV), according to the International Federation of Gynecology and Obstetrics (FIGO) classification and protocol.<sup>20</sup> All tissues removed during surgery were examined by a pathologist; tumor grade (G1: well-differentiated tumor, G2: moderately differentiated tumor, and G3: undifferentiated tumor), histological type, and the presence of metastases were assessed and classified according to FIGO and World Health Organization classifications.<sup>20</sup> The histological classification of the malignant ovarian tissue collected revealed that all cases were serous carcinoma.

All patients were submitted to adjuvant chemotherapy (carboplatin and paclitaxel). Even the 12 with early disease were classified as stage cystic fluid (IC) from the FIGO.

None of the patients were submitted to surgery interval, even when the surgeries were considered incomplete, for not staying with tumors larger than 3.0 cm in its largest diameter. In these cases, we chose to follow-up every 6 months with imaging and measurement of CA-125 after adjuvant chemotherapy.

Patients with ovarian cancer were followed postoperatively according to the protocol of the gynecologic oncology at the Hospital das Clínicas, with quarterly reviews in the first year and half since then. Besides the history and physical examination, the patients were followed up with computed tomography (CT) and serum marker CA-125. Recurrences were diagnosed when changes appeared in the imaging methods or at least 2 serial measurements of the marker CA-125 showed increase.

The control group patients, when indicating hysterectomy for benign disease (fibroids and endometrial hyperplasia), by

**Table 1.** Characteristics of the Patients With EOC (Study Group) and Gynecological Patients With No Ovarian Disease (Control Group).

|                    | Normal Ovary,<br>n = 32 | EOC, n = 44         |                       | <i>P</i> <sup>a</sup> |
|--------------------|-------------------------|---------------------|-----------------------|-----------------------|
|                    |                         | Stages I/II, n = 12 | Stages III/IV, n = 32 |                       |
| Age                | 62.3 ± 1.4              | 54.7 ± 1.5          | 60.1 ± 1.3            | .135                  |
| Parity             | 2.1 ± 0.3               | 1.6 ± 0.2           | 2.1 ± 0.3             | .236                  |
| CA-125 (U/mL)      | –                       | 327.2 (82.3-3195)   | 353.2 (48.7-605)      | .699                  |
| Tumor grade        |                         |                     |                       | <b>.001</b>           |
| G1                 | –                       | 2 (16.7%)           | 0                     |                       |
| G2                 | –                       | 10 (83.3%)          | 16 (50%)              |                       |
| G3                 | –                       | 0                   | 16 (50%)              |                       |
| Cytoreduction      |                         |                     |                       | <b>.001</b>           |
| Optimal (<1 cm)    | –                       | 12 (100%)           | 10 (31.2%)            |                       |
| Suboptimal (>1 cm) | –                       | 0                   | 22 (68.8%)            |                       |
| Recurrence         |                         |                     |                       | <b>.001</b>           |
| No                 | –                       | 12 (100%)           | 14 (43.8%)            |                       |
| Yes                | –                       | 0                   | 18 (56.3%)            |                       |
| Death              |                         |                     |                       | <b>.003</b>           |
| No                 | –                       | 12 (100%)           | 18 (56.3%)            |                       |
| Yes                | –                       | 0                   | 14 (43.7%)            |                       |

Abbreviations: CA-125, serum marker for epithelial ovarian tumors; G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, undifferentiated tumor; EOC, epithelial ovarian cancer.

<sup>a</sup>The bold face values signify the differences between I/II stages and III/IV stages for tumor grade, cytoreduction, recurrence, and death.

patient desire (high-risk family history), by the surgeon option (intraoperative findings), or for technical reasons (bleeding vascular pedicle and without possibility of maintaining the gonad), were submitted to oophorectomy.

### Cytokines and CA-125 Analysis

Peripheral blood samples in the presence of EDTA Vacutainer (BD Pharmingen, San Diego, California) as anticoagulant were collected in both the groups of patients, before anesthesia and surgery. Aliquots of intracystic and peritoneal fluids of patients with EOC were also collected. Blood samples, intracystic, and peritoneal fluids were centrifuged and stored at –80°C until laboratory analysis was performed. Serum CA-125 concentrations were determined using an immunoassay (ChemiLumi ACS-CA 125 II; Bayer Medical Co Ltd, Tokyo, Japan) and expressed in U/mL. Disease progression was defined by a CA-125 ≥35 U/mL value found in 2 or more occasions, observation of growing lesions, or presence of new lesions on a CT scan or at the patient's death.<sup>21</sup>

Preserved tissue samples were macerated in liquid nitrogen and resuspended in 1 × phosphate-buffered saline. Then, protein inhibitors (phenylmethanesulfonylfluoride and EDTA) were added. The BD cytometric bead array (CBA) human Th1/Th2/Th17 cytokine kit was used to analyze cytokine levels in all samples. The dilution curve was made according to the standard from the kit and assay diluents and then all the 7 beads corresponding to 7 cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFN-γ) were pooled. All samples were thawed in a water bath (37°C) and then centrifuged, and the supernatant was recovered. Subsequently, the supernatant of the samples and the standard curve were added to the bead

mixture and reagent B (detection reagent which is a mixture of phycoerythrin [PE]-conjugated antibodies, providing a fluorescent signal in proportion to the amount of bound analyte). The samples were incubated for 3 hours at room temperature. Sandwich complexes (capture bead + analyte + detection reagent) were formed. The intensity of PE fluorescence of each sandwich complex revealed the concentration of each cytokine. Complexes were measured using flow cytometry (FacsCalibur, San Jose, California) and data analysis was made using the BD CBA analysis software. The concentrations of cytokines were expressed in pg/mL.

### Statistical Analysis

All statistical analyses were performed using the computational software of SPSS 18.0 (SPSS Inc, Chicago, Illinois). Median (50th percentile) and 25th and 75th percentiles were calculated for the CA-125 values and cytokine levels were measured in the 4 distinct locations. The CA-125, IL-6, IL-10, and IFN-γ levels were divided into 2 groups according to the median (cut-off point) and data were analyzed by chi-squared analyses and by the Fischer exact test. Comparison between unpaired groups was made using the Mann-Whitney test or the Kruskal-Wallis test. The recurrence and survival curves were drawn with the product-limit estimator according to Cox regression. Data were expressed as mean ± standard error of mean, unless otherwise stated. Differences between means were calculated by the Student *t* test. *P* < .05 was considered significant.

### Results

There were no differences between the groups regarding age and parity (*P* = .135 and *P* = .256, respectively; Table 1).

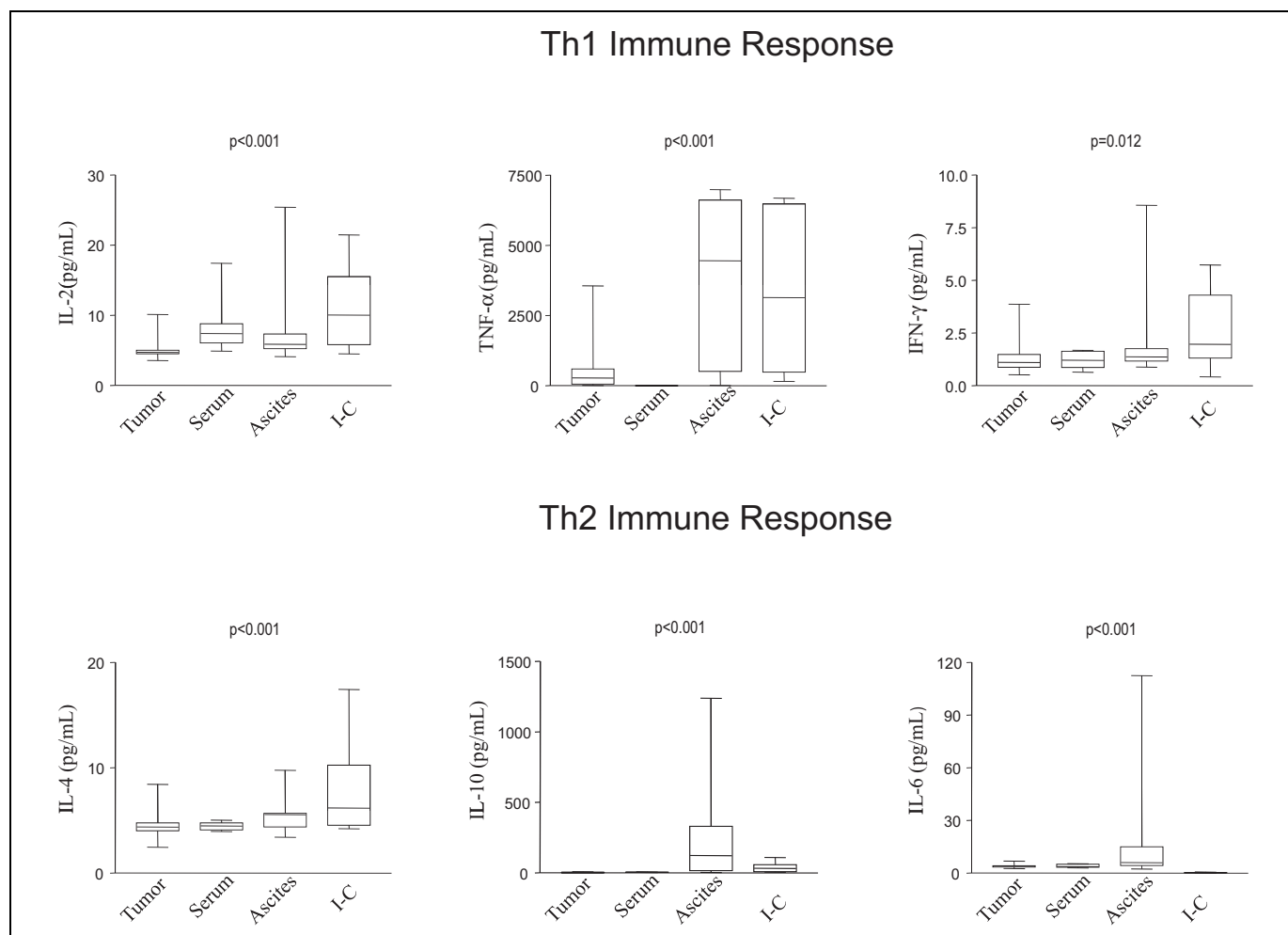


**Figure 1.** Interleukin (IL)-2, IL-4, IL-6, IL-10, IFN- $\gamma$ , and TNF- $\alpha$  levels (50th, 25th, and 75th percentiles) in the malignant ovarian tissue, intracystic fluid, peritoneal fluid, and serum of patients with EOC (IC indicates cystic fluid; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; EOC, epithelial ovarian cancer).

In the group of patients with EOC, 12 (27.2%) had stage I/II and 32 (72.7%) had stage III/IV ovarian cancer. Among these patients, intraperitoneal and intracystic fluid was observed in 38 (86.3%) and in 20 (45.5%) patients, respectively. The distribution of patients according to the tumor grade is shown in Table 1. There was an association between the grade of the tumor and the ovarian cancer stage, with a higher percentage of patients in stages III/IV with tumors G2 and G3 ( $P = .001$ ; Table 1). Optimal cytoreduction occurred in 12 patients with stages I/II. In stages III/IV, optimal cytoreduction was observed in only 10 of the 32 patients with EOC. No recurrence or death was observed in stages I/II, whereas in stages III/IV, 18 recurrences (56.3%) and 14 deaths (43.7%) were registered at 18-month follow-up.

The IL-17 levels were detected in 6 (15.7%) peritoneal fluid samples and in 2 (10%) samples of the intracystic fluid of women with EOC. There were no detectable concentrations of IL-17 in the ovarian tissue samples and in the serum of patients with EOC. In the control group, IL-17 was detected in 5 (15%) of the serum samples, but not in the healthy ovarian

tissue samples. Thus, this parameter was excluded from the statistical analysis. The detection rate of TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-4, IL-6, and IL-10 in patients with EOC was above 90% for the ovarian tissue, peritoneal fluid, intracystic fluid, and serum.

Higher concentrations of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, and IL-10 were detected in the intracystic fluid, when compared to those measured in the serum and in the ovarian tissue. This difference was more pronounced for TNF- $\alpha$  ( $P < .001$ ). Moreover, the concentrations of TNF- $\alpha$ , IL-4, IL-6, and IL-10 in the peritoneal fluid were significantly higher than in the serum and tumor sites (Figure 1).

The TNF- $\alpha$  ( $P < .001$ ), IL-4 ( $P < .001$ ), IL-6 ( $P = .018$ ), and IL-10 ( $P < .001$ ) concentrations in the ovarian tissue and TNF- $\alpha$  ( $P = .048$ ) serum levels were significantly higher in patients with EOC (Table 2). There were no differences in the seric levels of IFN- $\gamma$ , IL-4, IL-6, and IL-10 between EOC and the control groups. The IL-2 levels in the serum and IFN- $\gamma$  levels in the ovarian tissue were higher in the control group (Table 2).

The IL-10 levels in the peritoneal fluid and in the malignant ovarian tissue were higher in patients with stage III/IV ovarian

**Table 2.** Comparison Between Control Group and EOC Patients: Concentrations of IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ /IFN- $\gamma$  Cytokines in the Tumor and Serum Sites.

| Cytokine      | Control       | EOC                | <i>p</i> <sup>a</sup> |
|---------------|---------------|--------------------|-----------------------|
| <b>Tumor</b>  |               |                    |                       |
| IL-2          | 4.7 (4.1-5.1) | 4.7 (4.5-5.1)      | .863                  |
| IFN- $\gamma$ | 1.3 (0.9-2.0) | 1.1 (0.9-1.5)      | <b>.024</b>           |
| TNF- $\alpha$ | 3.1 (2.8-3.6) | 275.3 (56.3-597.9) | <b>&lt;.001</b>       |
| IL-4          | 3.8 (3.7-4.1) | 4.4 (4.0-4.8)      | <b>&lt;.001</b>       |
| IL-6          | 3.5 (3.2-3.7) | 4.0 (3.8-4.2)      | <b>.018</b>           |
| IL-10         | 2.7 (2.5-3.0) | 3.5 (2.9-5.6)      | <b>&lt;.001</b>       |
| <b>Serum</b>  |               |                    |                       |
| IL-2          | 7.7 (7.6-9.8) | 7.4 (6.1-8.8)      | <b>.001</b>           |
| IFN- $\gamma$ | 1.3 (1.1-1.6) | 1.2 (0.9-1.6)      | .151                  |
| TNF- $\alpha$ | 4.2 (4.1-4.4) | 23.5 (9.2-42.7)    | <b>.048</b>           |
| IL-4          | 4.1 (4.0-4.2) | 4.5 (4.1-4.8)      | .199                  |
| IL-6          | 3.5 (3.3-4.5) | 3.6 (3.4-5.1)      | .083                  |
| IL-10         | 3.7 (3.4-4.4) | 4.8 (3.7-4.9)      | .113                  |

Abbreviations: IC, cystic fluid; IL, cytokine; IFN, interferon; TNF, tumor necrosis factor; EOC, epithelial ovarian cancer.

<sup>a</sup>The bold face values signify the differences between the groups and show higher levels of type 2 cytokines in the EOC patients and higher levels of type 1 cytokines in the control group.

cancer. The TNF- $\alpha$  levels in the peritoneal fluid ( $P = .028$ ) and serum ( $P = .027$ ) were higher in stages III/IV. The levels of IL-4, IL-6, and IL-10 in the peritoneal and intracystic fluid showed no differences according to the ovarian cancer stage (Table 3).

Cytokine levels of patients with EOC according to ovarian cancer stage, cytoreduction, and tumor grade are shown in Table 3. Higher Th1-mediated immune response was observed when the cytoreduction was considered optimal. This was confirmed by the higher concentrations of IFN- $\gamma$  ( $P < .001$ ) in the ovarian cancer tissue and higher IL-2 levels ( $P < .001$ ) in serum (Table 3).

Higher concentrations of Th2 cytokines in the 4 sites studied were associated with unsatisfactory first cytoreductive surgery and undifferentiated tumors. Patients with unsatisfactory cytoreduction had higher levels of IL-4 ( $P = .033$ ) and IL-10 ( $P = .017$ ) in the peritoneal fluid, IL-10 ( $P = .030$ ) in the intracystic fluid, IL-4 ( $P < .001$ ), IL-6 ( $P = .005$ ), and IL-10 ( $P < .001$ ) in the tumor site, and IL-6 ( $P = .035$ ) and IL-10 ( $P = .048$ ) in the serum (Table 3). In patients with undifferentiated tumors, higher levels of IL-10 were observed in the peritoneal fluid. Furthermore, higher levels of IL-4, IL-6, and IL-10 were observed in the intracystic fluid of patients with undifferentiated tumors, with higher levels of IL-6 also being observed in the tumor site.

There was an association between higher levels of CA-125 and higher concentrations of IL-10 in the intracystic fluid ( $P = .004$ ). Moreover, elevated serum levels of TNF- $\alpha$  were associated with higher levels of CA-125 (Table 4).

We did not find an association between cytokine levels in different study sites and the rates of disease-free survival. Nonetheless, a lower overall survival rate was seen for patients with EOC with levels of IL-6  $\geq 6$  and  $\geq 4$  pg/mL in the peritoneal fluid and in the malignant ovarian tissue, respectively (Figure 2A and B).

Similarly, patients with IL-10  $\geq 3.5$  and  $\geq 123$  pg/mL levels in the tumor and in the peritoneal fluid, respectively, had a lower overall survival rate (Figure 3A and B). Analysis of the intracystic fluid of patients with EOC showed that significantly higher overall survival over time was achieved when IFN- $\gamma$  levels were equal or superior to 1.9 pg/mL (Figure 4).

## Discussion

The present study evaluated the levels of cytokines that could potentially be used as tumor markers for detecting ovarian cancer. Our study investigated the cell-mediated immune response profile in 4 different types of samples and the presence of key extracellular cytokines was assessed. The analysis of compartmentalized and systemic immune response could contribute to a better understanding of the conflicting results reported in the literature on the association between cytokines levels and EOC. Except for the higher levels of TNF- $\alpha$  observed in the ovarian tissue and serum of patients with EOC, higher concentrations of Th1 cytokines were found in the normal ovarian tissue (IFN- $\gamma$ ) and serum (IL-2) of the control group, when compared to patients with ovarian cancer. This observation has been reported earlier by other authors.<sup>22,23</sup>

In a recent study, no significant differences in the levels of the cytokines tested were observed between the patients with EOC and the control group.<sup>22</sup> In patients with EOC, higher concentrations of cytokine levels were observed in the intracystic and peritoneal fluid when compared to those present in the blood or in the ovarian tissue, suggesting the occurrence of a more intense immune response at sites of tumor growth and spread, which agrees with other studies.<sup>22,24</sup>

In the present study, TNF- $\alpha$  levels were significantly higher in patients with advanced EOC when compared to the control group. High values of this cytokine in the blood, ovarian cancer tissue, and presence of peritoneal fluid and CA-125  $\geq 350$  U/mL were significantly associated with unsatisfactory tumor cytoreduction (that are associated with a poor diagnosis). Corroborating with these findings, a study with an ovarian cancer model found that TNF- $\alpha$  is an important component of a malignant cell-autonomous network of inflammatory cytokines, including the cytokine IL-6 and macrophage inhibitory factor as well as vascular endothelial growth factor.<sup>6,14</sup> Also, it is known that TNF- $\alpha$  plays a role in the interactions between tumor cells and macrophages, which leads to increased tumor cell invasion and the generation of a tumor-associated macrophage phenotype that has been associated with tumor development and poor prognosis.<sup>25</sup>

The Th1 response is activated by IFN- $\gamma$ , whereas Th2 response is activated by IL-4 and IL-10. Polarized macrophages differ in terms of the production and function of cytokines and in their receptor expression. The tumor inflammatory microenvironment facilitates the breakage of the basal membrane in the process of cancer invasion of the surrounding tissues and metastases. Both IL-6 and IL-10 have been shown to inhibit inflammatory responses and crosstalk between innate and adaptive immune responses in human cancers, thereby favoring unrestrained tumor growth.<sup>5,26</sup>

**Table 3.** Comparison Between Concentrations of IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , IFN- $\gamma$  and Staging, Cyto reduction in Primary Surgery, and Tumor Grade in the 4 Different Sites.

| Cytokine       | Stages                |                          |                | Optimal Cyto reduction |                        |                 | Tumor Grade |                       |                        |
|----------------|-----------------------|--------------------------|----------------|------------------------|------------------------|-----------------|-------------|-----------------------|------------------------|
|                | I/II                  | III/IV                   | P <sup>a</sup> | No                     | Yes                    | P <sup>a</sup>  | I           | II                    | III                    |
| <b>Ascites</b> |                       |                          |                |                        |                        |                 |             |                       |                        |
| IL-2           | 4.7 (4.6-5.0)         | 4.9 (4.0-6.2)            | .371           | 6.1 (4.6-13.8)         | 5.9 (5.3-7.0)          | .859            | —           | 5.9 (5.7-10.7)        | 6.1 (4.5-7.3)          |
| IFN- $\gamma$  | 1.1 (0.9-1.5)         | 0.9 (0.8-1.1)            | .063           | 1.3 (1.1-2.3)          | 1.4 (1.2-1.8)          | .859            | —           | 1.3 (1.1-1.7)         | 1.4 (1.2-2.5)          |
| TNF- $\alpha$  | 106.4 (23.5-395.8)    | 335.1 (61.1-597.9)       | <b>.028</b>    | 6207.2 (4215.9-6610.7) | 778.9 (67.9-5669.2)    | <b>.012</b>     | —           | 3717.1 (512.9-6319.9) | 6235.3 (1576.6-6610.7) |
| IL-4           | 4.3 (4.0-5.7)         | 4.40 (4.0-4.5)           | .309           | 5.6 (5.2-5.9)          | 4.9 (4.2-5.5)          | <b>.033</b>     | —           | 5.6 (4.4-5.6)         | 5.4 (4.4-5.9)          |
| IL-6           | 3.9 (3.5-4.1)         | 4.1 (3.3-5.1)            | .309           | 5.7 (3.9-22.9)         | 6.5 (4.8-14.9)         | .492            | —           | 6.1 (4.1-11.5)        | 8.36 (4.5-29.1)        |
| IL-10          | 3.0 (2.9-4.8)         | 3.6 (3.1-5.6)            | <b>.003</b>    | 178.5 (61.6-561.3)     | 46.60 (3.8-255.3)      | <b>.017</b>     | —           | 82.9 (10.3-310.9)     | 214.9 (66.8-654.3)     |
| <b>I-C</b>     |                       |                          |                |                        |                        |                 |             |                       |                        |
| IL-2           | 7.4 (6.1-8.8)         | 5.4 (4.5-6.1)            | .284           | 10.9 (8.3-16.8)        | 6.4 (5.1-14.8)         | .284            | —           | 11.8 (10.1-18.5)      | 6.4 (5.1-14.8)         |
| IFN- $\gamma$  | 1.2 (0.9-1.4)         | 0.9 (0.8-0.9)            | .721           | 1.7 (1.3-4.3)          | 2.2 (1.1-4.1)          | .617            | —           | 1.97 (1.4-5.1)        | 2.22 (1.1-4.1)         |
| TNF- $\alpha$  | 8.6 (8.0-9.5)         | 31.0 (12.2-42.7)         | .721           | 3261.2 (254.1-6493.1)  | 3132.9 (1894.4-6420.7) | .617            | —           | 5961.1 (561.3-6670.4) | 3132.9 (1894.4-6420.7) |
| IL-4           | 4.5 (4.4-4.8)         | 4.6 (4.4-4.9)            | .284           | 8.1 (4.8-15.7)         | 6.2 (4.5-7.3)          | .284            | —           | 6.2 (4.5-7.3)         | 10.3 (5.8-17.4)        |
| IL-6           | 3.8 (3.4-5.1)         | 4.2 (3.6-6.7)            | .721           | 8.9 (4.1-13.8)         | 7.6 (5.8-11.1)         | .721            | —           | 7.6 (5.8-11.1)        | 12.1 (5.7-14.4)        |
| IL-10          | 4.3 (3.7-4.8)         | 5.0 (4.3-5.8)            | .153           | 38.6 (10.4-82.8)       | 25.5 (7.6-32.9)        | <b>.030</b>     | —           | 18.9 (32.1-33.1)      | 38.6 (10.4-82.8)       |
| <b>Tumor</b>   |                       |                          |                |                        |                        |                 |             |                       |                        |
| IL-2           | 10.9 (8.3-16.8)       | 6.4 (5.1-14.8)           | .931           | 4.8 (4.2-6.16)         | 4.6 (4.5-5.0)          | .693            | —           | 4.7 (4.0-4.9)         | 4.7 (4.5-5.6)          |
| IFN- $\gamma$  | 2.2 (1.3-4.6)         | 1.7 (1.1-4.1)            | .730           | 1.1 (0.9-1.4)          | 1.4 (0.8-1.9)          | <b>.007</b>     | —           | 0.9 (0.7-1.4)         | 1.3 (1.0-1.7)          |
| TNF- $\alpha$  | 3261.2 (254.0-6493.1) | 3132.9 (1894.4-6420.6)   | .121           | 292.5 (47.3-547.2)     | 56.3 (191.8-841.9)     | <b>&lt;.001</b> | —           | 292.5 (27.6-517.6)    | 293.4 (58.5-780.9)     |
| IL-4           | 8.1 (4.8-15.1)        | 6.1 (4.5-7.3)            | .762           | 4.8 (4.0-5.5)          | 4.4 (3.8-4.8)          | <b>&lt;.001</b> | —           | 4.4 (4.0-5.0)         | 4.3 (3.9-4.5)          |
| IL-6           | 8.9 (4.7-13.8)        | 7.6 (5.8-11.1)           | .196           | 4.3 (3.5-5.1)          | 3.9 (3.5-4.8)          | <b>.005</b>     | —           | 3.7 (3.5-4.0)         | 4.3 (4.1-4.9)          |
| IL-10          | 25.5 (7.6-32.9)       | 38.6 (10.4-82.8)         | <b>.047</b>    | 3.7 (3.1-6.6)          | 3.2 (2.8-4.6)          | <b>&lt;.001</b> | —           | 2.9 (2.7-6.6)         | 3.6 (3.2-5.4)          |
| <b>Serum</b>   |                       |                          |                |                        |                        |                 |             |                       |                        |
| IL-2           | 5.8 (4.8-12.9)        | 6.3 (4.2-6.8)            | .463           | 7.3 (5.8-10.9)         | 7.5 (7.4-7.7)          | <b>&lt;.001</b> | —           | 7.4 (6.1-8.8)         | 7.1 (6.4-8.2)          |
| IFN- $\gamma$  | 1.4 (0.93-3.2)        | 1.0 (0.9-1.8)            | .141           | 1.0 (0.9-1.2)          | 1.3 (0.9-1.6)          | .955            | —           | 1.3 (0.9-1.6)         | 0.8 (0.6-1.1)          |
| TNF- $\alpha$  | 1045.0 (118.4-5018.5) | 5572.06 (636.80-6610.69) | <b>.027</b>    | 23.5 (4.2-42.7)        | 23.5 (11.5-41.1)       | .955            | —           | 23.5 (9.2-42.7)       | 19.5 (8.5-36.3)        |
| IL-4           | 4.8 (4.0-6.7)         | 5.6 (4.8-5.7)            | .141           | 4.3 (4.1-4.5)          | 4.6 (4.3-4.8)          | .211            | —           | 4.5 (4.1-4.8)         | 5.2 (3.3-8.1)          |
| IL-6           | 5.4 (3.9-19.1)        | 6.4 (4.9-14.9)           | .462           | 3.9 (3.4-5.1)          | 3.2 (3.1-3.5)          | <b>.035</b>     | —           | 3.6 (3.4-5.1)         | 4.1 (3.8-5.9)          |
| IL-10          | 10.3 (4.3-88.4)       | 178.4 (61.6-561.3)       | .141           | 4.8 (3.8-4.9)          | 4.2 (3.5-4.9)          | <b>.048</b>     | —           | 4.8 (3.9-4.9)         | 5.8 (4.2-6.3)          |

Abbreviations: IC, cystic fluid; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; EOC, epithelial ovarian cancer.

<sup>a</sup>The bold face values signify the differences between type I and type 2 cytokines and show in the four sites a higher response in type 2 cytokines for the patients with advanced stages, unsatisfactory debulking, and undifferentiated tumors.

**Table 4.** Comparison Between IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , and IFN- $\gamma$  and Serum Levels of Tumor Marker CA-125 in Different Sites (Tumor, Cystic Fluid, Ascites, and Serum).

|          | CA-125                |                        |                |
|----------|-----------------------|------------------------|----------------|
| Cytokine | <350                  | ≥350                   | P <sup>a</sup> |
| Ascites  |                       |                        |                |
| IL-2     | 5.7 (4.7-9.1)         | 5.9 (5.6-7.2)          | .445           |
| INF-γ    | 1.5 (1.2-2.1)         | 1.3 (1.1-1.4)          | .556           |
| TNF-α    | 1045.0 (244.8-6731.7) | 5572.1 (2176.9-6319.9) | .638           |
| IL-4     | 5.2 (4.2-5.6)         | 5.6 (4.8-6.1)          | .078           |
| IL-6     | 6.9 (4.6-28.7)        | 5.4 (4.4-11.5)         | .158           |
| IL-10    | 82.9 (10.3-49.5)      | 155.8 (37.9-310.9)     | .860           |
| I-C      |                       |                        |                |
| IL-2     | 7.6 (6.4-10.1)        | 12.6 (5.3-18.5)        | .363           |
| INF-γ    | 1.9 (1.3-2.2)         | 3.5 (1.3-5.1)          | .363           |
| TNF-α    | 2391.8 (151.6-5961.1) | 6377.1 (561.3-6551.5)  | .069           |
| IL-4     | 6.4 (4.5-17.4)        | 6.2 (4.6-10.1)         | .585           |
| IL-6     | 11.2 (3.5-14.4)       | 7.6 (5.7-11.1)         | .716           |
| IL-10    | 3.9 (3.8-18.9)        | 38.6 (32.1-73.9)       | .004           |
| Tumor    |                       |                        |                |
| IL-2     | 4.7 (4.3-4.9)         | 4.9 (5.5-5.9)          | .121           |
| INF-γ    | 1.3 (0.9-1.4)         | 1.1 (0.9-1.7)          | .737           |
| TNF-α    | 309.6 (56.3-557.8)    | 191.8 (46.9-654.3)     | 1.000          |
| IL-4     | 4.4 (3.9-4.8)         | 4.3 (4.1-4.5)          | .867           |
| IL-6     | 3.8 (3.5-4.1)         | 4.0 (3.3-4.9)          | .401           |
| IL-10    | 3.5 (2.9-5.3)         | 3.5 (2.9-8.4)          | .742           |
| Serum    |                       |                        |                |
| IL-2     | 7.4 (7.2-7.5)         | 7.7 (5.8-10.9)         | .569           |
| INF-γ    | 1.3 (1.2-1.3)         | 1.1 (0.8-1.6)          | .569           |
| TNF-α    | 45.6 (42.7-48.6)      | 12.2 (7.9-27.3)        | .004           |
| IL-4     | 4.5 (4.4-4.5)         | 4.6 (4.1-4.5)          | .569           |
| IL-6     | 3.5 (93.4-3.6)        | 3.9 (3.4-5.1)          | .254           |
| IL-10    | 4.2 (3.5-4.8)         | 4.8 (3.9-4.9)          | .254           |

Abbreviations: IC, cystic fluid; IL, interleukin; INF, interferon; TNF, tumor necrosis factor; EOC, epithelial ovarian cancer.

<sup>a</sup>The bold face values signify higher levels of IL-10 and TNF-alpha in cystic fluid and serum sites when CA-125 serum levels were above 350 u/mL.

A microenvironment with elevated concentrations of anti-inflammatory/immunosuppressive cytokines such as IL-10-producing macrophages occurs in cancer cells that inhibit macrophage activation.<sup>27</sup> The macrophage secreting these cytokines is part of a distinct activation mechanism of the Th2 immune response.<sup>28</sup> Our data revealed significantly higher concentrations of the cytokine IFN- $\gamma$  in normal ovaries in the control group when compared to the tumor site. It was also shown that patients with ovarian cancer with higher levels of IFN- $\gamma$  in the IC had higher overall survival, indicating that high levels of IFN- $\gamma$  are associated with a better prognosis. In fact, the use of cytokines in cancer therapy has been previously evaluated.<sup>29</sup> Also, it is known that IFN- $\gamma$  can augment antitumor response and thereby is considered a promising agent in cancer therapy.<sup>30</sup> A Th1 IFN, IFN- $\alpha$ , is approved for the treatment of malignant melanoma and kidney cancer.<sup>28</sup> Granulocyte-macrophage colony-stimulating factor in combination with recombinant IFN- $\gamma$  and carboplatin in a phase

II trial has been shown to have a reasonable response against recurrent platinum-sensitive ovarian cancer.<sup>29,31</sup>

The present study showed that a positive association exists between factors associated with poor prognosis (such as residual tumor in primary surgery greater than 1 cm, CA-125 levels above 350 IU/mL, and undifferentiated tumors) and higher levels of IL-6 and IL-10. Patients with ovarian cancer with higher levels of IL-6 and IL-10 in the tumor and ascites had lower overall survival (Figure 2). Similar findings have been reported in the previous studies in which elevated IL-6 concentration in sera of patients with ovarian cancer correlated with poor overall survival.<sup>32,33</sup> In fact, our choice of measuring IL-6 in the microenvironment in which the malignant cells reside may provide better results than measuring IL-6 levels in the serum because this cytokine is released by the peritoneal mesothelial cells, tumor cells, and leukocytes present in the tumor (solid or cystic), and thus may be more rapidly cleared from circulation.<sup>14,34</sup> In support of this idea is the fact that higher levels of IL-6 in the tumor microenvironment are associated with resistance to chemotherapy drugs such as taxol. Higher levels of IL-6 in the tumor microenvironment are also associated with earlier disease recurrence.<sup>35</sup>

Interleukin 6 is directly involved with ovarian cancer angiogenesis and may be important in progression of ovarian cancer.<sup>36</sup> Interleukin 6 promotes the shift from Th1 to Th2 cell subtypes, contributing to the immune suppressive status in the tumor environment. This IL-6-dependent shift toward the formation of Th2 cells promotes apoptosis of tumor-associated macrophages and spreading of the tumor to the stroma of the ovary and potentially to other parts of the body (metastasis formation).

As mentioned earlier, we found that patients with EOC with higher levels of IL-10 in the tumor and ascites had lower overall survival (Figure 2). This finding is supported by many studies. For instance, it has been proposed that IL-10 modulates immunosuppressive response by itself or in conjunction with other cytokines, including TGF- $\beta$ . Also, IL-10 is known to play a role in downregulating the expression of Th1 cytokines, thus augmenting the immunosuppressive activity. The IL-10 suppresses the T-cell immune response against cancer cells by downregulating the expression of MHC class I and class II.<sup>8</sup> Interleukin 10 also downregulates costimulatory molecules such as CD40 expression on DCs in the tumor microenvironment and suppresses their maturation and functions. Dendritic cells derived from patients with progressive metastatic disease induce anergy in Th cells. Overexpression of IL-10 in IL-10 transgenic mice has been demonstrated to limit the ability of mice to control the growth of immunogenic tumors.<sup>37</sup> Data from clinical trials in which vaccines were tested against melanoma and other studies on IL-10 immunoadhesins support the critical role of IL-10 in the tumor microenvironment in modulating antitumor immune response.<sup>38,39</sup>

When comparing cytokine levels in the different sample sites, the present study identified an important trend for Th1 and Th2 immune responses in the control group and in the EOC patient group, respectively. This was also clear among the patients with EOC, with more advanced stages of the disease being associated with a more exacerbated Th2 cell response.

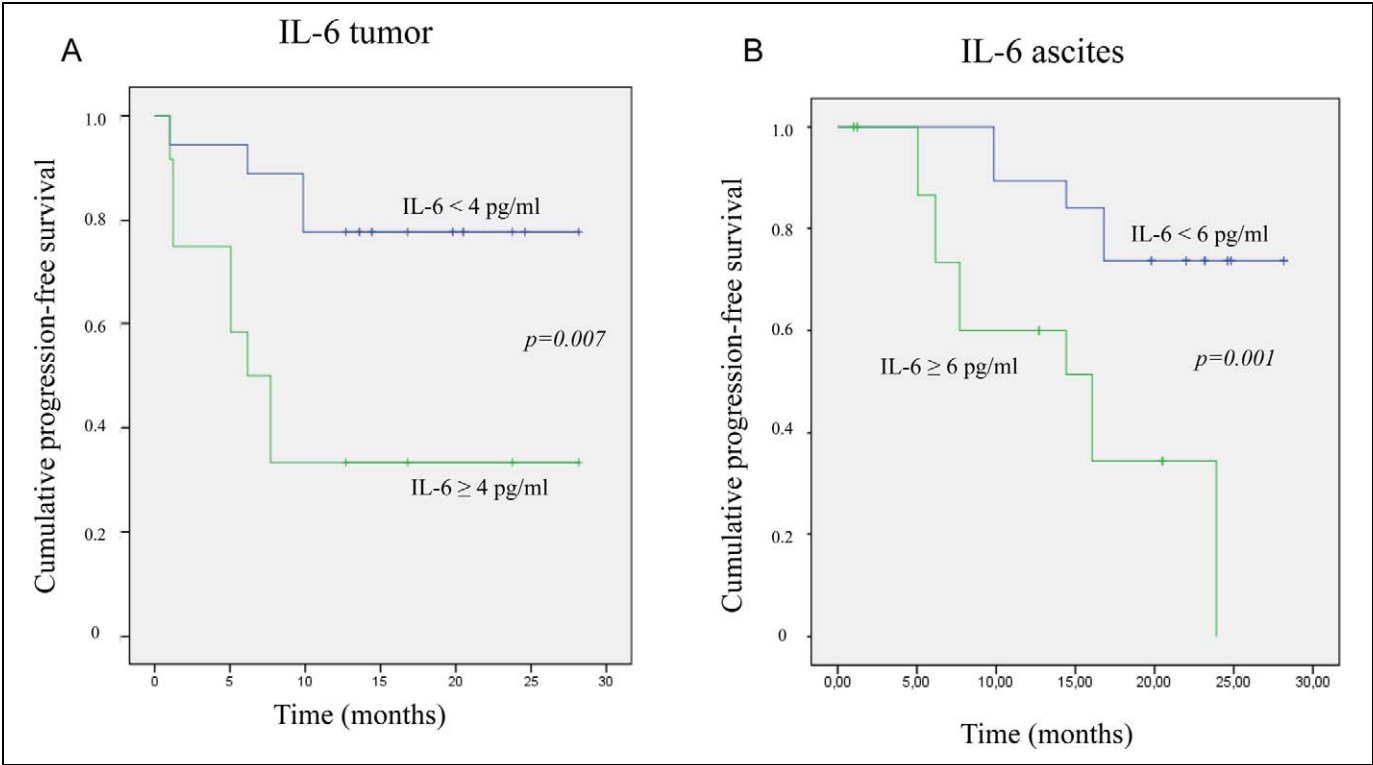


**Figure 2.** Curves of the overall survival of patients with EOC according to the levels of IL-6 in the malignant ovarian tissue and in the peritoneal fluid. Results are expressed as percentage of overall survival and follow-up time (months). The cutoff point for the concentration of cytokine was established and measured by the median (pg/mL). EOC indicates epithelial ovarian cancer; IL, interleukin.

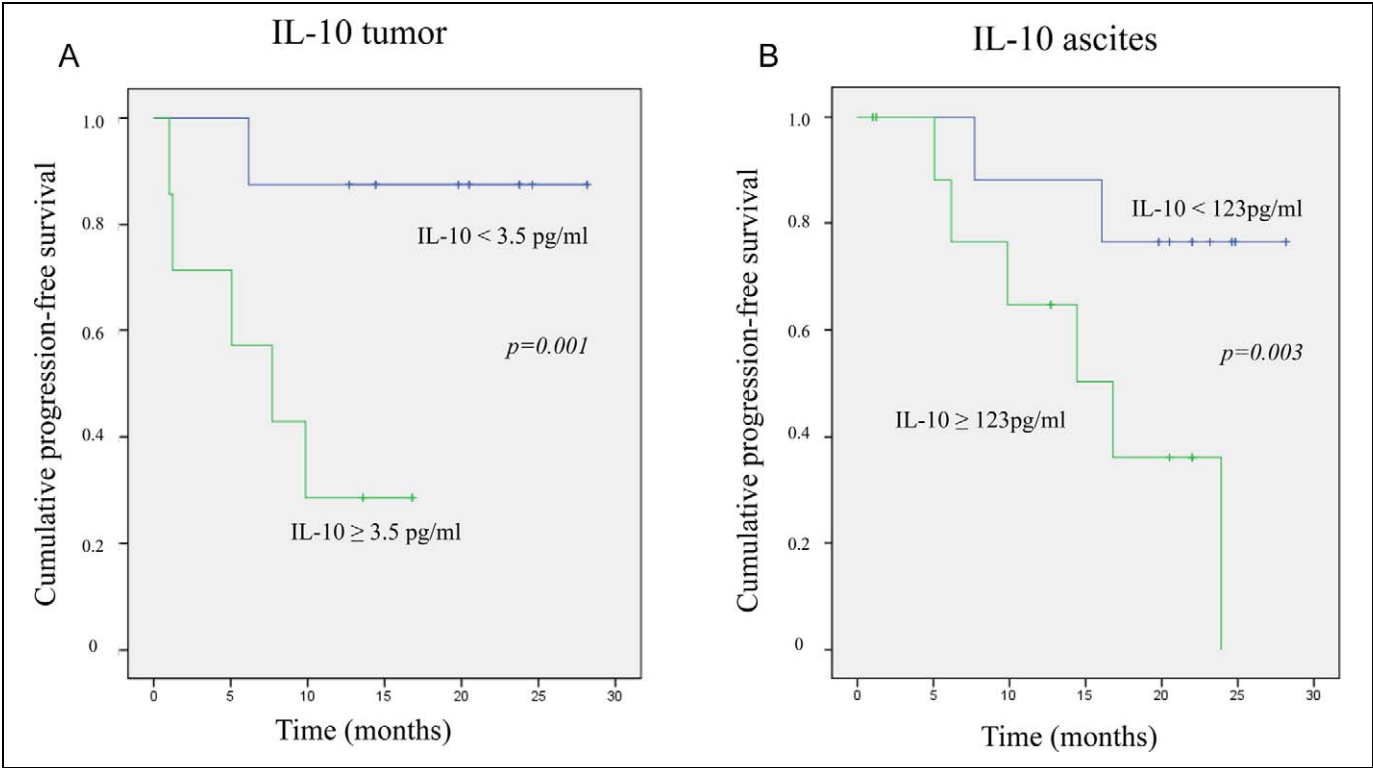


**Figure 3.** Curves of the overall survival of patients with EOC according to the levels of IL-10 in the malignant ovarian tissue and in the peritoneal fluid. Results are expressed as percentage of overall survival and follow-up time (months). The cutoff point for the concentration of cytokine was established and measured by the median (pg/mL). EOC indicates epithelial ovarian cancer; IL, interleukin.

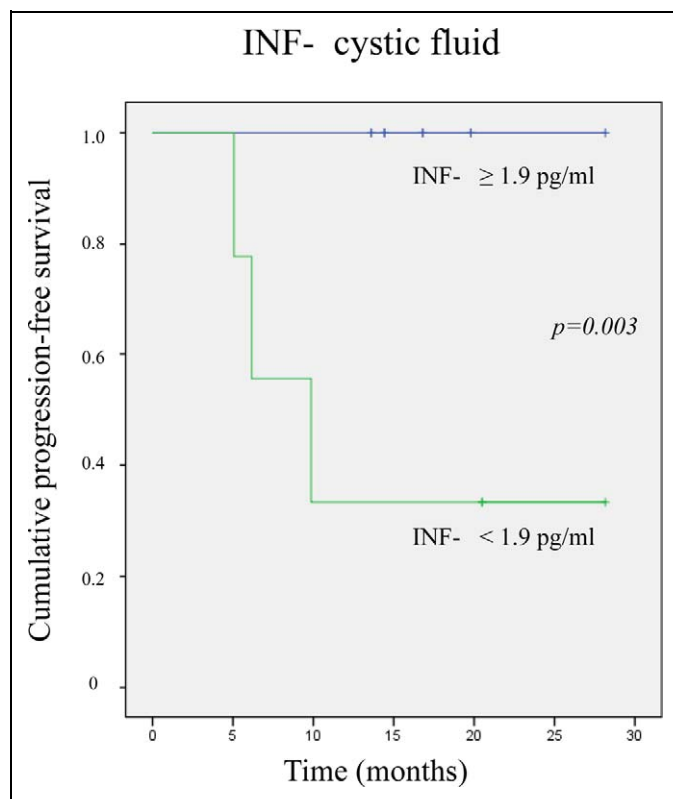


**Figure 4.** Curve of the overall survival of patients with EOC according to the levels of IFN- $\gamma$  in the cystic fluid. The cutoff point for the concentration of cytokine was established and measured by the median (pg/mL). EOC indicates epithelial ovarian cancer; IFN, interferon.

There was a significant association between the proinflammatory (IL-6 and TNF- $\alpha$ ) and the immunosuppressive (IL-10) cytokines and other factors directly related to poor prognosis in ovarian cancer (tumor grade, cytoreduction in primary surgery, and high serum levels of CA-125). Our main findings were that high IFN- $\gamma$  levels were associated with higher overall survival and high IL-6 and IL-10 levels were associated with a lower overall survival, with the former cytokine being correlated to Th1 immune response and the latter being correlated to Th2 immune response. The current study showed that women with EOC had a predominant Th2 response, showing that IL-6, IL-10, and IFN- $\gamma$  could be involved in the prognosis of patients with EOC. Further studies with a larger number of patients and a therapeutic approach are required to evaluate the importance of these components in the development of ascites and drug resistance mechanisms. Controlling the mechanisms that lead to increased IL-10 and IL-6 levels and to lower the levels of IFN- $\gamma$  in the tumor microenvironment may help develop target therapies for ovarian cancer and increase the effectiveness of cancer vaccines. The role of the immune response in the process of carcinogenesis represents a line of research with great potential in oncology.

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