OncoTargets and Therapy

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ORIGINAL RESEARCH

Assessment of selected cytokines, proteins, and growth factors in the peritoneal fluid of patients with ovarian cancer and benign gynecological conditions

This article was published in the following Dove Press journal:
OncoTargets and Therapy
23 February 2015
Number of times this article has been viewed

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Objectives: The ovarian tumor microenvironment, ie, the peritoneal fluid, is an intriguing research subject. The goal of this study was to assess the behavior of selected cytokines and growth factors within the peritoneal fluid in pathologies associated with ascites and to assess the relationship between the levels of these substances and select prognostic factors of ovarian cancer.

Methods: A total of 74 patients were enrolled in the study, including 36 patients with ovarian cancer and 38 patients with benign gynecological conditions. Peritoneal fluid collected during surgical procedures was used to assess the levels of interleukin (IL)-6, IL-8, stem cell factor (SCF), dickkopf-1, growth differentiation factor-15 (GDF-15), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), osteoprotegerin (OPG), osteopontin, osteonectin, and human epididymis protein 4. The median levels of these factors were compared between the two groups, and the levels of selected factors were assessed in the ovarian cancer group with regard to the clinical stage of cancer, tumor differentiation, presence of peritoneal spread and positive peritoneal fluid cytology results. The diagnostic value of the analyzed proteins within the peritoneal fluid was also assessed.

Results: Differences were observed between the patients with ovarian cancer and the patients with benign gynecological conditions associated with ascites with regard to the levels of IL-6, IL-8, GDF-15, SCF, osteopontin, osteonectin, and OPG. There were no differences in dickkopf-1, TRAIL, and human epididymis protein 4 levels between the two study groups. Cancer stage affected only the mean SCF and OPG levels, with lower SCF values and higher OPG values in advanced cancers compared to less-advanced cancers. Tumor differentiation was associated with significantly lower SCF values in the group of poorly differentiated tumors. A significant reduction in SCF values and a significant increase in OPG and IL-6 values were also observed within cancer cell-positive peritoneal fluid. Peritoneal spread was associated with higher levels of TRAIL, osteonectin, and IL-6 in ovarian cancer patients.

Conclusion: On the basis of the conducted studies, it appears that of the studied factors, GDF-15, SCF, and OPG deserve special attention in the context of future research on the tumor microenvironment. With regard to diagnostics, attention should be given primarily to GDF-15, IL-6, and osteonectin.

Keywords: ovarian cancer, ascites, cytokines, tumor growth factor, GDF-15, SCF

Introduction

Ascites fluid is a relatively late but very characteristic symptom of ovarian cancer. The accumulation of peritoneal fluid in this type of cancer is related primarily to

the micronodular spread along the peritoneum, leading to absorption disturbances due to the occlusion of lymphatic vessels and excess production of fluid by cancer cells. The main factor responsible for the increased production of fluid is vascular endothelial growth factor (VEGF). A number of other involved substances have also been described, including cytokines produced by both abdominal organ cells, particularly stimulated mesothelial cells, and by the cancer cells themselves. The peritoneal fluid is a unique tumor microenvironment, and there is no doubt that its composition affects the course of the disease.[1–7] Ovarian cancer is a very heterogeneous group of tumors; according to recent reports, it is diverse not only in histopathology but also in etiology.[1,8–10] The highest rates of massive, clinically significant ascites are observed with high-grade serous ovarian carcinomas.[10] The presence of ascites is an unfavorable prognostic factor due not only to the associated stage of the disease but also to its inhibition of apoptosis induced by the drugs used in the treatment of ovarian cancer.[11–13] Most studies on this mechanism of action were conducted in relation to interleukin (IL)-6, which has been detected in peritoneal fluid.[9–12,14–19] It appears, however, that the mechanism is more complex, and the effect of ascites on the disease course depends on a large number of elements, such as growth factors, cytokines, and signal transduction proteins.

The knowledge of the tumor microenvironment, ie, the cancer cell-positive peritoneal fluid, is very important as it might be an important step in the pursuit of new approaches to cancer therapy, with the intraperitoneal method of treatment gaining increasing support.

The goal of this study was to assess the expression of selected cytokines and growth factors in the ascites of different pathologies and to assess the levels of these substances in ovarian cancer effusions according to selected prognostic factors.

Materials and methods

Populations examined

A total of 74 patients, aged 18 to 89 years, were included in this study. The study group consisted of a successive sequence of patients who presented to the Clinic of Surgical Gynecology and Gynecological Oncology for Adults and Adolescents for various gynecological conditions and in whom fluid was detected within the peritoneal cavity. Upon admission to the clinic, all patients provided informed consent.

Peritoneal fluid was collected during surgery in a total volume of 5–6 mL distributed into three test tubes. The peritoneal fluid samples were collected, centrifuged for 10 minutes at 1,000× *g* to remove debris and aliquoted into two microfuge tubes; the samples were frozen immediately at −70°C. For the multiplex assay, the studied aliquots were thawed completely at room temperature, mixed well by vortexing and centrifuged prior to use in the assay to remove particulates. The study material was stored for several months. The remaining third tube was used to assess human epididymis protein 4 (HE4) marker levels on the day of collection. After the histopathologic results were obtained, the study group was divided into two arms. Table 1 presents the detailed characteristics of the patients.

The patients were divided into two groups:

- A – patients with malignant epithelial tumors, n=36;
- B – patients with benign gynecological conditions, n=38.

Peritoneal fluid levels were determined for IL-6, IL-8, stem cell factor (SCF), dickkopf-1 (DKK-1), growth differentiation factor-15 (GDF-15), osteoprotegerin (OPG), osteopontin (OPN), osteonectin, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and HE4.

The mean expression levels of HE4, OPG, OPN, osteonectin, IL-6, IL-8, SCF, GDF-15, DKK-1, and TRAIL were compared between the study groups. The means for the following subgroups were also determined but without comparisons due to the small numbers of patients within the individual subgroups: group A (serous ovarian tumors and tumors of other histopathological types) and group B (borderline tumors, functional ovarian cysts, benign epithelial tumors, dermoid tumors, and endometrioid cysts). The potential relationships and correlations between the examined proteins were examined, and the peritoneal fluid levels were assessed for diagnostic importance. In the group of ovarian cancer patients, assessments were performed to examine whether the mean expression levels differed depending on the clinical stage of cancer, tumor grade, peritoneal spread and the presence of cancer cells within the peritoneal fluid.

Multiplex immunoassay

Osteonectin, OPG, DKK-1, GDF-15, OPN, SCF, TRAIL, IL-6, and IL-8 concentrations in the peritoneal fluid were quantified by multiplex fluorescent bead-based immunoassays (Luminex Corporation, Austin, TX, USA) using the commercial Human Cancer Metastasis Biomarker Magnetic Bead Panel and Human Circulating Cancer Biomarker Magnetic Bead Panel 1 (Merck Millipore, Billerica, MA, USA). A total of 25 µL of each standard, control and samples was added to the plate together with multiplex antibody capture bead

Table 1 Patient characteristics

Study group	Mean age (range) years	Number of patients
All patients	50.77 (18–89)	74
Premenopausal	34.74 (18–50)	35
Postmenopausal	65.15 (52–89)	39
Epithelial ovarian cancer patients	60.8 (30–89)	36
– Serous	62.12 (40–88)	25
– Endometrioid	59.75 (46–74)	6
– Mucinous	49.66 (30–66)	5
FIGO I, II	51.1 (30–74)	10
FIGO III, IV	65.85 (4–89)	26
Grade 1	47.25 (30–66)	6
Grade 2	61.38 (46–82)	10
Grade 3	60.26 (41–88)	20
Peritoneal carcinomatosis, Yes	65.63 (47–89)	25
Peritoneal carcinomatosis, No	51.1 (30–74)	11
Presence of neoplastic cells in peritoneal fluid	63 (47–88)	19
Absence of neoplastic cells in peritoneal fluid	58.64 (30–89)	17
Benign gynecological conditions	41.26 (18–89)	38
– Functional cysts	30.3 (19–61)	6
– Ovarian endometrioma	39 (32–44)	6
– Mature teratoma	34.43 (18–67)	7
– Borderline epithelial tumor	55.5 (53–58)	2
– Benign epithelial and gonadal tumors	53.3 (18–89)	10
– Myoma	41.75 (31–46)	4
– Inflammation	39	1
– Ovary without pathology	30.5 (18–43)	2

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.

solution, and the plate was incubated with agitation at 4°C overnight. The following day, each well was washed with 200 µL of washing buffer three times using a hand-held magnet. A total of 25 µL of the detection antibody cocktail was pipetted into each well, and the plate was sealed and incubated with agitation at room temperature for 1 hour. After this step, 25 µL of a streptavidin-phycoerythrin mixture was added to the plate and incubated with agitation for 20 minutes in the dark. Finally, after washing, the microspheres contained within each well were resuspended in 100 µL of sheath fluid and agitated at room temperature for 5 minutes. The plate was read and analyzed on the Luminex analyzer and the analyte concentrations were determined from five different standard curves showing the median fluorescence intensity versus (vs) protein concentration.

HE4 assay

Peritoneal fluid HE4 concentrations were measured with the Elecsys ECLIA assay from Roche using the Cobas E 601 analyzer. The measurement range was 15.0–1,500 pmol/L; samples exceeding the upper range were diluted with Elecsys Diluent Multiassay. The manufacturer's instructions were followed, and the control samples were within the normal range. The normal upper limit range for serum was less than 70 pmol/L.

Statistical analyses

Statistical analyses were conducted using the Statistica 9.1 PL software package. Means, medians and ranges were determined for the study arms, and the results were compared using non-parametric Mann–Whitney *U*-tests. The means values and standard deviations for the studied proteins are presented in the corresponding figures.

To determine the relationship between the analyzed markers, scatter diagrams of the empirical points values were plotted, Pearson's linear correlation coefficients were calculated and the linear regression function was estimated.

The receiver operating characteristic (ROC) curves were obtained, and the area under the curve (AUC) was calculated with 95% confidence intervals according to the nonparametric method of DeLong.[20] This method was also used to compare the AUCs. The level of significance was taken as <0.05.

Results

Table 2 presents the comparisons of the peritoneal fluid levels of the studied proteins between the study arms. The mean levels of IL-6, IL-8, GDF-15, OPN, OPG, and osteonectin were significantly higher in the group of ovarian cancer patients compared with the patients with

Table 2 Levels of examined cytokines and growth factors in the effusion fluid in group A (patients with ovarian cancer) and group B (patients with benign gynecological diseases)

	IL-6	IL-8	SCF	GDF-15	HE4
Group A, n=36	379/528.13	270.2/133	28.8/24.1	1,272/1,069	4,438.8/2,049.5
Mean/median (range)	(9.7–528.1)	(15.3–1,148)	(13.3–80.7)	(342–3,458)	(223–30,000)
Group B, n=38	137.8/17.9	137.2/13.1	31.31/24.8	825.6/441	4,191.2/2,516
Mean/median (range)	(0.08–528.1)	(2.4–663)	(9.2–76.6)	(148–3,545)	(174.8–20,420)
P-value	0.00001	0.004	0.54	0.0125	0.602
	DKK-1	**OPN**	**Osteonectin**	**TRAIL**	**OPG**
Group A, n=36	423.6/119	27,726.7/24,720	478.25/447.5	41.4/19.4	1,321.9/1,045
Mean/median (range)	(29.9–4,174)	(3,230–83,440)	(158–1,347)	(3–537)	(172–5,364)
Group B, n=38	272.99/86.85	21,997/15,580	246/153	56.1/19.5	796.1/467
Mean/median (range)	(16.4–2,278)	(2,252–148,262)	(9.8–990)	(3.6–447)	(93.7–4,272)
P-value	0.2518	0.0324	0.00001	0.7105	0.0287

Abbreviations: IL, interleukin; SCF, stem cell factor; GDF-15, growth differentiation factor-15; HE4, human epididymis protein 4; DKK-1, dickkopf-1; OPN, osteopontin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; OPG, osteoprotegerin.

benign gynecological conditions. The results are presented in Figure 1.

In group A, 25 of the 36 patients suffered from serous ovarian carcinoma, whereas the remaining eleven cases included endometrioid and mucinous carcinomas. Of all studied factors, only IL-8 showed significant differences in peritoneal fluid levels between serous carcinomas (202.17 pg/mL) and the remaining histopathological types (456.03 pg/mL), P=0.0048. The mean values of the remaining factors observed between the serous carcinoma subgroup and the group of remaining tumor types were as follows: TRAIL, 46.59 pg/mL vs 32.43 pg/mL; IL-6, 391.09 pg/mL vs 344.13 pg/mL; SCF, 27.39 pg/mL vs 32.93 pg/mL; OPN, 28,577.88 pg/mL vs 27,222.3 pg/mL; GDF-15, 1,222.04 pg/mL vs 1,430.90 pg/mL; DKK-1, 357.96 pg/mL vs 609.09 pg/mL; osteonectin, 504.6 ng/mL vs 442.4 ng/mL; OPG, 1,489.36 pg/mL vs 971 pg/mL; and HE4, 5,293.17 pmol/L vs 2,642.62 pmol/L. These differences were not significant.

The following levels (mean values) of studied proteins were determined for the histopathological subgroups of group B: borderline tumors: TRAIL 3.6 pg/mL, IL-6 528.13 pg/mL, IL-8 285 pg/mL, SCF 18.6 pg/mL, OPN 9,669 pg/mL, GDF-15 1,116 pg/mL, DKK-1 76.45 pg/mL, osteonectin 273.5 ng/mL, OPG 971 pg/mL, and HE4 1,054.35 pmol/L; functional cysts: TRAIL 22.1 pg/mL, IL-6 45.12 pg/mL, IL-8 31.36 pg/mL, SCF 34.35 pg/mL, OPN 22,138.5 pg/mL, GDF-15 594.21 pg/mL, DKK-1 203.39 pg/mL, osteonectin 199.68 ng/mL, OPG 475.91 pg/mL, and HE4 4,694.92 pmol/L; benign epithelial tumors: TRAIL 157.57 pg/mL, IL-6 145.79 pg/mL, IL-8 228.36 pg/mL, SCF 25.06 pg/mL, OPN 15,456.29 pg/mL, GDF-15 1,591.75 pg/mL, DKK-1 709.8 pg/mL, osteonectin 250.51 ng/mL, OPG 1,120 pg/mL, and HE4 5,335.9 pmol/L; teratomas: TRAIL 30.36 pg/mL, IL-6 336.21 pg/mL, IL-8 285.47 pg/mL, SCF 32.76 pg/mL, OPN 40,172.14 pg/mL, GDF-15 716 pg/mL, DKK-1 1,15.81 pg/mL, osteonectin 330.86 ng/mL, OPG 810.29 pg/mL, and HE4 1,233.2 pmol/L; and endometrioid cysts: TRAIL 51.6 pg/mL, IL-6 40.22 pg/mL, IL-8 67.4 pg/mL, SCF 35.82 pg/mL, OPN 13,239.33 pg/mL, GDF-15 388 pg/mL, DKK-1 144.65 pg/mL, osteonectin 268.57 ng/mL, OPG 999.17 pg/mL, and HE4 5,474.67 pmol/L.

Figure 2 presents the ROC plots for individual cytokines and growth factors to determine the diagnostic value of these substances within the peritoneal fluid. The highest AUC values were noted for IL-6 (0.829), osteonectin (0.806), GDF-15 (0.752), IL-8 (0.747), and OPG (0.713). The lowest values of less than 0.5 were observed for TRAIL (0.482) and SCF (0.445). Figure 3 presents significant linear correlations between the studied proteins. Other relationships are not illustrated in the figures, as the criteria for significance were not met. In most cases, the correlations are characterized by a low power and $r<0.5$, with only the correlation between OPG and osteonectin presenting the features of a strong correlation, with r=0.5006.

A detailed analysis was performed in the group of ovarian cancer patients. Cytokines and growth factors were assessed for the relationships between their peritoneal fluid levels and the selected prognostic factors. Significant differences between high (International Federation of Gynecology and Obstetrics [FIGO] III, IV) and low (FIGO I, II) clinical staging were observed only for OPG, with higher mean peritoneal fluid levels in advanced cancers (1,524 pg/mL) compared with less advanced cancers (862.7 pg/mL; P=0.0125), and for SCF, with high mean levels (35.5 pg/mL) observed in non-advanced cancers and low mean levels (28.7 pg/mL) observed in advanced cancers (P=0.0077). The values of IL-6 and GDF-15 approached significance (Table 3).

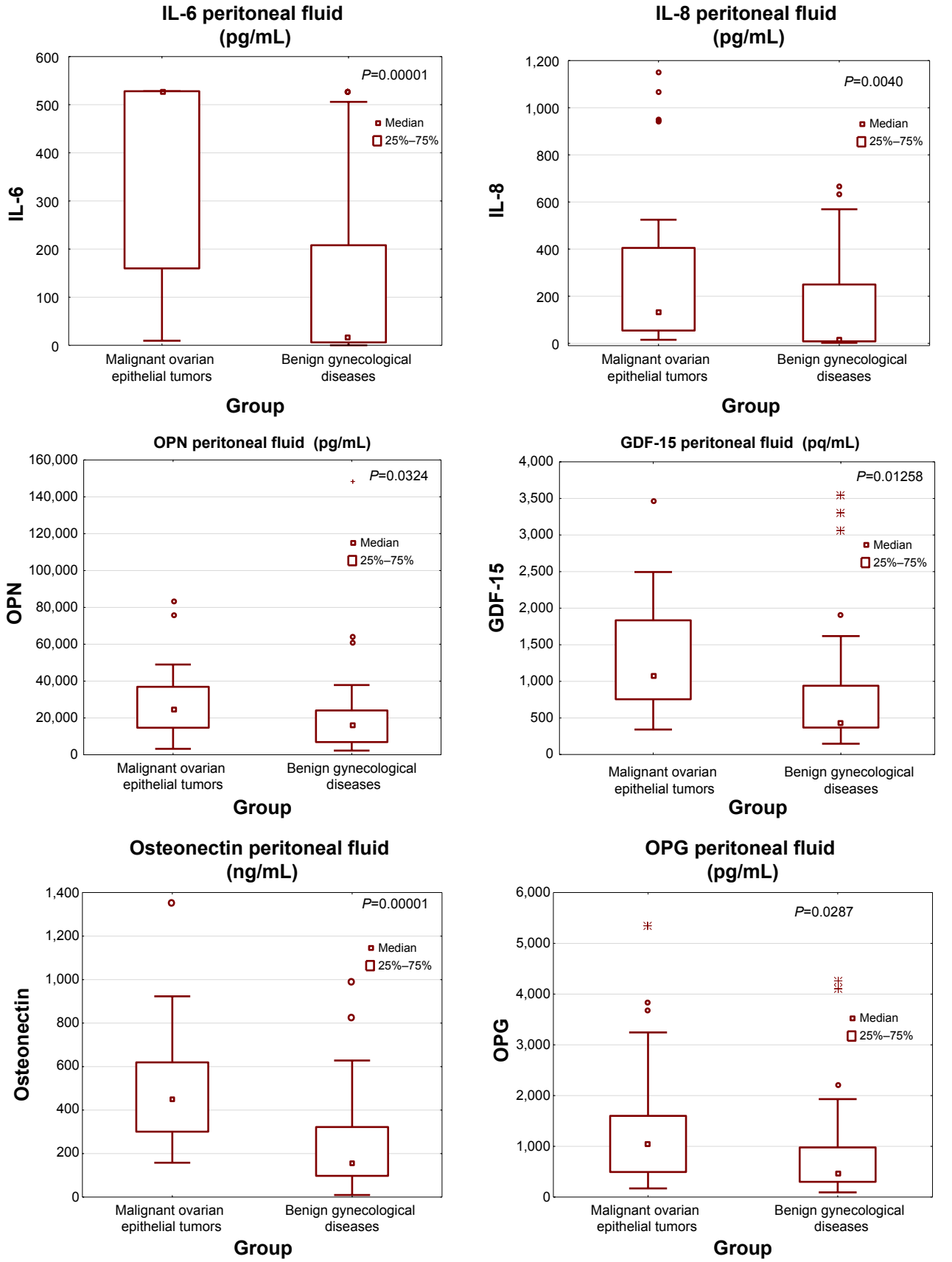

Figure 1 Comparison of examined cytokines and growth factors between patients with ovarian cancer and patients with benign gynecological conditions.
Abbreviations: IL, interleukin; OPN, osteopontin; GDF-15, growth differentiation factor-15; OPG, osteoprotegerin.

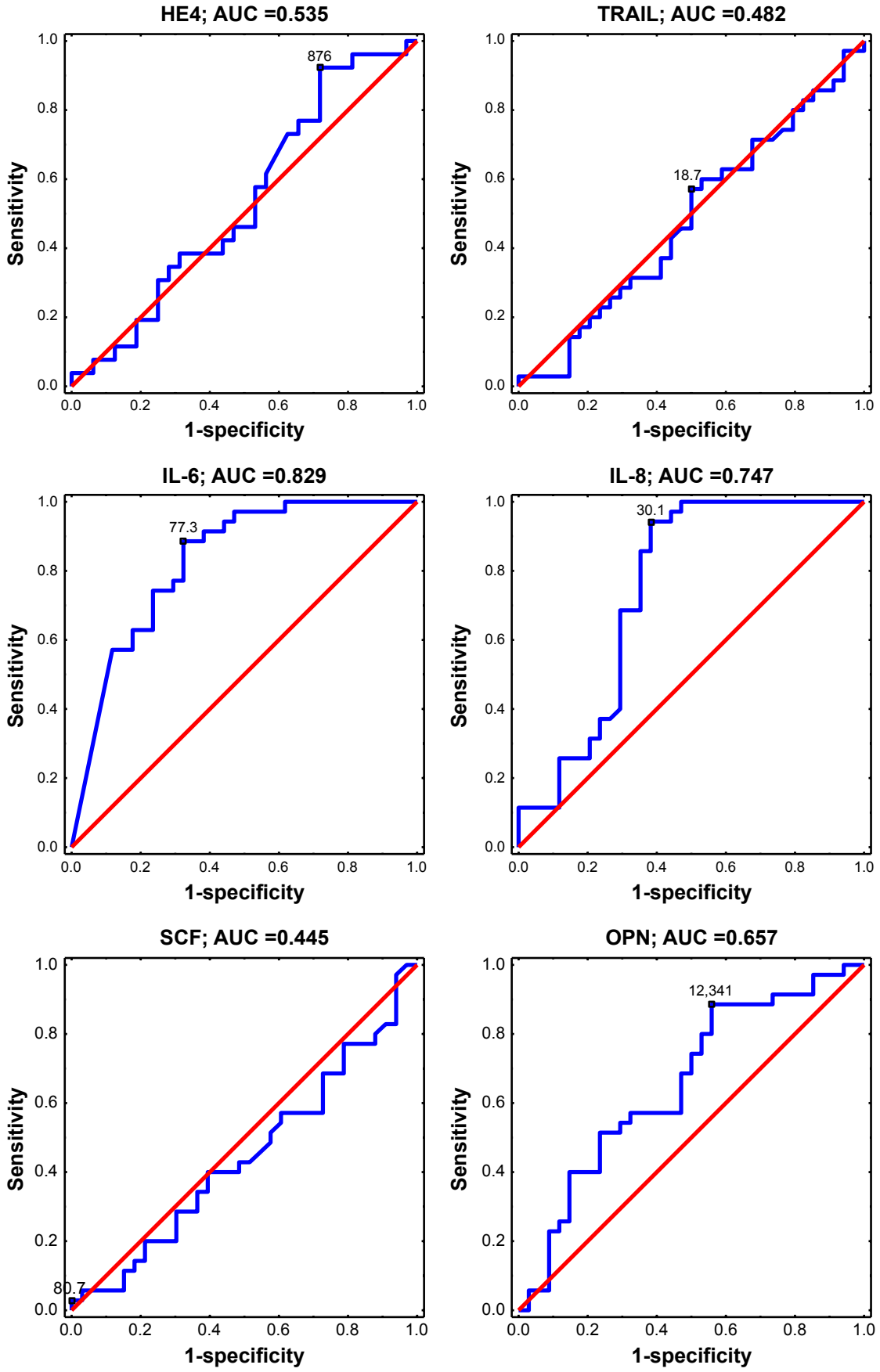

Figure 2 (*Continued*)

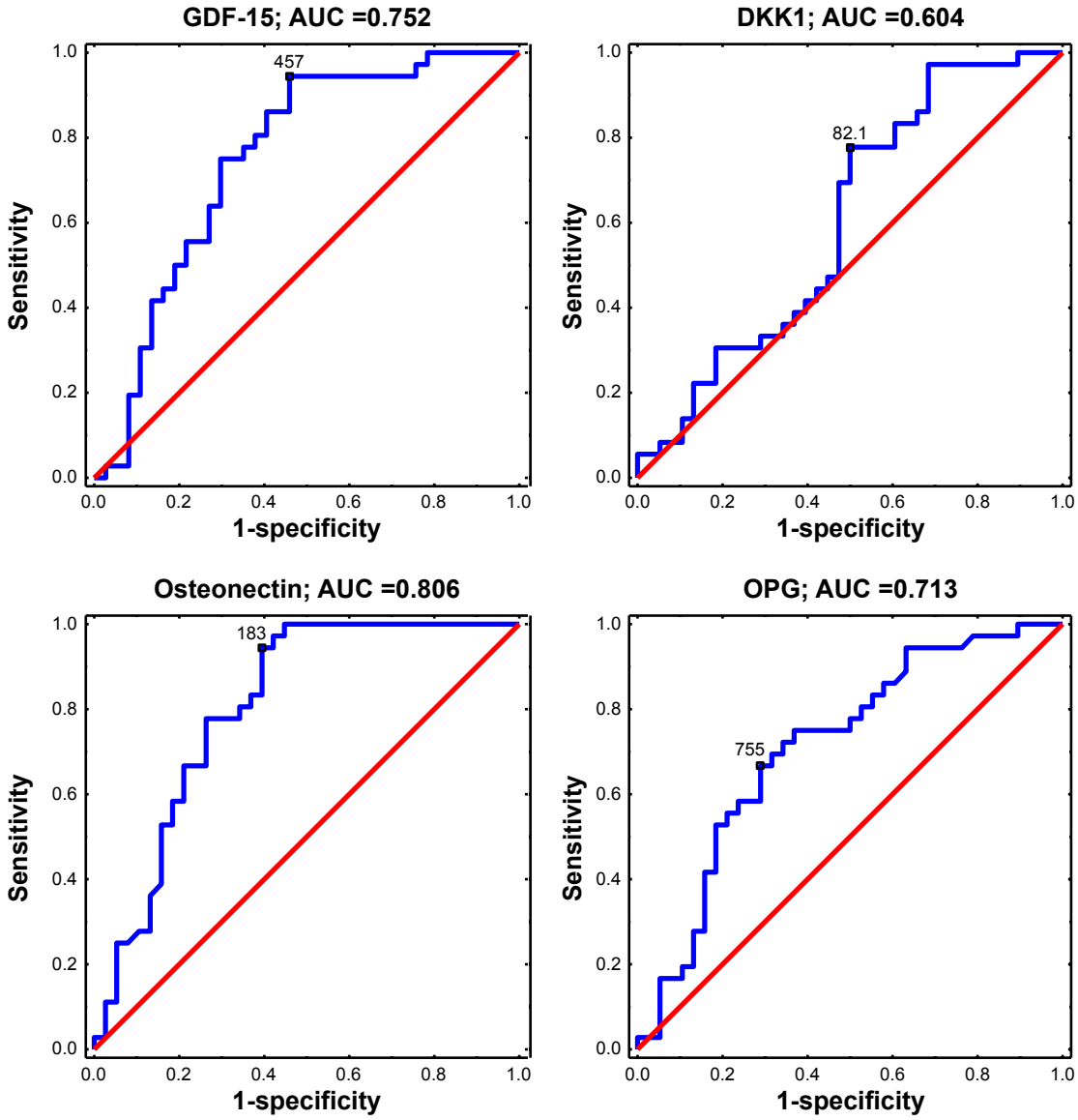

Figure 2 ROC curves for the examined cytokines and growth factors.
Abbreviations: ROC, receiver operating characteristic; HE4, human epididymis protein 4; AUC, area under the curve; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IL, interleukin; SCF, stem cell factor; GDF-15, growth differentiation factor-15; DKK-1, dickkopf-1; OPN, osteopontin; OPG, osteoprotegerin.

A comparison of the levels of selected substances within the ascites in group A patients according to tumor differentiation stage is presented in Table 4. Only the SCF values differed depending on the biological aggressiveness of the tumor, and this showed a negative correlation; less differentiated, more aggressive tumors had lower SCF values (27.5 pg/mL) than more highly differentiated tumors (37.3 pg/mL, P=0.0208). Results approaching significance were also observed for IL-6, with values of 290.2 pg/mL for grade-1 and -2 cancers and 411.9 pg/mL for grade-3 cancers (P=0.0779). The presence of cancer cells within the peritoneal fluid in group A patients had an effect on the levels of SCF, OPG, and IL-6. Higher mean levels of OPG and IL-6 and lower mean levels of SCF were observed in peritoneal fluids with positive cytology. No differences were observed for the remaining proteins (Table 5). Peritoneal spread in the group of ovarian cancer patients influenced the levels of selected proteins. Significantly higher levels of TRAIL, osteonectin, and IL-6 were observed within the peritoneal fluid for cases of concomitant peritoneal spread (Table 6).

Discussion

IL-6 and IL-8 have long been identified as cytokines involved in the neoplastic process leading to the development of ovarian cancer.[21] Because this analysis focused on the levels of cytokines and growth factors within the peritoneal fluid of

ovarian cancer patients, it included typical representatives of this class. It is known that the levels of these cytokines are substantially higher within the peritoneal fluid than in serum.[2] Both ILs, as well as their receptors, are expressed at different levels within the epithelial cells of ovarian cancer of various malignancy grades.[16,21,22] The tumor-promoting effect of these cytokines involves enhancement of anchorage-independent growth, proliferation, and angiogenesis as well as cellular adhesion and invasion.[17,23] Numerous authors have analyzed various molecular mechanisms and consider the functional

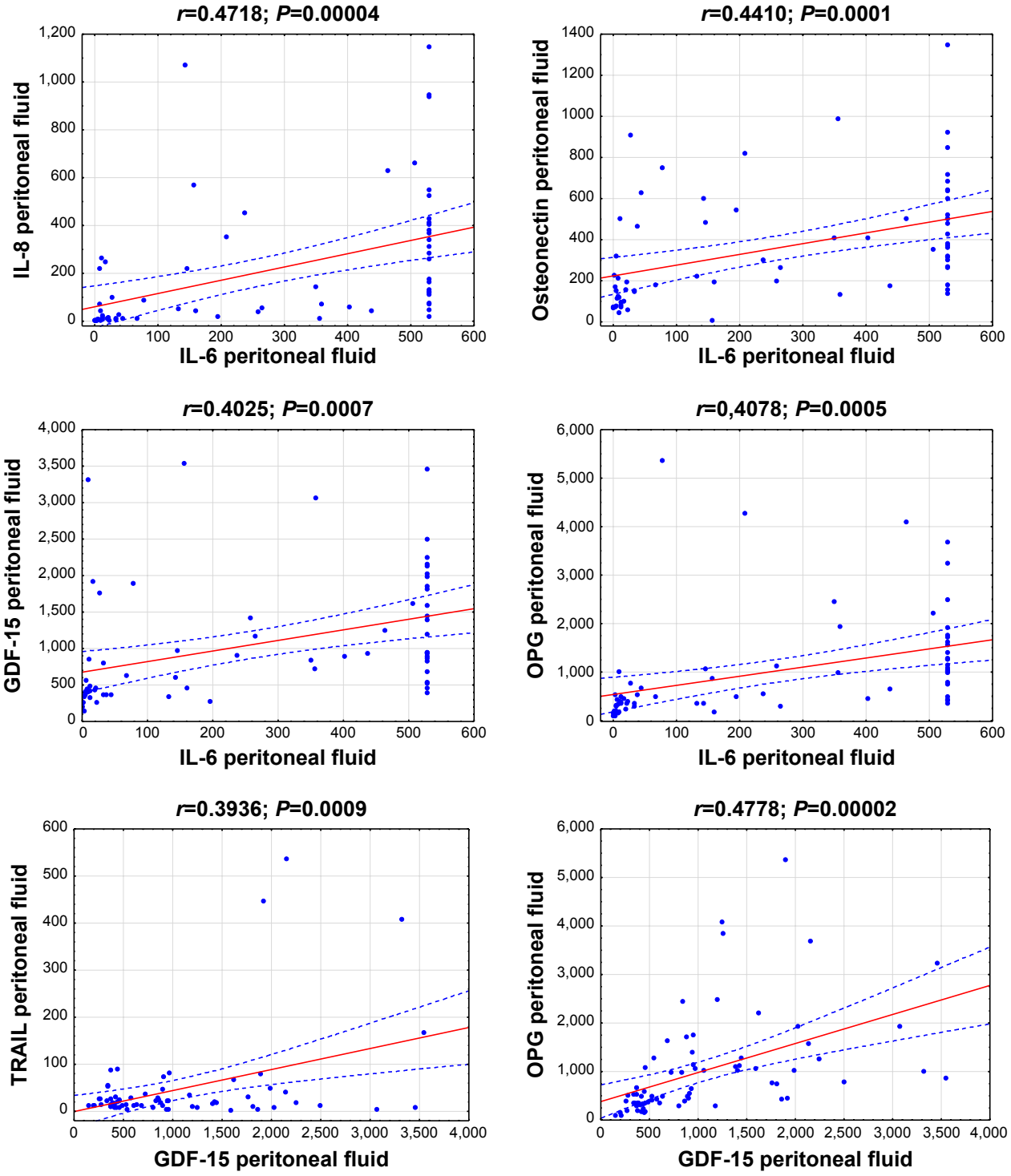

Figure 3 (*Continued*)

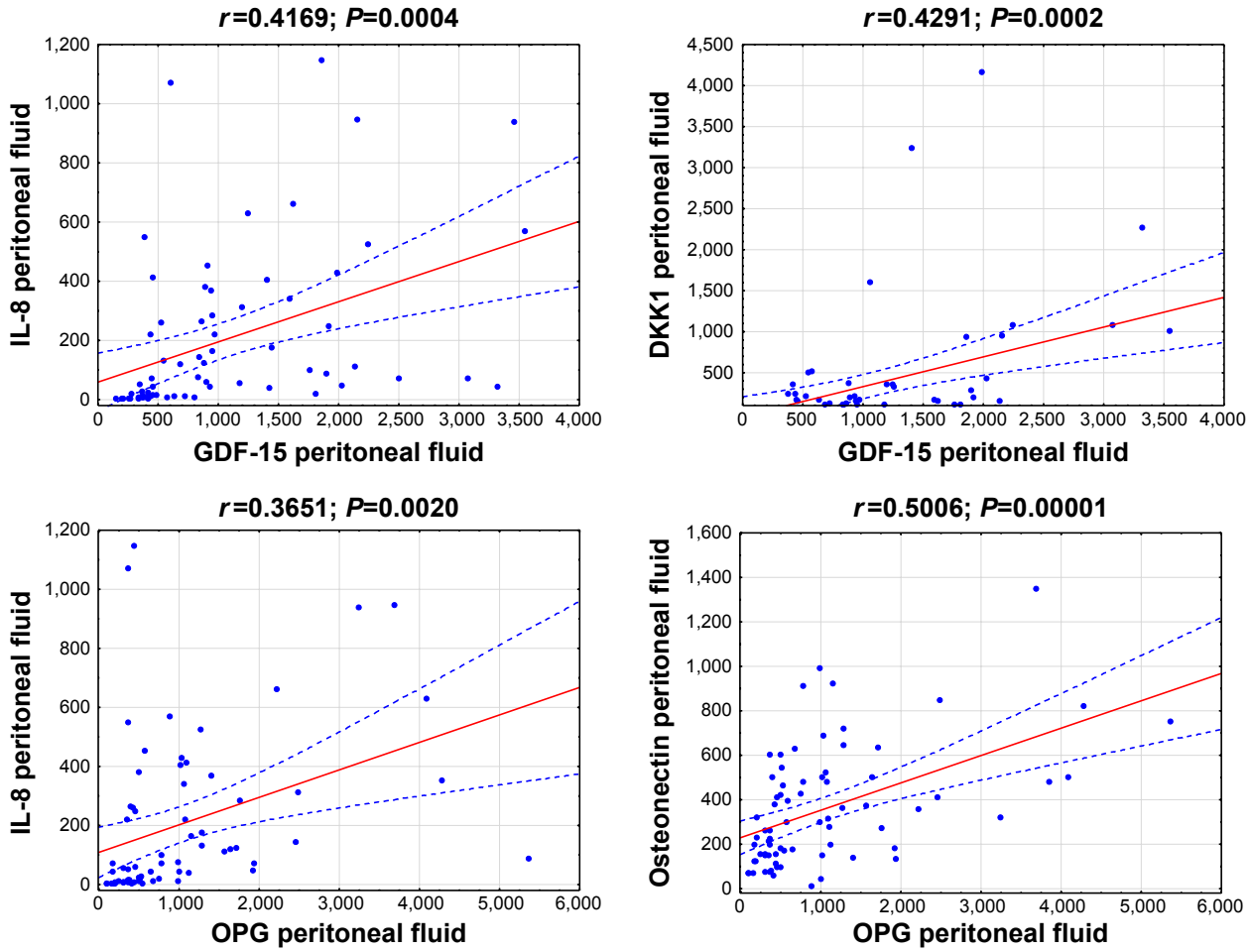

Figure 3 Pearson's correlations between the examined cytokines and growth factors.
Abbreviations: IL, interleukin; GDF-15, growth differentiation factor-15; OPG, osteoprotegerin; DKK-1, dickkopf-1; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

inhibition of IL-6 and IL-8 to be a potential keystone for novel therapy, such as for the treatment of ovarian cancer.[7,18,19] Dijkgraaf et al[7] suggest that COX inhibitors and/or IL-6 receptor blockers may enhance the therapeutic effect of platinum-based chemotherapy. High levels of IL-6 are associated with a poor prognosis in patients with ovarian cancer resistant to primary treatment. This situation is most likely due to the increased expression of multidrug resistance-related genes (*MDR1* and *GSTpi*) and the increased expression of inhibitors of apoptosis (*Bcl-2*, *Bcl-xL*, and *XIAP*).[12] Our studies

Table 3 Levels of examined cytokines and growth factors in the effusion fluid of the ovarian cancer group according to FIGO stage

	IL-6	IL-8	GDF-15	TRAIL	SCF
FIGO I and II, n=10	284.5/146	456.5/264	1,243/858	35.02/23.1	35.5/29.7
Mean/median (range)	(9.7–528.1)	(15.3–1,148)	(1,342–3,458)	(5.1–88.5)	(21.9–80.7)
FIGO III and IV, n=26	409.5/528.1	184/113	1,284.8/1,194	46.5/19.4	28.7/21.9
Mean/median (range)	(26.6–528.13)	(18.8–948)	(13.3–101)	(3–537)	(13.3–101)
P-value	0.0531	0.2193	0.079	0.2336	0.0077
	DKK-1	**OPN**	**Osteonectin**	**OPG**	**HE4**
FIGO I and II, n=10	629.5/94	25,982/21,652	398.6/382	862.7/441	2,192.5/1,500
Mean/median (range)	(29.9–4,174)	(3,230–46,051)	(158–687)	(361–3,245)	(101–7484)
FIGO III and IV, n=26	333/119	27,389/24,720	513.3/481	1,524/1,123	3,694/1,500
Mean/median (range)	(42.9–3,241)	(101–83,440)	(178–1,347)	(172–5,364)	(101–30,000)
P-value	0.7846	0.8948	0.6285	0.0125	0.4316

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; IL, interleukin; GDF-15, growth differentiation factor-15; SCF, stem cell factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DKK-1, dickkopf-1; OPN, osteopontin; HE4, human epididymis protein 4; OPG, osteoprotegerin.

Table 4 Levels of examined cytokines and growth factors in the effusion fluid of the ovarian cancer group according to cancer grade

	IL-6	IL-8	GDF-15	TRAIL	SCF
Grades 1 and 2, n=16	290.2/247.5	363.9/263.5	1,434.9/1,196.5	30.93/21.75	37.3/29.9
Mean/median (range)	(9.7–528.1)	(15.3–1,070)	(457–3458)	(9–82.7)	(14.8–80.7)
Grade 3, n=20	411.9/528,1	216.2/116	1,190.5/1,061.5	35.7/23.5	27.5/22.1
Mean/median (range)	(37.1–528.1)	(18.8–1,148)	(342–2,494)	(3–537)	(13.3–101)
P-value	0.0779	0.3011	0.584	0.8551	0.0208
	DKK-1	**OPN**	**Osteonectin**	**OPG**	**HE4**
Grades 1 and 2, n=16	539.4/106.5	33,200.5/33,702.5	427.3/373	1,044.7/900.5	2,183.8/1,672.3
Mean/median (range)	(29.9–4,174)	(3,230–76,276)	(183–911)	(361–3,245)	(101–7484)
Grade 3, n=20	365.7/137.5	23,838.7/18,695	503.7/474	1,460.5/1,076.5	3,758.9/1,362.5
Mean/median (range)	(48.6–3,241)	(101–83,440)	(158–1,347)	(172–5,364)	(101–30,000)
P-value	0.4058	0.2315	0.3508	0.2828	0.4621

Abbreviations: IL, interleukin; GDF-15, growth differentiation factor-15; SCF, stem cell factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DKK-1, dickkopf-1; OPN, osteopontin; HE4, human epididymis protein 4; OPG, osteoprotegerin.

demonstrate unambiguously that both ILs are characterized by significantly higher levels in the peritoneal fluid of ovarian cancer patients compared with the peritoneal fluid from patients with other gynecological diagnoses. Clinical stage was associated with higher and nearly significant levels of IL-6 in the subgroup of patients with advanced cancer. No similar relationship was observed for IL-8. Peritoneal spread and the presence of cancer cells within the malignant peritoneal fluid had a significant impact on the increase in IL-6 levels but not IL-8 levels. As shown by the results of Lane et al[24] peritoneal fluid levels of IL-6 and IL-8 depend on the history of chemotherapy, the histopathological type of the tumor and cellular differentiation. The diagnostic value of IL-6 and IL-8 in the peritoneal fluid was examined by ROC curve analysis. We obtained very good results for both cytokines, but in particular for IL-6 (0.829), which makes these proteins potentially important diagnostic biomarkers, especially when taking into account the similar results by other authors. A significant correlation between IL-6 and IL-8 in the peritoneal fluid may indicate the involvement of cytokines with similar mechanisms associated with malignancies.

SCF is a cytokine produced under physiological conditions by ovarian granulosa cells, and it plays an important role in the dynamics of oocyte growth and the development of ovarian follicles.[25] SCF expression has also been observed in ovarian, breast, and lung cancers.[26,27] More than 70% of ovarian cancer epithelial cells co-express Kit and SCF, suggesting the contribution of an autocrine feedback in the development of ovarian cancer.[28] In addition, as demonstrated by Liu et al[29] SCF may also stimulate proliferation of ovarian cancer cells secondary to various mechanisms, both MEK-1-dependent and MEK-1-independent, that play an important role in disease development. Studies by Lawicki et al[30] demonstrated differences in serum SCF levels between patients with ovarian cancer and healthy women, although the cytokine did not meet the criteria for a useful diagnostic test. We were also unable to confirm the diagnostic role of SCF in the peritoneal fluid because its associated AUC was

Table 5 Levels of examined cytokines and growth factors in the effusion fluid of the ovarian cancer group according to the results of cytologic examination of the peritoneal fluid

	IL-6	IL-8	GDF-15	TRAIL	SCF
Neoplastic cells in peritoneal fluid – Yes, n=19	436.1/528.1	273.6/133	1,194.2/952	49.7/13.4	27.5/22,2
Mean/median (range)	(26.6–528.13)	(18.8–1,070)	(459–2949)	(3–537)	(13.9–72.9)
Neoplastic cells in peritoneal fluid – No, n=17	304.8/307.5	263.2/183	1,163.7/1,069.5	36.7/23.9	37.2/27.8
Mean/median (range)	(9.7–528.13)	(15.3–1,148)	(368–2,244)	(5.1–101)	(13.3–101)
P-value	0.0872	0.9632	0.9131	0.088	0.2495
	DKK-1	**OPN**	**Osteonectin**	**OPG**	**HE4**
Neoplastic cells in peritoneal fluid – Yes, n=19	220.3/114	28,477.6/25,123	632.7/602	1,245/1,094	5,613/2,184
Mean/median (range)	(48.6–951)	(5,179–76,276)	(172–3,690)	(172–3,690)	(101–30,000)
Neoplastic cells in peritoneal fluid – No, n=17	770.2/148.5	18,569/16,194	376.6/397	1,064.4/832	2,042.5/1,167.5
Mean/median (range)	(47.3–4,174)	(101–46,051)	(295–3,845)	(295–3,845)	(101–7,484)
P-value	0.4194	0.1816	0.0077	0.2564	0.2513

Abbreviations: IL, interleukin; GDF-15, growth differentiation factor-15; SCF, stem cell factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DKK-1, dickkopf-1; OPN, osteopontin; HE4, human epididymis protein 4; OPG, osteoprotegerin.

Table 6 Levels of examined cytokines and growth factors in the effusion fluid of the ovarian cancer group according to peritoneal spread

	IL-6	IL-8	GDF-15	TRAIL	SCF
Peritoneal carcinomatosis – Yes, n=25	416.7/528.1	172.8/107	1,300.5/1,222	45.4/19.1	29.3/22.1
Mean/median (range)	(26.6–528.1)	(18.8–948)	(459–2,494)	(3–537)	(13.3–101)
Peritoneal carcinomatosis – No, n=11	260.1/144.5	401.7/263.5	1,021.5/730.5	37.6/23.9	36.9/31.2
Mean/median (range)	(9.7–528.1)	(15.3–1,148)	(342–2,244)	(5.1–88.5)	(24.1–80.7)
P-value	0.0476	0.1765	0.1677	0.1763	0.0103
	DKK-1	**OPN**	**Osteonectin**	**OPG**	**HE4**
Peritoneal carcinomatosis – Yes, n=25	345.1/137.5	26,488.5/22,423.5	522.2/481	1,564/1,132	3,841.6/1,500
Mean/median (range)	(47.3–3,241)	(101–83,440)	(178–1,347)	(172–5,364)	(101–30,000)
Peritoneal carcinomatosis – No, n=11	689.49/110.5	25,165.9/19,530.5	406.5/424.5	624.5/428	2,261.8/1,464.3
Mean/median (range)	(60.6–4,174)	(3,230–46,051)	(158–687)	(361–1,272)	(101–7,484)
P-value	0.9548	0.9844	0.3074	0.0006	0.4315

Abbreviations: IL, interleukin; GDF-15, growth differentiation factor-15; SCF, stem cell factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DKK-1, dickkopf-1; OPN, osteopontin; HE4, human epididymis protein 4; OPG, osteoprotegerin.

only 0.445, which excludes SCF as a diagnostic marker. In addition, despite the fact that we were unable to identify significant differences in mean SCF levels between the cancer cell-positive peritoneal fluid and the fluid collected from women with benign gynecological disorders, the behavior of this cytokine within the ovarian cancer-patient group is interesting per se, as we observed that the more advanced and the less differentiated the tumor characterized by peritoneal spread, the more significant the reduction in the SCF levels within the peritoneal fluid. A difference, albeit not significant, was also observed between individual histopathological types, with lower SCF values in the peritoneal fluid of patients with serous carcinomas. The lack of correlation between SCF and other examined cytokines might indicate a completely different mechanism of action. Due to the low volume of studies published to date on this cytokine and the relatively small study group of our research, further research is required to understand this behavior.

GDF-15 is a growth factor belonging to the TGF-β family. Because it was initially identified in macrophages, it is also referred to as macrophage-inhibitory cytokine-1 (MIC-1).[31] Under physiological conditions, greater amounts of GDF-15 are produced only in the placenta, but significant increases are observed in inflammation, acute disorders, and cancer conditions.[32,33] Cytoplasmic expression of GDF-15 in ovarian cancer cells is high, at approximately 97%. GDF-15 is also found, but to a lesser extent, in prostate, pancreas, colon, and kidney cancers. GDF-15 regulates cellular processes, including cell cycle control, proliferation, differentiation, and apoptosis. It is also called stress-related cytokine because its transcription is induced by tumor necrosis factor-α, IL-1β, IL-2 and macrophage colony-stimulating factor. GDF-15 is a versatile growth factor capable of regulating carcinogenesis by affecting apoptosis, cell death, and tumor invasiveness.[33,34] Stimulation of ovarian cancer cell lines with GDF-15 has been shown to promote growth, invasion, and the increased activity of metalloproteinases and VEGF, whereas neutralization of GDF-15 by m-TOR inhibitors is associated with inhibition of tumor invasion and growth.[34] Similar results have been observed with the non-steroidal anti-inflammatory drug sulindac sulfide, which caused GDF-15-mediated inhibition of ovarian cancer cell growth in vitro.[35] The diagnostic and prognostic applicability of GDF-15 has been reported with increasing frequency in recent years.[36–39] Staff et al[36] reported on the diagnostic utility of GDF-15 in uterine carcinoma. Significantly higher levels of GDF-15 correlated with high stage, low differentiation, non-endometrioid histopathological type, hormonal status and lymph node involvement.[36] In light of studies conducted by Trovik et al[37] GDF-15 appears to be a very good marker for the differentiation of benign mesenchymal tumors from uterine sarcomas. To date, only one report has been published with regard to the behavior of GDF-15 within the peritoneal fluid.[39] In that report, the authors demonstrated that the levels of GDF-15 within the peritoneal fluid of patients with ovarian cancer ranged from 353 to 190,082 ng/L (median, 5,277 ng/L). Higher levels were observed in patients after chemotherapy compared with treatment-naïve patients. GDF-15 expression, mostly noted within the cytoplasm, was detected in 97% of cancer cells collected from peritoneal fluid samples. No expression was observed in reactive non-cancer mesothelial cells. Expression in cancer cells correlated with cytokine levels within the peritoneal fluid.[39] In our study, similar to the study conducted by Bock et al[39] we determined the GDF-15 levels in the peritoneal fluid of patients with ovarian cancer patients and patients with non-malignant conditions. The point values

and ranges observed in our study were significantly higher (after conversion from pg/mL to ng/L). In our study, the median GDF-15 level in the peritoneal fluid of patients with cancer was 1,069 pg/mL compared with 5,277 ng/L in the study by Bock et al.[39] Even the median GDF-15 levels from the non-cancerous peritoneal fluid was higher in our study (441 pg/mL) than in the study conducted by Bock et al.[39] The difference in GDF-15 levels between groups A and B was significant, P=0.0125, although no differences between GDF-15 levels were observed within the cancer group according to the tested prognostic factors. In our study, no differences were observed in the level of the studied cytokine according to the status of cancer cells in the peritoneal fluid, whereas in the study by Bock et al[39] the fluid levels correlated with cellular expression of the cytokine. It should be noted that the study group in the Bock et al[39] study was markedly larger, as the analyses were conducted in 195 ovarian cancer patients. However, it appears that such significant differences are mostly due to completely different analytical methods used in both studies. Staff et al[36] concluded that GDF-15 may act as a new marker for ovarian cancer. In our study, in assessing the diagnostic utility of GDF-15 in the peritoneal fluid by ROC curve analysis, the AUC was found to be 0.752, which leaves open the possibility that this cytokine may have use as a diagnostic marker. Correlation analysis of the GDF-15 concentrations with other cytokines in our study showed that the concentrations of this cytokine correlate with OPG, TRAIL, DKK-1, and IL-8, which confirms its interaction and participation in carcinogenesis. In summary, the growth factor GDF-15 is a promising protein to be used in future analyses and likely also in clinical practice.

DKK-1 is the best-studied member of the DKK protein family and a direct inhibitor of the Wnt transduction pathway.[40] Most studies suggest that the activity of *DKK-1* is that of an oncogene rather than that of a suppressor gene. High DKK-1 expression has been observed in numerous tumors, including gynecological tumors.[41–44] The mechanism of action of DKK-1 in the neoplastic process of serous ovarian carcinoma was shown to likely involve activation of P-JNK1 in cancer cells, leading to formation of actin filaments and filopodia.[41] When analyzing the serum levels of DKK-1 in patients with various types of gynecological malignancies, the highest utility of the factor was observed in cervical carcinoma, particularly squamous cell carcinoma, and in endometrial carcinoma.[45] In the present study, despite the fact that the mean serum levels of DKK-1 in ovarian cancer patients were higher than in healthy patients, the difference was not significant. In our study, we observed a tenfold increase in mean DKK-1 levels in the peritoneal fluid compared with serum levels, as reported by Jiang et al.[45] The DKK-1 levels within the peritoneal fluid of patients with uterine cancer (423.6 pg/mL) were found to be higher than in the fluid of patients without cancer (272.9 pg/mL); however, the difference was not significant. No differences were observed in DKK-1 levels among ovarian cancer patients according to the analyzed prognostic factors. The 0.604 AUC value for this cytokine indicates the probability that it is of little diagnostic value.

TRAIL is an interesting molecule described as a selective killer of cancer cells that maintains the function of healthy cells; according to numerous authors, it may constitute an ideal pathway for selective anticancer treatment.[46–49] In addition it has been found that TRAIL selectively promotes apoptosis in particular in cells with high c-MYC expression levels.[46] Interesting studies have been presented by Lane et al[11,49] who determined that ascites may activate the Akt pathway and focally enhance cellular adhesion in an integrin-mediated fashion, thus protecting tumor cells from the pro-apoptotic effects of TRAIL. In our studies, we observed no differences in the levels of the soluble forms of TRAIL in the peritoneal fluid of both groups. The levels of TRAIL within the peritoneal fluid also did not correlate with adverse prognostic factors. The area under the ROC curve for TRAIL was 0.482, indicating that TRAIL in the peritoneal fluid does not qualify as a marker for diagnostic significance.

HE4 is one of the newer and exceptionally useful tumor markers used in preoperative diagnostics for patients with ovarian cancer.[50,51] Despite numerous studies on its diagnostic applicability, its biological role has not been fully explained. In our studies, we were unable to observe any differences in the levels of HE4 within the peritoneal fluid, which is consistent with previously reported results.[52,53] The diagnostic value of HE4 protein in the serum of ovarian cancer patients is not subject to discussion, and at the present time, in combination with CA125, it has a very important role in predicting ovarian tumor malignancies. However, in our study, we found that the diagnostic utility of HE4 in the peritoneal fluid was very small, with an AUC of only 0.536. HE4 does not correlate with any other tested cytokines, which confirms its biological individuality.

Under physiological conditions, OPG is the main modulator of bone remodeling. Its activity is regulated by the nuclear factor (NF)-κB receptor (RANK) and its ligand, RANKL. RANKL is an activator of differentiation and osteoclastogenesis, whereas OPG is a dummy molecule that inhibits this differentiation. OPG Is produced within

matrix cells, megakaryocytes and endothelial cells. In bone metastases, the RANKL/OPG ratio is significantly increased, leading to bone destruction.[54] Other effects of this factor have also been demonstrated; secreted by endothelial cells, OPG may affect their migration and proliferation.[55] OPG may also regulate the neoplastic process in ovarian cancer. By activating integrins and enhancing local adhesion, OPG may inhibit TRAIL-induced apoptosis.[12] This process may develop in the condition of malignant ascites during the natural course of ovarian cancer.[12] The OPG levels in the peritoneal fluid of ovarian cancer patients described by Lane et al[12] were very diverse, ranging from 0.18 to 453 nM, with a median of 11.2 nM. In our study, the mean OPG level in malignant peritoneal fluid was 1,321 pg/mL, with a median of 1,045 pg/mL and a range of 172–5,364 pg/mL. Despite the fact that the mean value of OPG in group B was lower (796 pg/mL), the difference was not significant (P=0.287). By contrast, in the ovarian cancer group, a significant increase in OPG levels was observed in advanced cancers and cancers with peritoneal spread. This result might indirectly confirm the results obtained by Lane et al who demonstrated that high levels of OPG within the peritoneal fluid had the adverse effect of inhibiting cancer cell apoptosis.

OPN is a glycophosphoprotein expressed in numerous types of cells, such as osteoblasts, osteoclasts, mammary gland epithelial cells, smooth muscle cells, endothelial cells, macrophages, and others. OPN is involved in bone remodeling, inflammation, ischemia, and tumor progression. OPN is a mediator of cell adhesion; it has chemotactic, proangiogenic, and apoptosis-inhibitory properties.[56,57] OPN is ascribed with diagnostic and prognostic properties,[56,58–60] and some researchers note its possible use in cancer treatment.[61] The study by Kato and Motoyama[62] revealed a markedly higher expression of OPN in clear cell ovarian cancer compared with serous ovarian cancer. In 2011, Davidson et al[63] published a study that described the expression of OPN in ovarian cancer cells isolated from peritoneal fluid. OPN expression was detected in 74% of cancer cells; it was more common in more advanced and less differentiated cancers and yet was associated with a greater success of radical surgery and a better response to chemotherapy.[63] In our study, we observed significantly higher OPN levels within the peritoneal fluid of patients with ovarian cancer (27,726 pg/mL) compared with the control group (21,997 pg/mL; P=0.0324). Contrary to expectations, no differences were observed in the behavior of OPN within the peritoneal fluid of ovarian cancer patients according to stage, grade or peritoneal spread status.

Osteonectin (also known as SPARC) is an intercellular matrix glycoprotein present mostly in tissues subject to mineralization. Despite substantial evidence supporting the involvement of osteonectin in neoplastic processes, its role has not been fully elucidated, and studies have yielded contradictory results.[64–66] A study by Chen et al[66] revealed that osteonectin overexpression was associated with poor differentiation, high staging, lymph node involvement, and poor prognosis in patients with ovarian cancer. Blocking osteonectin inhibits the invasion and proliferation of cancer cells, while enhancing apoptosis. Said et al[67,68] and Said and Motamed[69] claimed that the absence of osteonectin in the peritoneal fluid is associated with extensive neoplastic spread. An interesting behavior of osteonectin was observed in our study group. Significantly higher values of osteonectin were observed in patients with ovarian cancer compared with the remaining patients (478.25 ng/mL vs 246 ng/mL), but among patients with ovarian cancer, high values were not related to tumor stage, grade or peritoneal spread status. Significantly higher osteonectin levels were observed in patients with positive-cytology peritoneal fluid (P=0.0077).

OPG, OPN, and osteonectin have high values of area under the ROC curve which presupposes that they can have diagnostic value, which requires further studies (OPG =0.713, OPN =0.657, osteonectin =0.806). We described the strong correlation between OPG and osteonectin (r=0.5006). Between OPN and osteonectin and OPG and OPN there were no significant correlations.

Conclusion

To conclude, only IL-6, IL-8, GDF-15, OPN, and osteonectin were characterized by significant differences in peritoneal fluid levels in patients with ovarian cancer compared with patients with other gynecological disorders. The levels of SCF and OPG, although not different between those with ovarian cancer and those with non-malignancy diseases, were significantly different depending on the analyzed prognostic factors. IL-6, GDF-15, and osteonectin levels showed the highest diagnostic value. GDF-15, SCF, and OPG deserve special attention in the context of future research on peritoneal fluid as a tumor microenvironment because precise understanding of the mechanisms involved may contribute to the development of newer, more effective diagnostic methods and new therapies for this very difficult-to-treat cancer.

Disclosure

The authors have no conflicts of interest to disclose.

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