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Vascular Endothelial Growth Factor (VEGF) Concentration in Sera and Tumor Effusions from Patients with Ovarian Carcinoma

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Abstract. *VEGF is an important angiogenic cytokine with a critical role in tumor angiogenesis. VEGF concentrations were measured using an ELISA assay, detecting $VEGF_{165}$ isoform, in tumor cyst and/or ascitic fluids and in sera of 86 patients with malignant neoplasms and in 53 patients with benign ovarian neoplasms. VEGF levels were significantly elevated in the sera and cyst fluids of carcinoma patients compared with patients who had benign neoplasms. In carcinoma patients, statistically higher VEGF levels were detected in tumor effusions than in corresponding sera. The differences between VEGF values in sera and tumor effusions in relation to histological subtypes of ovarian carcinoma and FIGO stages were statistically insignificant. High VEGF levels in ascitic fluids appeared to be significantly associated with shorter disease-free survival and overall survival. In multivariate analysis, besides FIGO stage and age of patients, only serum VEGF concentration was an independent prognostic factor for overall survival. The elevated VEGF levels in sera and tumor effusions of patients with FIGO stages I/II indicated that angiogenesis promoted by VEGF is a continuous process, independent of clinical advancement of the disease.*

Angiogenesis is a crucial event for the growth and metastasis of solid tumors, regulated by a balance between pro- and antiangiogenic factors (1,2). One of the key molecules in the regulation of angiogenesis is a multifunctional cytokine – vascular endothelial growth factor A (VEGF-A), a member of the vascular permeability family of proteins (3).

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VEGF promotes the formation of new blood vessels and is necessary for endothelial survival (1,3-7), acting through kinase receptors selectively, though not exclusively, located on the surface of vascular endothelial cells (2-4,7). Thus, VEGF plays an important role in tumor expansion. As a result of alternative splicing of mRNA, five isoforms of VEGF-A: 121,145,165, 189 and 206 amino acids, are generated from a single VEGF-A gene (8). Usually the $VEGF_{121}$ and $VEGF_{165}$ isoforms are predominant and secreted in soluble forms (8,9).

Epithelial ovarian carcinomas are the leading cause of death from gynecological cancers. The lack of early stage symptoms and poor understanding of ovarian tumor biology contribute to the diagnosis of ovarian carcinomas in advanced stages of the disease. The common pathway of progression in ovarian carcinomas is the accumulation of ascitic fluids, which often contain malignant cells. The significance of VEGF in the development of ovarian carcinoma has been extensively studied (6,7,10-16), as well as the presence of VEGF in tissue specimens (6,10,11,14,17) and in patient sera (10,12,13,15,18).

A growing body of evidence indicates that angiogenesis is an early event in ovarian cancer progression and also reflect poor outcome of patients (6,12,13,15,19). There are further reports that increased VEGF expression enhances vascular permeability and thus facilitates the dissemination of carcinomas beyond regional borders and formation of ascitic fluids (3,10,12,13,20). However, the clinical consequence of the difference in VEGF concentration in the tumor cyst and/or ascitic fluids and in sera of individual patients with ovarian carcinoma remains largely unclear (10,11,15).

For a better understanding of the relationship between VEGF levels and the biological behaviour of ovarian carcinomas, we analyzed and compared VEGF concentrations using an ELISA assay, which mainly detects the most

Table I. *Histological structure and FIGO stage in ovarian carcinoma patients (n=86).*

	FIGO Stages								
	I		II		III		IV		
Carcinoma	n	(%)	n	(%)	n	(%)	n	(%)	N
Serous	4	29	2	22	25	68	18	69	49
Endometrioid	5	36	6	67	6	16	3	12	20
Mucinous	3	21	1	11	0	0	0	0	4
Undifferentiated	2	14	0	0	6	16	5	19	13
Total	14	100	9	100	37	100	26	100	86

abundant $VEGF_{165}$ isoform (9), in the representative series of tumor cyst and/or ascitic fluids and in sera of individual patients with ovarian malignant and benign neoplasms, taking into account the conventional clinicopathological variables. The clinical usefulness of preoperative serum VEGF concentration in patients with ovarian carcinoma was also considered to assess the effect of VEGF levels on disease progression and survival of patients.

Materials and Methods

Patients. Eighty-six consecutive patients with newly diagnosed and histologically confirmed ovarian carcinomas and 53 patients with ovarian benign neoplasms were enrolled in this study. Informed consent was obtained from all patients. The patients were hospitalized and underwent laparotomy between the years 1997-2002 at the 1st Department of Gynecology, Wroclaw Medical University, Poland.

Cyst and ascitic fluids as well as serum samples were collected during primary surgical debulking prior to any other intervention. The clinical stage of disease was defined according to the FIGO staging system for ovarian carcinoma. Histopathological recognition and the clinical stages of the ovarian carcinomas are shown in Table I. In 49 serous carcinomas, 32 cases presented with serum and corresponding ascitic fluid, 8 patients with serum and cyst fluids and 8 subjects with serum, cyst and ascitic fluids. In the remaining patient, only serum was available. In 7 out of 20 patients with endometrioid carcinoma, serum and cyst fluids were available, in 9 patients serum and ascites and in 4 patients serum, cyst and ascitic fluids. In 1 out of 4 mucinous carcinomas, serum and cyst fluids were available, in 1 patient serum and ascitic in 2 patients' serum, cyst and ascitic fluids were available. Among 4 out of 13 patients with undifferentiated carcinoma, serum and cyst fluid were taken, in 8 patients serum and ascites were available and in 1 case only a serum sample. In general, cyst fluid samples were obtained from patients with FIGO I/II stages and ascites from patients with FIGO III/IV stages. The majority of carcinomas (78%) were poorly- or moderately- differentiated; 48% were classified as G_2 and 30% as G_3. The remaining carcinomas (22%) were well- differentiated (G_1). Thirty-two patients (37%) had a residual tumor mass less or equal to 2 cm after surgery. The ovarian benign neoplasms were histopathologically recognized as 9 serous, 10 mucinous cystadenomas and 34 simple ovarian cysts. In these patients only serum and cyst fluid samples were available.

Table II. *VEGF concentration in sera, cyst and ascitic fluids of patients with ovarian carcinoma.*

	Serum		Cyst fluid		Ascites	
Histological recognition	VEGF (pg/mg)					
	median	range	median	range	median	range
Serous cystadeno-carcinoma	396.8	96.4-1664.0	20000.0	2016.0->20 000	3465.0	387.0->20 000
Endometrioid cystadeno-carcinoma	443.8	83.2-1226.0	20000.0	127.3->20 000	2127.0	5.3->20 000
Mucinous adenocarcinoma	310.4	168.8-361.6	1934.0	1553.0->20 000	126.0	74.7-3451.0
Undifferentiated carcinoma	485.3	64.9-2416.0	10863.0	2101.0->20 000	2406.0	297.0->20 000
Total	386.8*	64.9-2416.0	20 000**	127.3->20 000	3035.0	5.3->20 000

*Significant difference in VEGF serum level between ovarian cancer and reference groups (<0.0001)
** Significant difference in VEGF cyst fluid level between ovarian cancer and reference groups (<0.00001)

Disease-free survival (DFS) was defined as the interval between surgery and recurrence/progression. Overall survival (OS) was defined as the time between diagnosis and death or the time at which the patient was last seen.

Assays. All tumor effusions and serum samples were centrifuged at 2000 rpm for 10 minutes and stored at -80˚C until analysis. For measurement of vascular endothelial growth factor a commercially available enzyme-linked immunosorbent assay was applied (Quantikine human VEGF, R&D Systems Minneapolis, USA). All vascular endothelial growth factor analyses were performed under similar experimental conditions.

According to the manufacturer, the VEGF assay measures the $VEGF_{165}$ isoform. The upper reference limit for VEGF based on healthy donors was 220 pg/mL. Cyst/ascitic fluids and serum samples were assayed in duplicate, undiluted or appropriately diluted.

Statistical analysis. For evaluation of differences between analysed groups the Mann-Whitney *U*-test and Wilcoxon rank test were performed. ROC curves were used to assess the sensitivity and specificity of VEGF serum and cyst fluid levels. The differences between the areas under curves (AUC) were estimated by the Wilcoxon test. The prognostic value of clinical parameters and VEGF levels in serum, cyst and ascitic fluids was evaluated by the Kaplan-Meier method and with the log-rank test. The evaluation of the independent prognostic factors was performed applying multivariate analysis using the Cox hazard model.

Table III. *VEGF concentration in sera and cyst fluids of patients with ovarian benign neoplasms (reference group).*

Histological recognition	Serum		Cyst fluid	
	VEGF (pg/ml)			
	median	range	median	range
Serous cystadenoma	355.3	67.3 - 656.2	1941.0	7.0 - >20 000
Mucinous cystadenoma	191.9	47.8 - 349.8	2944.0	636.4 - >20 000
Serous cyst	208.8	3.0 - 1325.0	241.5	20.0 - >20 000
Total	210.9	3.0 - 1325.0	636.4	7.0 - >20 000

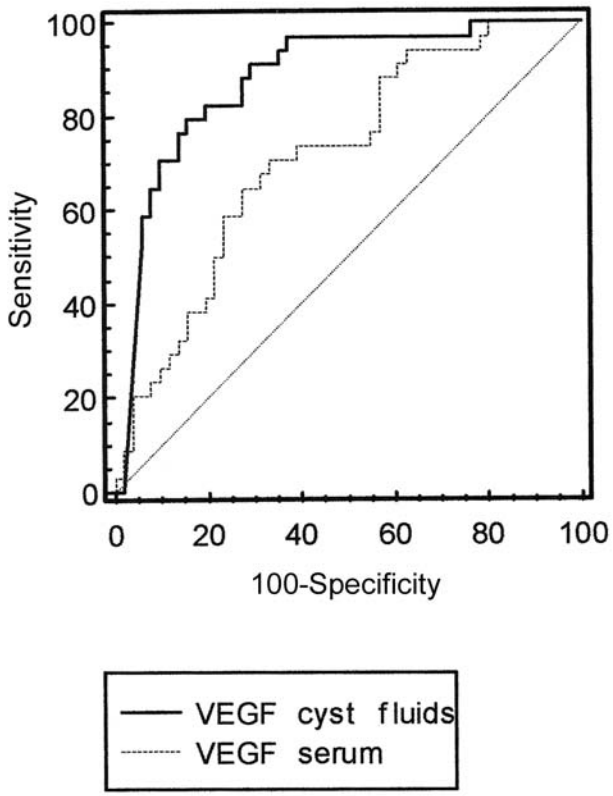

Figure 1. *ROC curves analysis of VEGF levels in sera and cyst fluids of patients with ovarian carcinoma (n=86) and benign neoplasms (n=53). Area under ROC curve; VEGF cyst fluid 0.878±0.066, VEGF serum 0.714±0.059, p=0.013.*

Results

The median values and ranges of VEGF levels in sera, cyst and ascitic fluids of patients with ovarian carcinoma and the reference group of patients with benign ovarian neoplasms are presented in Table II and Table III, respectively. Independent of the histological structure, VEGF concentrations were significantly higher in sera and cyst fluids of patients with ovarian carcinoma compared to the reference group.

The VEGF serum level exceeded the upper reference limit of 220 pg/mL in 71% and 47% of the patients with malignant and benign ovarian neoplasms, respectively ($p=0.005$). The cut-off values, calculated as 95th percentile concentration based on our reference group of benign ovarian neoplasms, appeared to be much higher, at 758 pg/mL for serum and 19058 pg/mL for cyst fluid. By using these calculated VEGF cut-off values, only 19.8% of patient sera and 55.9% of cyst fluids showed elevated VEGF concentration.

The ROC curves and AUCs analysis revealed that the measurement of VEGF in cyst fluids showed elevated values compared with patient sera, thus reflecting a differentiation between malignant and benign ovarian neoplasms (Figure 1). ROC analysis resulted in an elevation of serum VEGF cut-off (372 pg/mL) and this higher cut-off point gave a sensitivity and specificity of 55.8% and 76.5%, respectively. ROC analysis of the cyst fluid gave a VEGF cut-off value of 4368 pg/mL and this cut-off exhibited a sensitivity and specificity of 79.4% and 84.9%, respectively.

In cancer patients, the highest VEGF concentrations were found in cyst fluids, lower in ascites and the lowest in patient sera. In the reference group of benign neoplasms, the VEGF levels were also significantly higher in cyst fluids compared with patient sera ($p=0.001$) (Figure 2).

Neither in patient sera nor in tumor effusions were significant correlations between VEGF concentration and histological subtypes of ovarian carcinomas shown ($p=0.6$ for the sera, $p=0.4$ for cyst and $p=0.3$ for ascitic fluids, respectively) (Table II). Similarly, in ovarian benign neoplasms, no significant differences between serum VEGF concentration and histological subtypes were found (Table III). The VEGF levels were also comparable in cyst fluids of serous and mucinous cystadenomas ($p=0.3$). However, significantly lower VEGF values were observed in serous cysts than in mucinous cystadenomas ($p=0.001$), while the achieved difference between serous cystadenoma and serous cysts only indicated a tendency to significance ($p=0.07$).

Figure 3 presents VEGF concentrations in sera, cyst and ascitic fluids in relation to FIGO stages of individual patients with ovarian carcinoma. The differences between VEGF values in sera and tumor effusions in relation to FIGO stages I/II and III/IV were relatively small and statistically insignificant ($p=0.54$ for sera, $p=0.19$ for cyst and $p=0.15$ for ascitic fluids). No significant associations between the grade of differentiation and VEGF level in

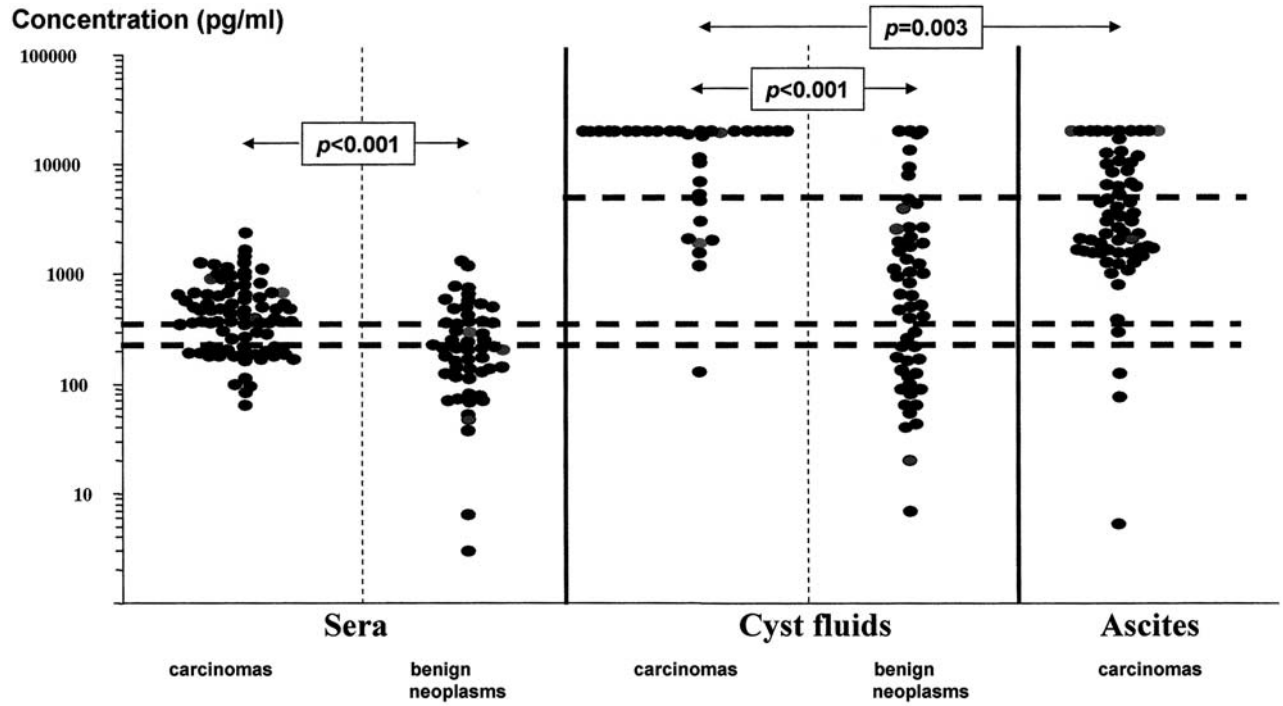

Figure 2. *Comparison of VEGF concentration in sera, cyst and ascitic fluids of individual patients with ovarian carcinoma and in sera and cyst fluids of patients with benign neoplasms. VEGF cut-off in serum and tumor effusions 220 pg/ml, after ROC analysis VEGF cut-off in serum 372 pg/ml and in tumor effusions 4368 pg/ml.*

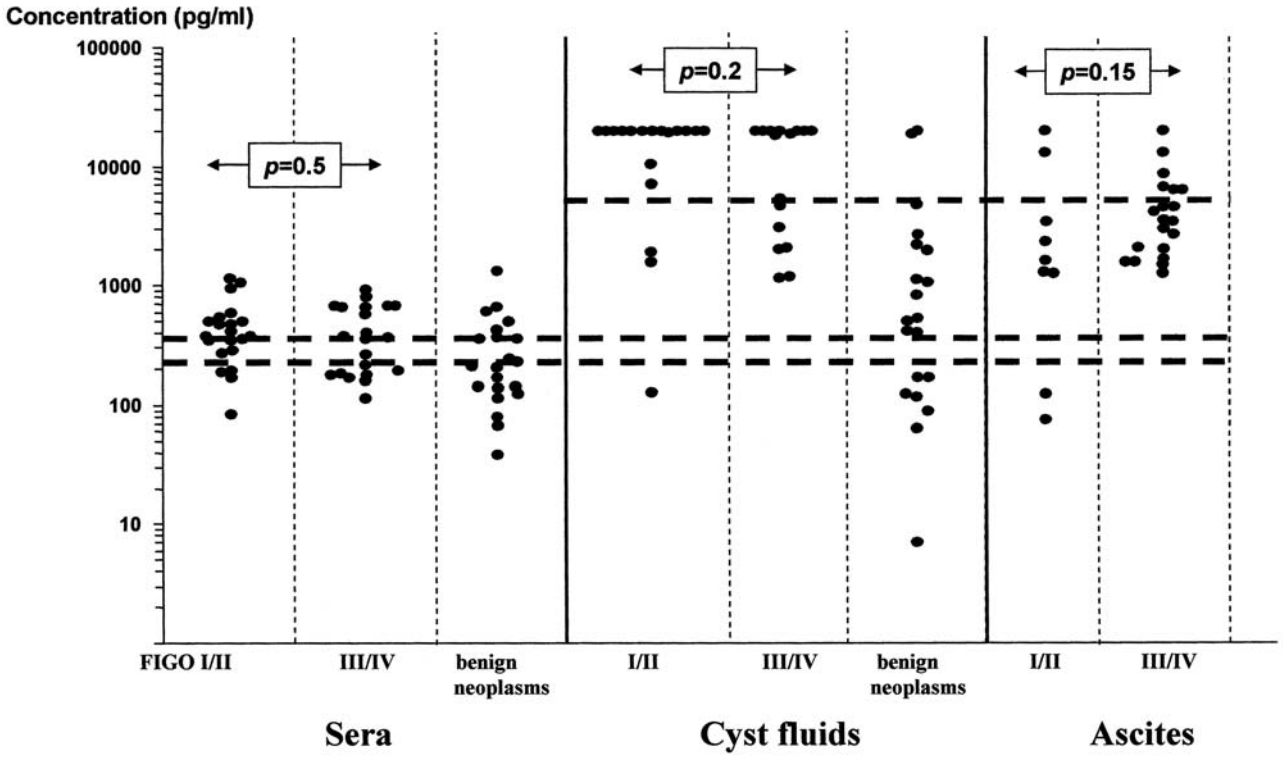

Figure 3. *Relationships between VEGF concentration and FIGO stage in sera, cyst and ascitic fluids of individual patients with ovarian carcinoma. VEGF cut-off in serum and in tumor effusions 220 pg/ml, after ROC analysis VEGF cut-off in serum 372 pg/ml and in tumor effusions 4368 pg/ml.*

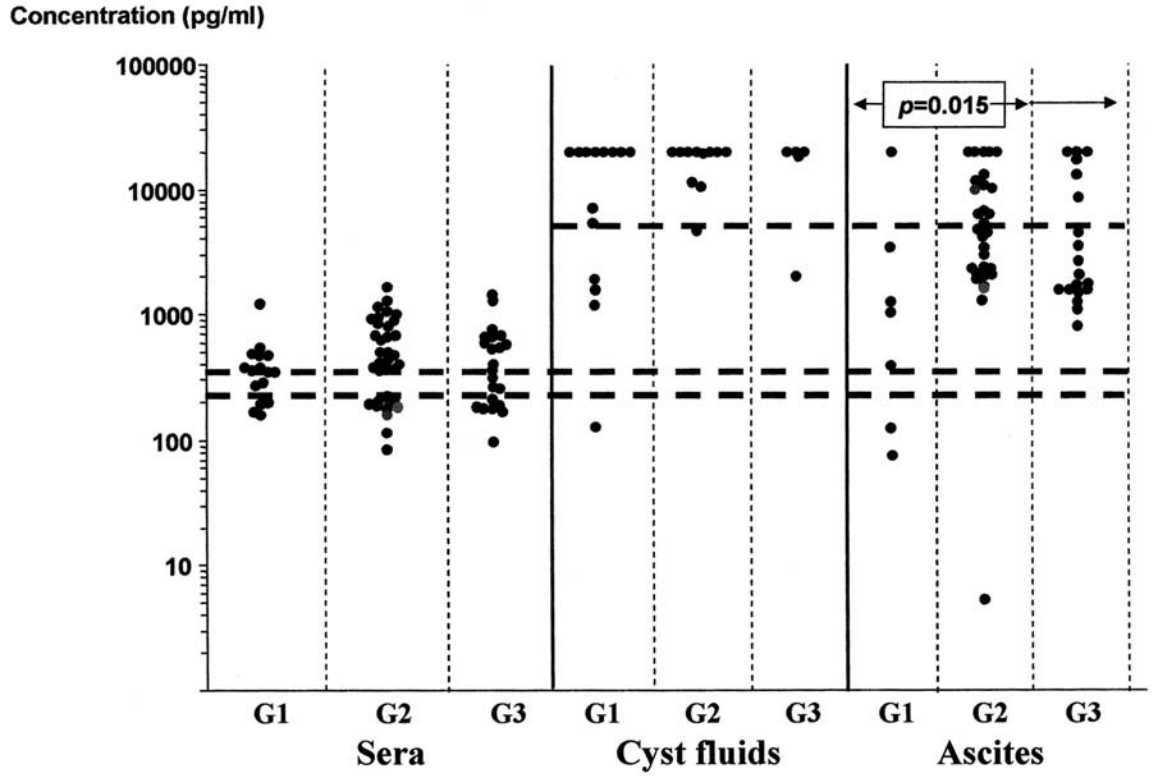

Figure 4. *Relationships between VEGF concentration and grade of differentiation in sera, cyst and ascitic fluids of individual patients with ovarian carcinoma. VEGF cut-off in serum and in tumor effusions 220 pg/ml, after ROC analysis VEGF cut-off in serum 372 pg/ml and in tumor effusions 4368 pg/ml.*

Table IV. *Univariate and multivariate disease-free survival analysis.*

Univariate analysis		Multivariate analysis		
Variable	*p* log-rank	Relative risk	95% CI	*p*
FIGO stage (I + II *vs.* III + IV)	0.0000	4.66	2.08 – 10.4	0.00018
Histological grade (1 *vs.* 2+3)	0.0001	-	-	-
Histology (others *vs.* serous)	N.S	-	-	-
Age (< 62 *vs.* > 62)	N.S	-	-	-
Residual disease (< 2 cm *vs.* > 2 cm)	0.0001	-	-	-
VEGF [pg/mL] serum (< 750 *vs.* > 750)	N.S	-	-	-
VEGF [pg/mL] ascites (< 2000 *vs.* > 2000)	0.0298	-	-	-

Table V. *Univariate and multivariate overall survival analysis.*

Univariate analysis		Multivariate analysis		
Variable	*p* log-rank	Relative risk	95% CI	*p*
FIGO stage (I + II *vs.* III + IV)	0.00066	4.08	1.44 - 11.55	0.008
Histological grade (1 *vs.* 2+3)	0.00079	-	-	-
Histology (others *vs.* serous)	N.S	-	-	-
Age (< 62 *vs.* > 62)	0.0478	2.20	1.09 – 4.43	0.0272
Residual disease (< 2 cm *vs.* > 2 cm)	0.00637	-	-	-
VEGF [pg/mL] serum (< 750 *vs.* > 750)	0.0169	2.35	1.14 – 4.84	0.0208
VEGF [pg/mL] ascites (< 2000 *vs.* > 2000)	0.00675	-	-	-

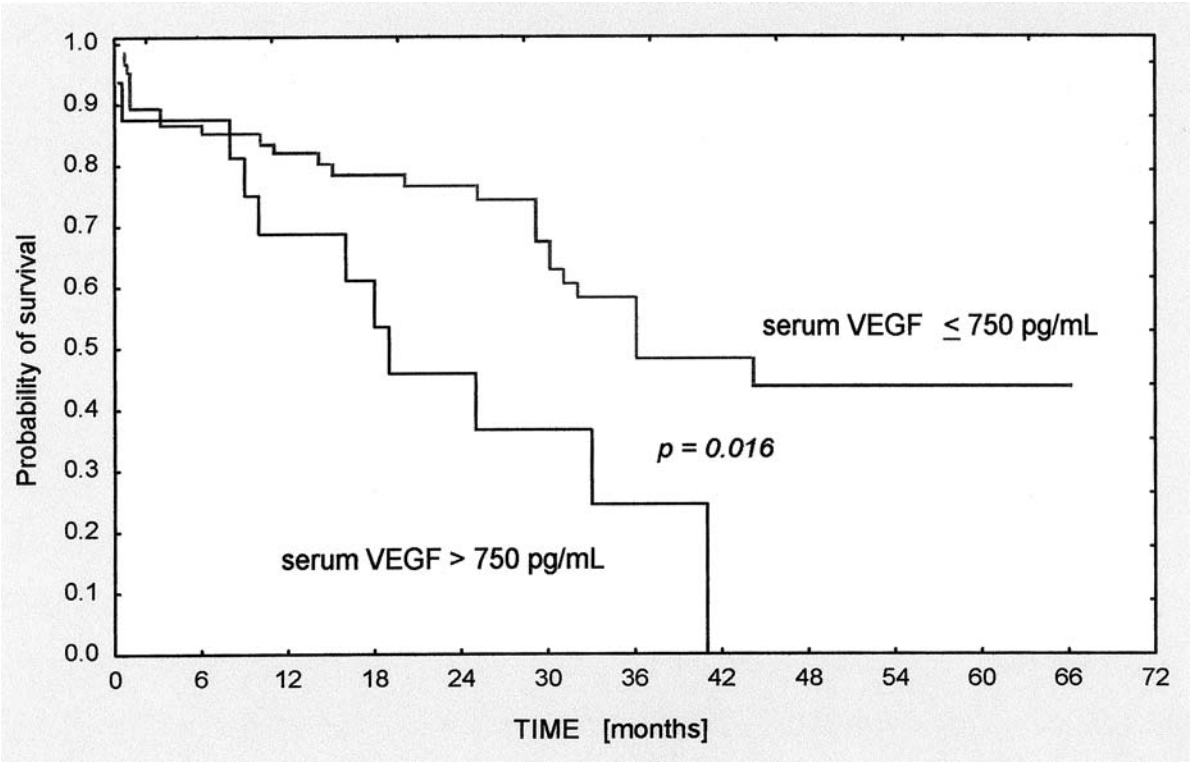

Figure 5. *Kaplan-Meier overall survival analysis of ovarian carcinoma patients according to the preoperative serum VEGF levels.*

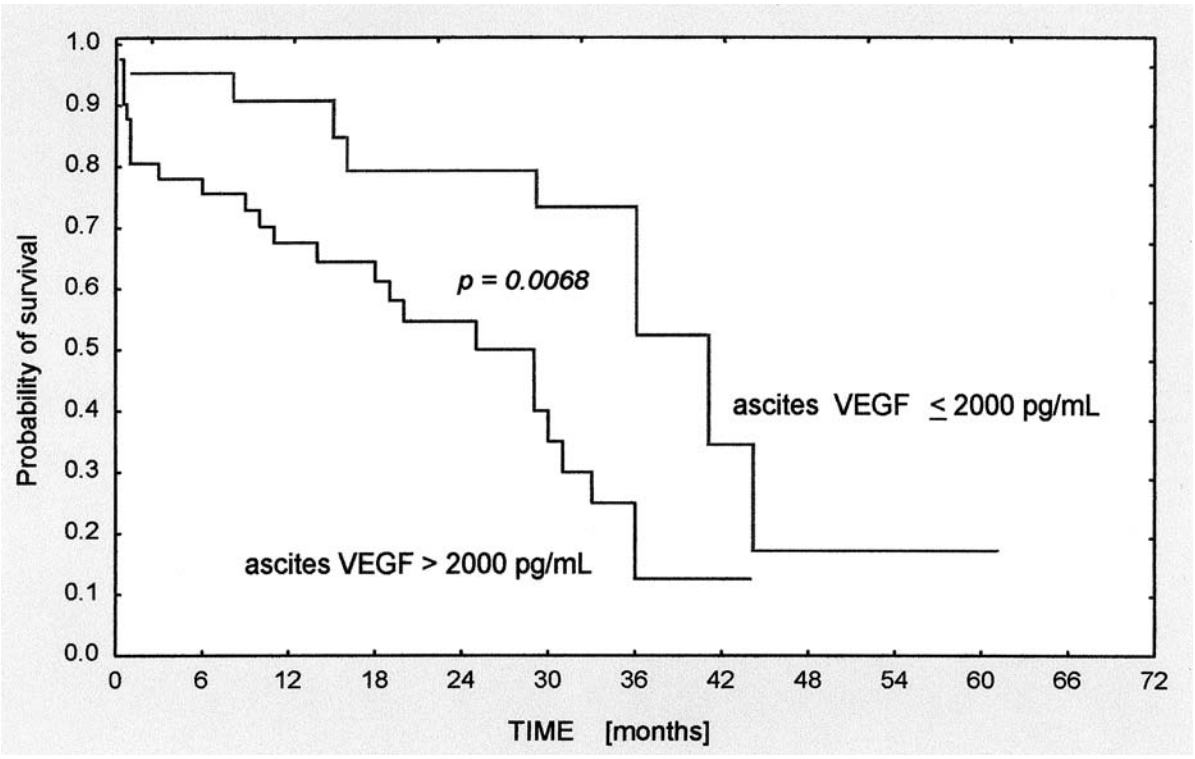

Figure 6. *Kaplan-Meier overall survival analysis of ovarian carcinoma patients according to VEGF levels in ascites.*

sera and cyst fluids could be demonstrated. However, a correlation between the subgroups, divided according to the degree of differentiation, G1 *versus* G2+G3, and VEGF concentration in ascitic fluid was observed ($p<0.015$) (Figure 4). The presence of ascitic fluid was found in 64 out of 86 (74.4%) ovarian carcinoma patients, but no difference in VEGF serum concentration was noted.

In univariate analysis, a significant correlation between disease-free survival and FIGO stage, grade of differentiation, residual disease and VEGF ascitic fluid was found. However, in multivariate analysis only the FIGO stage was an independent prognostic factor (Table IV). Independent factors for the assessment of overall survival in ovarian carcinoma patients were also determined by uni- and multivariate analysis. Univariate analysis revealed a significant correlation between overall survival and FIGO stage, grade of differentiation, age, residual disease and VEGF levels measured in sera and ascitic fluids (Table V). Using the Kaplan-Meier method, the probability of predicting an overall survival for ovarian carcinoma patients based upon VEGF levels in sera and ascites was calculated. The median overall survival for patients, with preoperative VEGF serum level <750 pg/mL and >750 pg/mL, was 36 and 18.5 months, respectively (Figure 5).

Considering the VEGF concentration in ascitic fluids, it appeared that the mean survival time was 36 months for patients with VEGF level <2000 pg/mL and 25 months in patients with VEGF concentration exceeding 2000 pg/mL (Figure 6). Multivariate analysis revealed that only FIGO stage, age of patients and serum VEGF level appeared to be independent prognostic factors for the assessment of overall survival in ovarian carcinoma patients (Table V).

Discussion

Ovarian cancer is usually associated with malignant ascites formation, the principal cause of patients' morbidity and mortality. VEGF-A seems to play a key role in ovarian cancer progression by promoting the neovascularization and increasing vascular permeability, facilitating induction of ascites (10,12,20-22). The quantitative determination of circulating VEGF in serum and cyst and ascitic fluids from individual patients with epithelial ovarian carcinoma, to evaluate the relationship between the ability to produce and release VEGF from the primary tumor into the blood circulation, have been described in a few reports (10,11,15). Our studies were performed using an ELISA test which detected mainly the predominant $VEGF_{165}$ isoform (9). According to Fujimoto *et al.* (6), this VEGF subtype is essential for all processes of ovarian carcinoma advancement, depending on angiogenic activity. In our studies, independent of the histopathological structure of ovarian carcinomas, significantly higher VEGF concentrations were measured in cyst and ascitic fluids than in corresponding patients' serum. Moreover, cyst fluids showed statistically higher VEGF levels compared with ascites. Ovarian cyst fluid is by nature more homogenous than tumor tissue and comparative analysis of VEGF concentration in cyst fluid and serum of individual patients provides important information regarding tumor biology (3,15). VEGF is not a tumor-specific marker and other factors may contribute to its serum concentration. It is known that inflammatory cells are a rich source of VEGF (3,4,14,23) and the measurement of VEGF in sera seem to be less valid than expected. The high intracystic levels of VEGF may also indicate the existence of a physiological barrier, at least partially restricting the release of the marker into the surrounding environment and circulating blood. The limited correlation between tumor effusions and serum CA125 and TPS levels in patients with ovarian carcinomas has been previously reported (24-27). Similarly to other data (10,13,15,18), our observation showed that carcinomas contained higher levels of VEGF than benign neoplasms both in sera and cyst fluids, indicating that VEGF is produced more actively in carcinomas. Moreover, leakage of VEGF from the highly permeable vascularized tumor compartment caused its elevation in the blood circulation (10).

Higher preoperative VEGF serum levels in ovarian carcinoma patients could suggest that VEGF may be considered as a circulating marker facilitating the discrimination between malignant and benign ovarian tumors (10,15). Our studies revealed, however, that 29% of ovarian carcinoma patients showed a VEGF level below the reference value and 47% of benign neoplasms had elevated VEGF serum levels. The application of ROC analysis facilitated the determination of the optimal cut-off points for the diagnostic specificity and sensitivity of the serum markers. Independently of the selected cut-off point, VEGF always demonstrated elevated values in some patients with benign ovarian neoplasms and also some carcinoma patients presented unexpectedly low VEGF levels. Our results revealed that, in ovarian carcinoma patients, the measurement of VEGF in cyst fluids showed higher value compared with patient sera, reflecting some degree of discrimination between malignant and benign ovarian neoplasms. Based on ROC analysis, the elevation of the VEGF upper reference limit to 4368 pg/mL for cyst fluids decreased the false-positive results for benign neoplasms from 66% to 15.3%. However, at the same time the percentage of positive results for carcinoma patients also decreased from 97% to 79.4% .The increase of the serum VEGF cut-off value to 372 pg/mL demonstrated only 55.8% sensitivity and 76.5% specificity.

Our results clearly indicated that, to distinguish malignant and benign ovarian neoplasms, the measurement of VEGF in cyst fluids showed higher diagnostic utility than its level in patients' sera. This growth factor, however, seems not to be a really valuable diagnostic indicator. Independently of its limited discrimination ability, cyst fluid is not available in each ovarian carcinoma patient and thus the follow-up of

all patients after surgery of the primary tumor is impossible. Serum VEGF levels are difficult to interpret, because its elevation may be caused by other cytokines regulating vascular permeability (10). Moreover, serum VEGF levels reflect not only the VEGF of tumor origin, but also that VEGF secreted by platelets and leukocytes (3,4,23). Mc Ilhenny *et al.* (23) reported that most of the plasma VEGF measurements were below the limit of detection using commercially available immunoassays.

According to results recently described by Boss *et al.* (15), individually differentiated VEGF levels in cyst fluids of patients with subgroups of benign ovarian neoplasms were reported. Higher VEGF values were observed in mucinous rather than in serous cystadenomas and serous cysts may reflect more extensive formation of blood vessels and/or increased vascular permeability. However, further studies were requested to confirm this observation.

The relationship between FIGO stages and VEGF content have not been definitely established and remain controversial. Yamamoto *et al.* (10) concluded that VEGF immunoreactivity strongly correlates with FIGO stage and positive peritoneal cytology.

Our study clearly showed that VEGF concentrations measured both in sera and tumor effusions were not associated with tumor stage. It indicates that, according to a majority of recently published results (6,12,13,28), the VEGF production is an early event in ovarian cancer development. Angiogenesis promoted by VEGF is a continuous process and independent of clinical advancement of the disease (12,13). The prognostic significance of VEGF levels in advanced disease (FIGO stages III/IV) seems to show the limited clinical value, but may be important in identifying high-risk patients with FIGO stages I/II (15).

The connections between histological grade of tumors and VEGF levels have been analysed in a few reports (12,13,15) and lower VEGF serum concentrations were measured in patients with G1 carcinomas compared with G2 and G3 carcinomas. Our results showed a trend toward higher VEGF values in patients with histological grade 2 and 3. However, a significant association between elevated VEGF concentration and histological grade 2 and 3 was only observed in ascitic fluids indicating that in peritoneal effusions a strong cell proliferation exists.

The role of VEGF as an independent factor in predicting the outcome of ovarian cancer patients is still unclear. Some reports state that the elevated pretreatment VEGF serum level (12,13), as well as its strong tissue expression (14), is an independent significant factor associated with shorter time to progression and poor survival. Yamamoto *et al.* (10) showed that patients with strong VEGF immunoreactivity had poorer survival rates than those with weak or no staining. The multivariate analysis, including clinicopathological parameters, revealed that this growth factor was not an independent prognostic indicator. The studies of Abendstein *et al.* (16), evaluating several serum markers in patients with recurrent ovarian carcinomas, did not demonstrate a significant relationship between VEGF and disease-free survival or overall survival. Our study revealed that, in ovarian carcinoma patients, the elevated VEGF level in ascitic fluids was significantly associated with a shorter disease-free survival. The high VEGF values measured in ascites and sera also correlated with shorter overall survival. Unfortunately, the prognostic significance of ascitic VEGF levels was lost in multivariate analysis considering tumor histology, grade of differentiation, FIGO stage, age of patients and residual disease. However, serum VEGF concentration, FIGO stage and age of patients appeared to be independent significant factors for overall survival in multivariate analysis. Our observations also suggest that high VEGF levels in cyst fluids might be associated with poorer clinical outcome of the patients. Unfortunately, the majority of our studied patients were in advanced stage of disease and the subgroup of patients with available tumor cyst fluid and low VEGF concentrations was too small to perform a proper statistical analysis. Similarly to our results, Hazelton *et al.* (29) demonstrated that ovarian cancer is associated with a dramatic elevation of VEGF levels in cyst fluid, indicating an important role of this angiogenic factor in tumor progression and potential antiangiogenic therapy.

The generated results were consistent with the known role of VEGF to induce angiogenic activity and its dissemination capacity (8,10,14,15,20-22,29). For the first time, it was shown that high VEGF levels in ascitic fluids are significantly associated with shorter disease-free survival and overall survival. However, multivariate analysis revealed that, besides FIGO stage and age of patients, only serum VEGF concentration is an additional independent prognostic factor predicting clinical outcome of ovarian carcinoma patients. Moreover, our study indicated that the measurement of VEGF in cyst fluids showed higher, but also limited value than its serum level in discrimination between malignant and benign neoplasms. A better understanding of VEGF dynamics in determining the biological behaviour of ovarian carcinoma, considering the possible role of other VEGF family members, should assist in the development of new forms of antiangiogenic therapy (30).

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