P R A C E O R Y G I N A L N E
ginekologia

Evaluation of IL-10 and TGF-beta levels and myeloid and lymphoid dendritic cells in ovarian cancer patients

Ocena stężenia IL-10 i TGF-beta oraz mieloidalnych i limfoidalnych komórek dendrytycznych u pacjentek chorych na raka jajnika

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Abstract

Aim: *The aim of the study was to evaluate IL-10 and TGF-beta levels in the peritoneal fluid (PF) and plasma of patients with ovarian cancer (n=104), serous cyst (n=32) or normal controls (n=20). IL-10 and TGF-beta levels were correlated to myeloid (M) and lymphoid (L) dendritic cells (DC).*

Material and Methods: *IL-10 and TGF-beta concentrations were evaluated using the enzyme linked immunosorbent assay (ELISA). The percentage of DC in mononuclear cells was quantified by using flow cytometry.*

Results: *The PF and plasma IL-10 concentrations were significantly higher in epithelial ovarian cancer (EOC) patients when compared to the women with serous cyst (the reference group). Plasma levels of IL-10 were elevated in EOC patients in comparison with the reference and control groups. There were significant differences in the PF and plasma IL-10 levels with respect to tumor stage, grade and histology. Significant negative correlations were found between the plasma IL-10 levels, MDC and LDC in the peripheral blood.*

TGF-beta levels were detected in PF of all EOC patients and were significantly lower when compared with plasma. Plasma levels of TGF-beta were elevated in EOC patients compared with the control group. No significant differences in the PF and plasma TGF-beta levels were noted between EOC patients and the reference group. The authors did not find a correlation between the plasma and PF TGF-beta levels and the percentage of MDC and LDC.

Conclusions: *IL-10 production in EOC patients depends on the tumor stage, grade and histological type of the tumor cells. IL-10 may have impact on the percentage of dendritic cells in EOC patients.*

Key words: **dentritic cell** / **ovarian cancer** / **IL-10** / **TGF-beta** /

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Otrzymano: **30.03.2011**
Zaakceptowano do druku: **20.05.2011**

Streszczenie

Cel: *Ocena stężenia interleukiny (IL)-10 i transformującego czynnika wzrostu beta (TGF-β) w osoczu i płynie otrzewnowym (PO) chorych na raka jajnika oraz ustalenie czy istnieje zależność pomiędzy ich stężeniem a odsetkiem mieloidalnych (M) i limfoidalnych (L) komórek dendrytycznych (DC).*

Materiał i metody: *Stężenie IL-10 i TGF-β oceniono u chorych na raka jajnika (n=104), z torbielą surowiczą jajnika (grupa referencyjna , n=32) oraz w grupie kontrolnej (n=20) metodą immunoenzymatyczną ELISA. Odsetek MDC i LDC oceniano metodą cytometrii przepływowej.*

Wyniki: *Stężenie IL-10 w PO chorych na raka jajnika było istotnie wyższe niż w grupie referencyjnej. Ponadto stężenie IL-10 w osoczu chorych na raka jajnika było wyższe niż w grupie referencyjnej i kontrolnej. Odnotowano istotne statystycznie różnice stężeń IL-10 w osoczu i PO chorych na raka jajnika w zależności od stopnia zaawansowania nowotworu wg FIGO, stopnia zróżnicowania histologicznego oraz typu histologicznego nowotworu.*

Stężenie IL-10 w osoczu chorych na raka jajnika istotnie ujemnie korelowało z odsetkiem MDC oraz LDC krwi obwodowej.

Stężenie TGF-β w osoczu i PO chorych na raka jajnika nie różniło się istotnie od odnotowanego w grupie referencyjnej. Stężenie TGF–β w osoczu chorych na raka jajnika jak i grupie referencyjnej było wyższe niż w grupie kontrolnej. Nie wykazano istotnej korelacji pomiędzy stężeniem TGF-β w osoczu i PO a odsetkiem MDC i LDC u chorych na raka jajnika.

Wnioski: *Zaobserwowana w badaniach istotna ujemna korelacja pomiędzy stężeniem IL-10 w osoczu chorych na raka jajnika a MDC oraz LDC krwi obwodowej może sugerować wpływ IL-10 na odsetek komórek dendrytycznych.*

Słowa kluczowe: **komórki dendrytyczne** / **rak jajnika** / **IL-10** / **TGF-beta** /

Introduction

Despite advancements in chemotherapy and surgical technique, morbidity and mortality rates for ovarian cancer are still very high [1]. It is well known that cancer cells develop many mechanisms which impede their identification and destruction by the immune system [2]. One such mechanism is production of factors modifying the function of antigen- presenting cells (APC). The fact that ovarian cancer cells produce immunosuppressive cytokines like IL-10 [3, 4], TGF-β [5, 6], vascular endothelial growth factor (VEGF) [7] or proinflammatory cytokines (IL-6) [8], which inhibit or decrease maturation and activation of APC [2] had been reported in literature. Progression of malignant tumors decreases the number of dendritic cells (DC), which are the most potent among antigen-presenting cells [9, 10]. The latest published data reported a significant role of IL-10 and TGF-β in the differentiation, maturation and function of DC [11,12]. *In vitro*, IL-10 /TGF-β - treated DC, (10/TGF-DC), revealed decreased expression of costimulatory molecules, class II major histocompatibility complex antigens (HLA-DR) and maturation markers (CD83) [13]. Furthermore, 10/TGF-DC induced tolerance in memory T-cells and the differentiation of regulatory T-cells (Tregs) [11].

Aim of the study

The aim of the study was to estimate IL-10 and TGF-β levels in the plasma and peritoneal fluid (PF) of ovarian cancer patients. In addition, the study aimed to evaluate the correlation between the IL-10 and TGF-β concentrations and the percentage of myeloid and lymphoid DC. In ovarian cancer patients, plasma and PF cytokine concentrations were analyzed in relation to International Federation of Gynecologists and Obstetricians (FIGO) stage, histopathological grade and type of the tumor.

Materials and methods

The study analyzed 104 ovarian cancer patients (median age 56 years; min. 22, max. 85 years), who had undergone laparotomy between 2004-2010 in the I Chair and Department of Oncological Gynaecology and Gynaecology, Medical University of Lublin.

Peripheral blood (PB) and peritoneal fluid were collected for the study. The reference group consisted of 32 patients with ovarian serous cysts (SC) (median age 28 years; min. 22 – max.78 years). The control group comprised of blood donors in good health condition (n=20), (median age 29 years; min. 21– max. 51 years).

Plasma was collected after immediate centrifugation (3.000 rpm for 10 min.) of 8ml PB drawn into heparinized tubes. PF was collected from peritoneal cavity during surgery. Centrifuged plasma and PF were stored at -80°C before being tested by ELISA (enzyme-linked immunosorbent assay).

Estimation of IL-10 and TGF-β concentration in plasma and PF

Concentrations of IL-10 and TGF-β were estimated using ELISA assay. Analysis was done with available kits: human IL-10 (R$D System, USA), sensitivity <3.9 pg/ml, human TGF-β1(Bender MedSystems, USA), sensitivity <9pg/ml, according to manufacturer's protocols. All samples were assayed in duplicate.

MDC and LDC were estimated by flow cytometry with the use of monoclonal antibodies: anti-BDCA-1 (CD1c) FITC, anti-BDCA-2 (CD303) FITC (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD19 Cy-Chrome, anti-CD123 PE (Pharmingen, San Diego, California, USA). Details on the method of DC isolation and identification were published in our earlier work, Wertel et. al., 2008 [14]. Results were presented as a percentage of MDC and LDC in mononuclear cells (PBMC).

Statistical analysis

Results were analyzed using Statistica 9.0. software and nonparametric tests. The Wilcoxon paired test was used to compare results in the PF and plasma. The Mann-Whitney U test was used to compare the studied groups. Spearman's rank test was used to assess the relationship between cytokines levels and DC. P value of less than 0.05 was considered statistically significant. Data were presented as medians and range.

Results

Estimation of IL-10 in ovarian cancer, serous cyst (SC) and healthy women

Results are presented in Table I. Concentration of IL-10 in the PF of ovarian cancer patients and in the SC group was significantly higher ($p<0.001$) than in plasma. Furthermore, plasma IL-10 levels in ovarian cancer group was higher ($p<0.001$) than in the reference and control group. No significant difference was found in the plasma IL-10 levels between the reference and control group. (Figure 1a). PF IL-10 concentration in ovarian cancer patients was higher ($p<0.001$) than estimated in the reference group. (Figure1b).

Significant differences in the ovarian cancer group were found in the plasma and PF IL-10 concentration in correlation to FIGO stage, histopathological grade and type of tumor. Results are presented in Table I.

Estimation of TGF-β in ovarian cancer, serous cyst (SC) and healthy women

Results are shown in Table II. The plasma TGF-β levels in ovarian cancer and SC patients were significantly higher ($p<0.001$) than estimated in PF. In the ovarian cancer and the SC group, the plasma TGF-β concentration was significantly higher ($p<0.001$) than in the control group. No significant difference was found in the plasma TGF-β level between the ovarian cancer and the reference group ($p=0.09$). (Figure 2a). Peritoneal fluid TGF-β concentration estimated in ovarian cancer patients and in the reference group presented the same level. (Figure 2b).

TGF-β concentration in the PF of patients with FIGO III stage of ovarian cancer was higher than in I, II and IV stage of tumor, but the difference was not statistically significant.

Estimation of TGF-β concentration in correlation to histopathological type of tumor did not reveal any differences. (Table II). The concentration of plasma TGF-β was higher in the endometrioid than serous type of tumor. (Table II).

Estimation of MDC and LDC percentage in the PF and PB of patients with ovarian cancer, serous cyst (SC) and healthy women

MDC and LDC were found in the peripheral blood and PF of all studied patients. Results are presented in Figure 3, 4.

Correlation between the plasma and PF IL-10 and TGF-β concentration and percentage of MDC and LDC in ovarian cancer patients

The plasma IL-10 concentration revealed significant negative correlation with percentage of peripheral blood MDC ($R=-0.20$, $t(N-2) =-1.94$, $p=0.05$) and LDC ($R=-0.23$, $t(N-2) =-2.26$, $p=0.02$). Furthermore, a positive correlation between PF IL-10 concentration and lymphoid DC percentage ($R=0.17$, $t(N-2)=1.79$, $p=0.07$) was found.

No significant correlation was found between the plasma IL-10 levels and percentage of MDC in PF.

No significant correlation was found between the plasma and peritoneal fluid TGF-β concentration and percentages of MDC and LDC in ovarian cancer patients.

Discussion

Cytokines, like IL- 10, IL- 6, TGF-β and VEGF, present in the cancerous microenvironment affect the antigen-presenting cells status [2, 15]. Supernatants obtained from the cancerous cell cultures have an adverse effect on differentiation of DC

Table I. Levels of IL-10 (pg/ml) in the plasma and PF of patients with ovarian cancer.

OVARIAN CANCER (n=104)	**PLASMA**		**PERITONEAL FLUID**	
	Median	Min.-Max.	Median	Min.-Max.
FIGO STAGE				
I (n=20)	18,11	5,73-481,46	114,50	24,83-517,79
II (n=10)	21,43	10,15-58,52	182,83	55,68-401,08
III (n=63)	**29,34***	4,95-112,99	**296,88***	8,16-677,19
IV (n=11)	16,79	6,75-36,07	**243,24***	66,66-607,41
GRADE				
G1,2 (n=47)	19,04	5,73-112,99	194,19	24,83-655,27
G3 (n=57)	**25,59****	4,95-481,46	**274,01****	8,16-677,19
HISTOLOGY				
Serous (n=49)	24,32	5,73-112,99	206,53	8,16-655,27
Mucinous (n=15)	12,57	7,36-58,52	**283,74*****	49,27-614,01
Endometrioid (n=15)	**16,79*****	4,95-481,46	168,21	36,15-567,39
Clear cell carcinoma (n=5)	10,69	10,15-15,33	37,16	36,51-149,88
Undifferentiated carcinoma (n=20)	**35,76******	11,58-100,89	**361,45*****	35,55-677,19

PLASMA
*p<0,05 in relation to FIGO I i IV
**p<0,05 in relation to (G1,2)
***p<0,05 in relation to clear cell carcinoma
****p<0,05 in relation to mucinous, endometrioid and clear cell carcinoma

PERITONEAL FLUID
*p<0,05 in relation to FIGO I
**p<0,05 in relation to (G1,2)
***p<0,05 in relation to endometrioid, serous and clear cell carcinoma
****p<0,05 in relation to clear cell carcinoma

from $CD34^+$ or $CD14^+$ precursors, and decrease expression of costimulatory molecules on their surface [16, 17].

The aim of our studies was to evaluate the concentrations of selected immunosuppressive cytokines as IL-10 and TGF-β in the plasma and peritoneal fluid obtained from women with ovarian cancer, as well as to establish whether there exists a relation between their concentration and the percentage of professional antigen-presenting cells, DC myeloid and lymphoid.

Our studies showed that both the PF and the plasma IL-10 levels in women suffering from ovarian cancer were significantly higher than those in patients with benign ovarian cysts. These observations are supported by results of Santin et al. [18], who found higher levels of IL-10 in the peritoneal fluid of patients with ovarian cancer in comparison to the plasma levels. Zou et al. [3] observed higher concentrations of IL-10 in the serum and peritoneal fluid of women suffering from ovarian cancer compared to both healthy subjects and patients with benign ovarian tumors. They also found higher concentrations of IL-10 in the peritoneal fluid than in serum. Additionally, the peritoneal fluid IL-10 levels were significantly higher in women with III/IV FIGO stages than in patients with the stage II of the disease. Unlike our results, the authors did not notice any differences in the concentration of IL-10 depending on the histological degree of the cancer [3]. Mustea et al. [19] also observed higher peritoneal fluid IL-10

Table II. Levels of TGF-β (ng/ml) in the plasma and PF of patients with ovarian cancer.

OVARIAN CANCER (n=104)	**PLASMA**		**PERITONEAL FLUID**	
	Median	Min.-Max.	Median	Min.-Max.
FIGO STAGE				
I (n=20)	8,39	3,03-28,44	2,97	1,05-9,65
II (n=10)	8,18	4,14-21,21	2,42	0,56-10,40
III (n=63)	9,97	1,96-100,0	**4,55**	0,61-19,90
IV (n=11)	11,59	5,97-32,27	2,0	0,14-15,36
GRADE				
G1,2 (n=47)	9,27	3,03-23,73	3,06	0,60-19,90
G3 (n=57)	9,97	1,96-100,0	**4,22**	0,41-16,32
HISTOLOGY				
Serous cystadenocarcinoma (n=49)	9,25	3,10-100,0	4,13	0,56-19,90
Mucinous cystadenocarcinoma (n=15)	8,68	3,03-21,21	2,73	0,90-9,06
Endometrioid cystadenocarcinoma(n=15)	**15,63***	**2,51-35,06**	2,48	0,60-10,63
Clear cell carcinoma (n=5)	6,67	5,90-15,88	3,55	1,80-4,50
Undifferentiated carcinoma (n=20)	10,29	1,96-32,57	4,22	0,41-16,32

OSOCZE

*p<0,05 in relation to serous cystadenocarcinoma

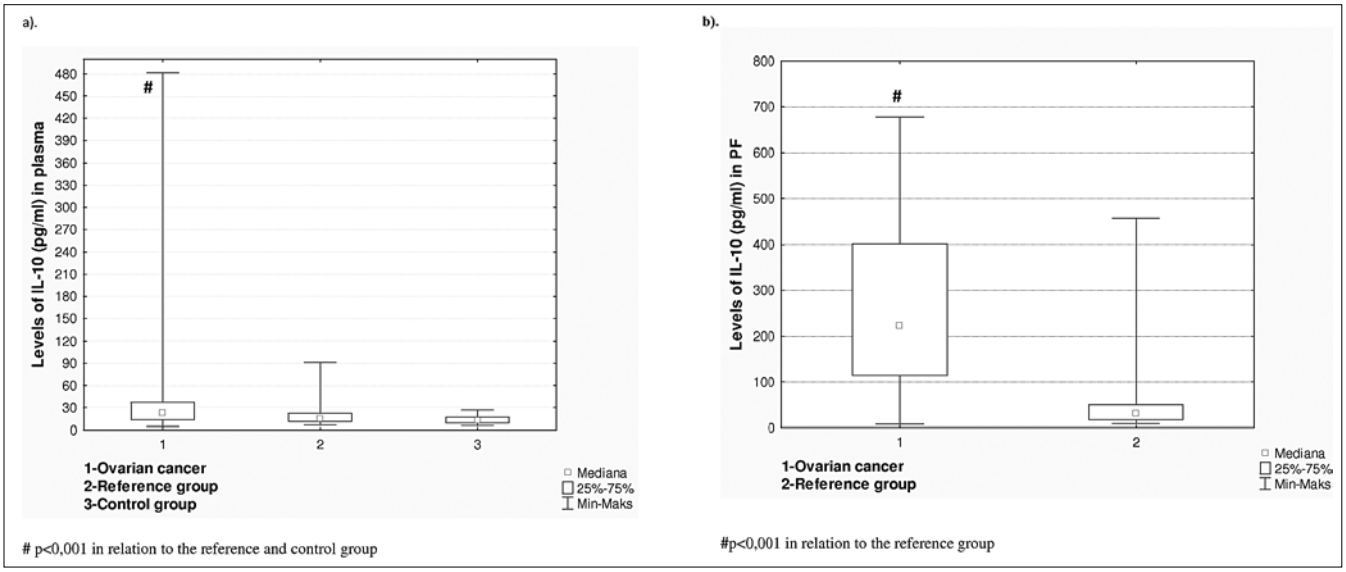

Figure 1ab.

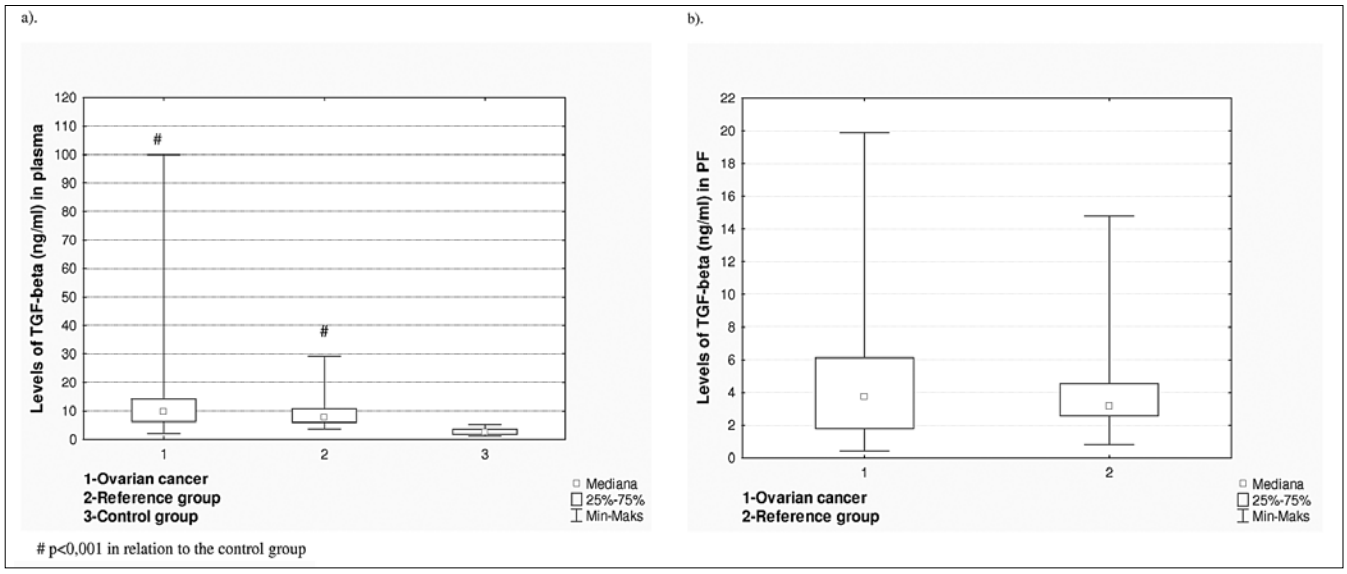

Figure 2ab.

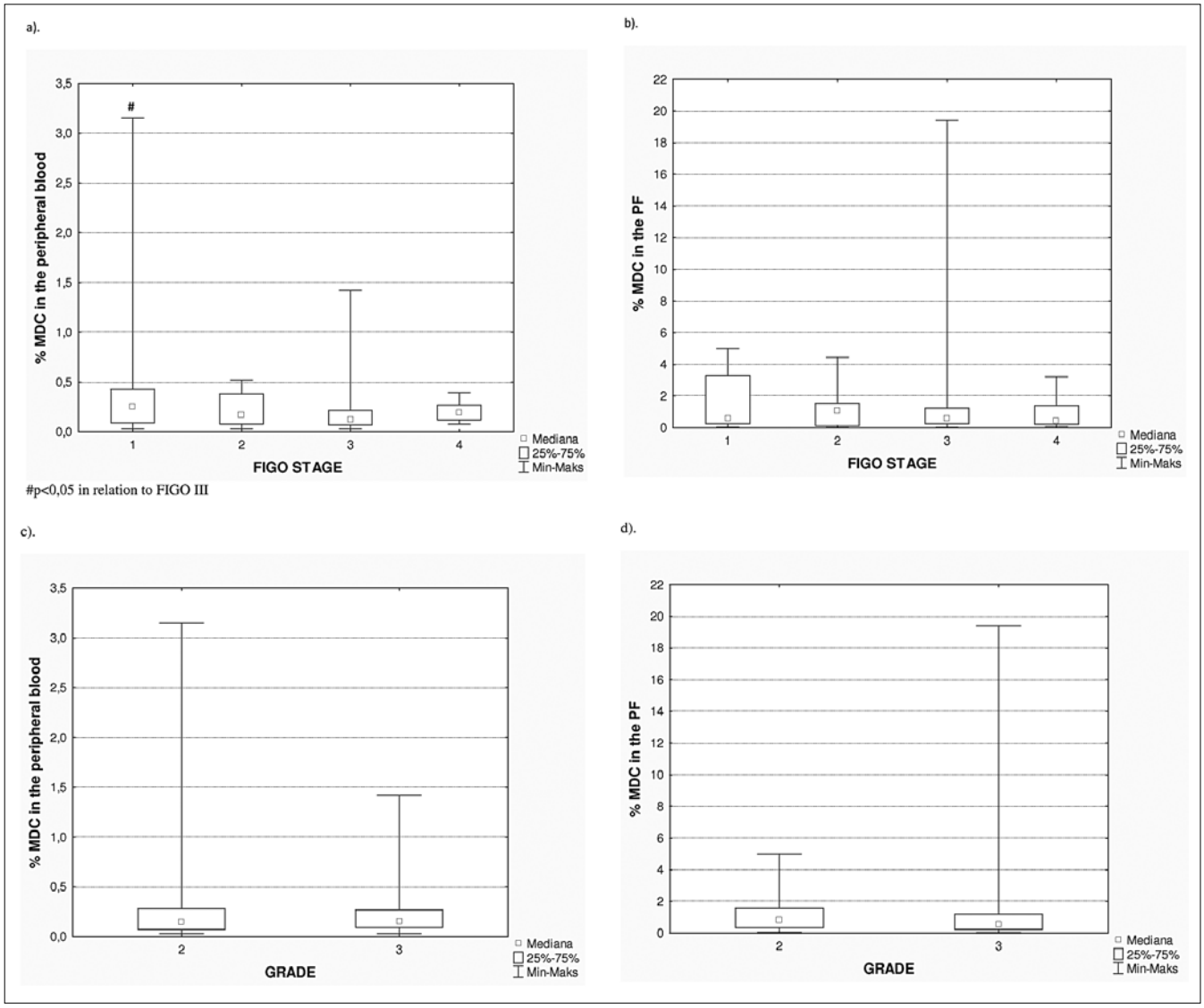

Figure 3abcd.

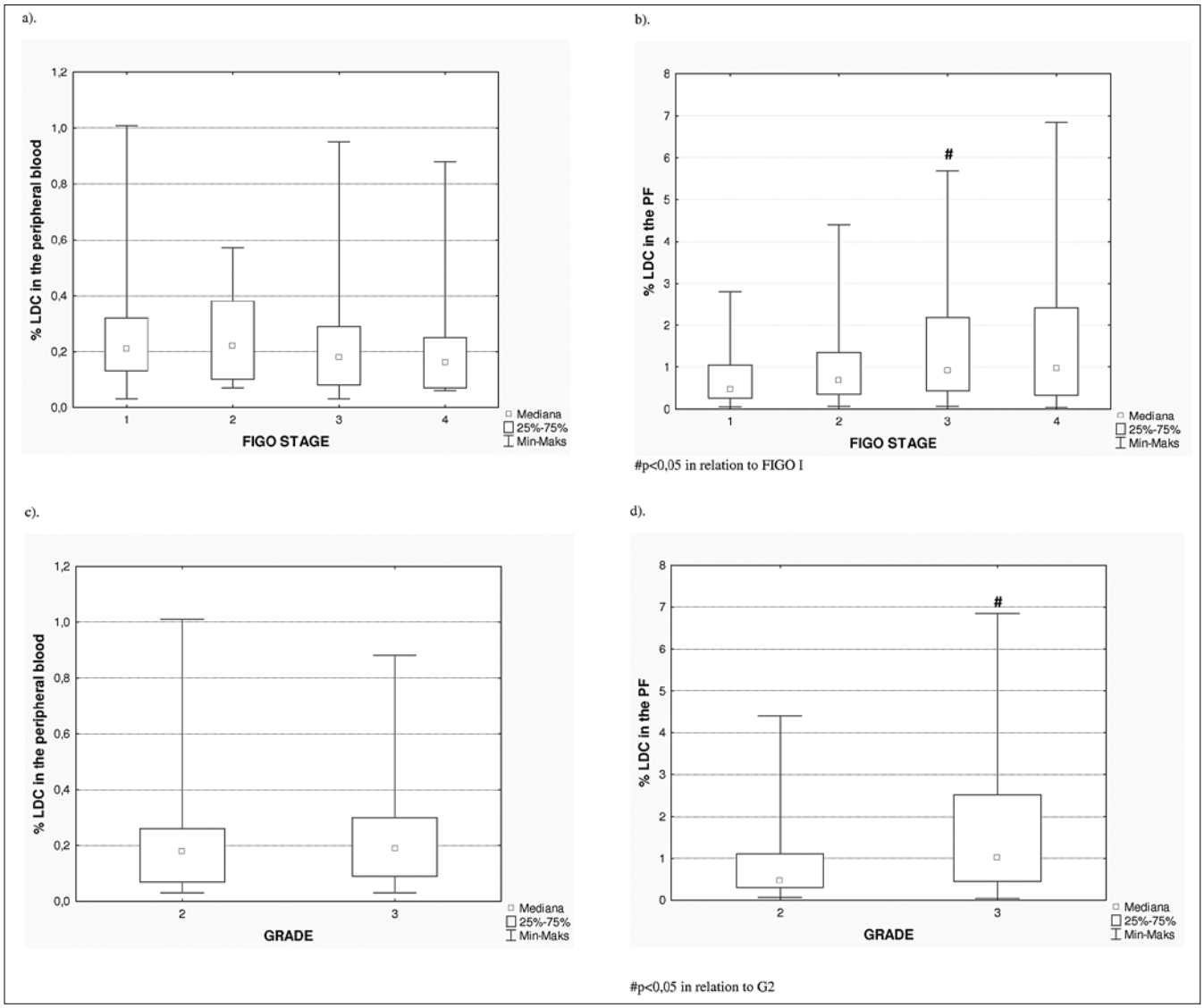

Figure 4abcd.

levels compared to serum of both patients with ovarian cancer and women with benign ovarian cysts. Concentrations of IL-10 correlated with the histological type and with the degree of differentiation of cancer. In the presented work we found higher plasma IL-10 concentrations in women with undifferentiated cancer when compared to mucinous, endometrioid, clear-cell and endometrioid or clear cell carcinoma. Like Mustea et al. [19], we observed a relationship between IL- 10 levels and histological degree of the cancer. (Table I).

Moreover, concentrations of IL- 10, both in the plasma as well as in the peritoneal fluid, correlated with the FIGO stages of ovarian cancer classification. (Table I).

Higher PF than plasma IL-10 levels in patients with ovarian cancer support the hypothesis of the local IL-10 production. However, numerous studies showed that not only cancerous cells [3, 4] but also macrophages, ovarian carcinoma infiltrating lymphocytes [18], dendritic cells [20] and regulatory T-lymphocytes (Tregs) [21] may be the source of IL-10. Consequently, it is difficult to determine the source of IL-10 in women suffering from ovarian cancer.

Many studies showed that IL- 10 influences the differentiation, maturation and DC functions *in vitro*. Allavena et al. [22] demonstrated that IL-10 inhibits monocytes differentiation into DC and promotes the development of macrophages-phenotype cells. Other investigators [20, 23] found that monocytes incubate with IL-10, differentiate to the new population of dendritic cells, DC-10, which produce high amounts of IL-10, but do not produce IL-12. Moreover, these cells stimulate differentiation of regulatory lymphocytes (Tr1) *in vitro*, which by producing IL-10 and TGF-β, inhibit immune response [12, 24].

A statistically significant relationship between plasma IL-10 concentrations in women suffering from ovarian cancer and peripheral blood MDC and LDC percentages, observed in our study, suggests the influence of IL-10 on the percentage of dendritic cells. Interestingly enough, the highest concentrations of IL-10 and the highest percentage of lymphoid DC (0.92% and

0.97%) were found in the PF of women with FIGO III and IV ovarian cancer stages. Moreover, higher IL-10 levels, as well as significantly higher LDC percentage were found in patients with G3 ovarian cancers comparing to patients with G2 degree (1.02% and 0.47%).

In 2004 Curiel et al. [25] demonstrated the accumulation of lymphoid DC in the peritoneal fluid of patients with ovarian cancer. These cells produced high concentrations of angiogenic cytokines like TNFα and IL-8, and inhibited the development of the immune response. High concentrations of IL-10 and increased percentage of lymphoid DC in the PF of women with advanced ovarian cancer, as reported by our study, may affect the development of the immune response.

TGF-β, which suppresses DC maturation and stimulates their differentiation into cells which promotes the immunologic tolerance, is the second immunosuppressive cytokine estimated in our study [26, 27]. Apart from the influence on DC, TGF-β inhibits the proliferation of T-lymphocytes, synthesis of antibodies through B lymphocytes, and suppresses cytotoxic activity of NK cells [18]. TGF-β also plays an important role in differentiation of Tregs [21].

Similarly to our results, Santin et al. [18] and Chen et al. reported significantly higher plasma TGF-β levels in comparison to PF concentrations, in patients suffering from ovarian cancer [28]. Like Chen et al. we did not observe significant differences in the plasma and peritoneal fluid TGF-β concentration between women with ovarian cancer and the reference group [27]. Our results seem to support the hypothesis of Santin et al., which suggests that the source of TGF-β in patients with ovarian cancer comprises of mononuclear cells rather, than cancerous cells [18].

Interestingly enough, it can be noted that the highest TGF-β concentrations, as well as the highest percentage of lymphoid DC (0.92%), were found in women with FIGO III stage of the disease. In addition, LDC percentages and TGF-β concentrations were higher in G3 ovarian cancer as compared to G2 degree (1.02% and 0.47%, respectively). However, the differences in TGF-β concentrations were not statistically significant.

Conclusions

1. The plasma and peritoneal fluid IL-10 levels are higher in women with ovarian cancer in comparison to patients with serous cysts. Concentrations of IL- 10 in women suffering from ovarian cancer and in the group of patients with the serous ovarian cysts were significantly higher in the peritoneal fluid than in plasma.
2. Both, the plasma and peritoneal fluid TGF-β levels, did not differ significantly between patients with ovarian cancer and those with the serous ovarian cysts.
3. A statistically significant negative correlation was found between the concentration of IL- 10 in the plasma and the peripheral blood MDC and LDC percentages in women with ovarian cancer.

The study was supported by the Polish Ministry of Science and Higher Education Grants NN 114036 and NN 160940.

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