# Clinical and Histopathological Parameters Correlate with Microvessel Density but Not with Vascular Endothelial Growth Factor Expression in Ovarian Cancer

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*Ovarian cancer malignancies have the worst prognosis among all gynecological malignancies. As angiogenesis represents a key step for tumor progression, vascular endothelial growth factor (VEGF) is one of the most discussed pro-angiogenic factors. VEGF expression was investigated in 62 cases of ovarian carcinomas. Microvessel density (MVD) was evaluated by correlating the results with clinical and histopathological parameters. Because of the controversial results reported in other studies, VEGF was assessed together with MVD. Our results suggest a more complex angiogenic mechanism in ovarian cancer based on the discrepancies between VEGF expression, microvessel density and their correlation with clinical parameters. The conflicting data arising from this study supports the implications of different growth factors, others than VEGF in ovarian cancer. This hypothesis is sustained by the lack of correlation between VEGF and clinical parameters, and by the significant correlation between microvessel density and clinicopathological parameters. Thus, further studies are needed for a complete evaluation of angiogenesis in ovarian cancer.*

*Keywords: VEGF, MVD, ovarian cancer, angiogenesis, growth factors*

Among all gynecologic malignancies, ovarian cancer has the worst prognosis, and represents the fifth leading cause of death due to malignant diseases in women. Despite standard treatment, cytoreductive surgery followed by platinum/paclitaxel-based chemotherapy, the overall survival rate in ovarian malignancies is only 35% [1]. The high mortality rate in ovarian cancer is due to the difficulty of detecting this malignancy at an early stage and the lack of effective therapeutic strategies in advanced stages. For a better understanding of ovarian cancer pathogenesis, the use of reliable early diagnostic markers and novel therapeutic targets is necessary.

A lot of data support the importance of angiogenesis in ovarian cancer progression. It has been shown that VEGF over-expression in ovarian cancer stimulates not only the formation of new blood vessels, but also induces malignant transformations in the normal epithelial cells of the ovarian surface.

As the most studied and the most effective pro-angiogenic factor, VEGF is known to induce endothelial cell proliferation, migration and survival. VEGF has been identified in a large variety of human malignancies, several evidences supporting its involvement in tumor angiogenesis. In most cases VEGF level of expression correlates not only with MVD but also with clinicopathological prognostic parameters. These observations have generated extensive laboratory studies, which led to the development of numerous specific inhibitors, out of which the humanized monoclonal antibody known as bevacizumab is the most renowned.

Approval of this antiangiogenic substance by the Food and Drug Administration prompted the initiation of several clinical trials, most of them being focused on ovarian cancer. Despite the promising results in early stages of ovarian cancer, advanced stages of this disease resulted in treatment failure without a plausible explanation. It seems that most of these clinical trials did not consider the angiogenic profile of primary tumors and/or peritoneal metastasis as selection criteria when patients were included in the study.

VEGF production in the normal ovarian tissue during the fertile period is accepted by many authors [2]. The detection rate for VEGF is about 7% in postmenopausal women, but is increased up to 42% in ovarian cancer patients [3]. However, no direct connection has been found between VEGF and microvessel density (MVD), nor between VEGF and the heterogeneous pattern of vascularization [4].The complexity of this issue is due to the presence of VEGF165 and 121 in both normal and malignantly transformed ovarian tissue at the same levels as VEGF [5].

The rather low detection rate for VEGF in ovarian carcinomas is unable to explain the high values of MVD for immature vessels. A possible explanation could be related to the presence of VEGF- B (167 and 186 forms) that is capable of stimulating angiogenesis and tumor progression [6]. The correlation between increased VEGF detection rate and ovarian tumor progression seems more accurate in clear cell carcinomas. Based on these findings, many authors advocate for adjustment of therapy to the angiogenic profile of each patient individually [7-10].

MVD evaluation is the first and probably the most useful method for assessing tumor angiogenesis. MVD evaluation techniques were applied in many scientific papers for

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almost all types of human and experimental tumors and, for almost 10 years period of time, it was the only method that generated mathematical results, thus making statistical analysis possible. In a large number of studies a statistically significant relation between the number of vessels (calculated using the method proposed by Weidner et al 1993), local tumor progression and the risk of distant metastasis was found [11]. However, MVD especially draws attention only on the number of vessels at a certain point in the evolution of the tumor and not on the angiogenic profile of the tumor cells. Under these circumstances, the controversial results published by different authors are explainable, even after the standardization of this procedure in terms of working methodology.

In the normal and malignantly transformed ovary this issue becomes significantly more complicated, mainly due to the impact of ovarian hormones on the number and distribution of vessels. In this regard, hormonal therapy, that aims to reduce the levels of circulating gonadotropins, may prolong remission in ovarian cancer by extending the dormant feature of the tumor [12]. These observations suggest that certain hormonal combinations may have either inhibiting or stimulatory effects on angiogenesis in ovarian tumors. MVD analysis in primary ovarian tumors and in metastatic peritoneal tumors showed no correlation between this parameter and clinicopathological features such as the age of the patient, tumor stage, histological type, preoperative CA125 levels and survival rate [13].

Our study aims to evaluate MVD by correlating the results with the patient's age (with special reference to menopausal status), tumor stage, histological type and degree of differentiation, that has been very poorly studied until now in terms of MVD, and VEGF expression.

## Experimental Part

*Material and methods*

Patient selection.

62 female patients diagnosed with ovarian carcinomas were retrospectively selected during a four-year period of time. All patients had complete clinicopathological and postsurgical evaluation data. The ovarian carcinomas were accurately characterized regarding local and distant invasion and surgical protocols applied for each patient. A signed informed consent was obtained from each patient prior to their inclusion in the study.

All procedures were carried out according to the principles embodied in the Declaration of Helsinki and were approved by the Institutional Review Board of Victor Babes University of Medicine and Pharmacy, Timi'oara, Romania.

Description of specimens and primary histopathological processing methods

Tumor specimens were surgically removed and carefully selected by retrieving the most representative parts, including both the tumor area and the normal adjacent ovarian tissues. Tumor areas containing necrosis and extensive hemorrhage were avoided. 10x10x3 mm tumor tissues biopsies were washed in saline solution followed by 10% buffered formalin fixation for 24 h. Tissue specimens were then paraffin embedded. 5μm serial sections were taken from each paraffin embedded specimen and mounted on silanized slides. One slide from each case was stained using routine haematoxylin and eosin method for histopathologic evaluation and case selection for immunohistochemical procedures. DAKO LSAB2/HRP system was used for immunohistochemical evaluation and Bond Polymer Refine Detection System (Leica Biosystems, Newcastle uponTyne, UK) was used for visualization.

We investigated the immunohistochemical expression of VEGF clone VG1 in the selected cases. The correlation between our results and the clinical and histopathological available data was analyzed. VEGF expression was scored from 0 to 3 by assessing positive tumor cells and the staining intensity. Cases scored between 0 and 2 were considered negative while cases scored between 3 and 6 were considered positive.

MVD was evaluated on CD34 stained sections, based on the fact that CD34 selectively identifies only endothelial cells both in the normal ovarian tissue and in the tumor stroma which facilitates blood vessels counting. The evaluation was performed for intratumoral and peritumoral areas by selecting three fields with maximum vascular density at low magnification. The mean of the three fields was then calculated for each case. The intratumoral area was considered the area containing compactly arranged tumor cells, and vessels were counted only in case they were located within the tumor area. The obtained data was correlated with the histopathological types of ovarian cancer included in the study and to the quasinormal ovarian tissue adjacent to the tumor.

Thrombospondin 1 was assessed using the same immunohistochemical procedure.

Statistical analysis was performed using the commercially available SPSS version 17.0. We applied Student's test and a $<0.05$ $p$ index value was considered statistically significant.

## Results and discussions

In the specimens containing normal ovarian tissue vessels were identified both in medulla and in the cortex stroma, with similar appearance in all cases. All vessels had well-defined borders, regular lumen with or without luminal content. Constantly, the vessels from the medulla were larger than those located in the cortex. We noticed particular features for the vessels found in the corpus albicans. The corpus albicans presented peripherally located vessels that were similar to those identified in the cortex while the corpus area presented rare, small and irregular vessels. The number of vessels found in normal ovarian stroma ranged between 16 and 35, with a mean of 22.34 vessels.

In ovarian tumors, the lowest MVD values were obtained in Brenner tumors and Sertoli cells tumors. However, the results were not significantly different from those obtained for the normal ovary. Despite being a benign lesion, the Sertoli cells tumor, presented heterogeneously distributed blood vessels with variable caliber only in the peritumoral area, in the connective tissue septa located between the nests of proliferating Sertoli cells. In the other tumor cases, we observed a direct correlation between the types of vessels and the investigated area. Thus, in the peritumoral area, blood vessels were consistently larger, with a wider lumen and a thin, regular wall, occasionally presenting emerging angiogenic sprouts. Unlike the peritumoral area, the intratumoral vessels were smaller, with a narrow lumen, irregular contour and were located among the tumor cells. All investigated tumors presented increased microvessel density values for the peritumoral areas. Serous adenocarcinomas showed a relatively increased variability in both the distribution and density of blood vessels. Intratumoral areas that presented rare vessels were excluded from MVD evaluation. In most areas, however, numerous vessels were present in both the peritumoral and intratumoral areas, ranging from 22 to 68 and from 16 to 44 respectively. The vessels were extremely variable in size and the identification of vascular structures without

an apparent lumen potentially indicates the presence of immature vessels. These features have been evident especially in the intratumoral areas whereas in the peritumoral areas they were rarely found. We noticed a particular aspect in four cases of serous adenocarcinoma, which regards the presence of numerous blood vessels exhibiting a plexiform layout in the peritumoral area. No blood vessels were identified in the intratumoral adjacent area. Higher magnification analysis showed that most vessels were dilated and contained blood elements within their lumen. Immature or intermediate types of vessels were either rare or absent. Due to the difficulties encountered when attempting to evaluate the number of vessels in these areas, we only chose to count the points of emergence. Moreover, we noticed some peculiarities apparently dependent on the histological type of the tumor. Thus, in the proliferating tumor areas with papillary differentiation, the vessels were strictly located within the connective tissue. In the solid tumor area however, the vessels were disposed between the malignant cells. In the clear cell carcinoma type, blood vessels were often situated in direct contact with the malignant cells that were arranged in nests and presented numerous irregular cytoplasmic processes. In the endometrioid carcinoma type, tumor cells were often disposed around fine connective tissue axes which, under low magnification, showed a large number of blood vessels.

The values of MVD statistical analysis associated with clinicopathological prognostic parameters revealed a statistically significant correlation between MVD, tumor stage ($p<0.00021$) and degree of differentiation ($p < 0.0032$). We found no statistically significant correlation with the patient's age ($p = 0.33$), nor with the histopathological type of ovarian cancer ($p <0.24$). The associations between MVD values and the histopathological types of ovarian cancer are presented in table 1. Based on these data, we noticed that the values were similar for the classical types of ovarian carcinomas, except for the mucinous carcinoma where the values were slightly lower, but not statistically significant.

Following the surgical procedure, a number of patients presented residual disease. By analyzing the relationship between MVD, residual disease and age, we did not obtain statistically significant correlations, neither in univariate nor in multivariate analysis, as it is shown in table 2. These results could be explained through the evaluation of MVD using specimens taken from the primary tumor and by the fact that during the second-look intervention fragments with uncertain relevance to this type of investigation were taken for processing.

The reaction for thrombospondin1 was positive in 43 cases (69.35%) out of the total number of 62 cases. The results were statistically analyzed in association with MVD values, tumor grade, tumor stage (FIGO), histopathological type and menopausal status. For the statistical analysis we considered both the mean and the maximum MVD values.

When comparing serous and non-serous ovarian tumors, we did not obtain any statistically significant correlation between MVD and TSP-1 expression. Also, we did not obtain statistically significant correlations neither with the

| Form / MVD | Peritumoral / stroma | Intratumoral |
|---|---|---|
| Normal ovary | 22.34 (16-35) | NA* |
| Serous adenocarcinoma | 42.25 (24-68) | 33.5 (16-44) |
| Endometrioid carcinoma | 46.76 (36-97) | 41.20 (35-48) |
| Mucinous carcinoma | 31.66 (21-49) | 26.33 (19-38) |
| Clear cell carcinoma | 43.33 (39-54) | 33.67 (24-51) |
| Undifferentiated carcinoma | 44.66 (35-59) | 41.00 (28-61) |
| Brenner tumor | 22.5 (15-36) | 1.66 (0-6) |
| Sertoli cells tumors | 25.33 (17-29) | 0 |

**NA: not applicable*

**Table 1**
MVD VALUES IN THE NORMAL OVARY AND IN THE HISTOPATHOLOGICAL TYPES OF OVARIAN CARCINOMAS

| | | Univariate Analysis | | | Multivariate Analysis | | |
|---|---|---|---|---|---|---|---|
| Variables | Score | Odds ratio | 95% CI | p | Odds ratio | 95% CI | p |
| MVD | | 1.35 | 1.01-1.8 | 0.03 | 1.37 | 1-1.89 | 0.05 |
| Residual disease | 0<br>1 | 2.4 | 0.48-11.97 | 0.2 | 0.58 | 00.1-35.44 | 0.7 |
| Age | 0<br>1 | 1.5 | 0.34-6.94 | 0.5 | 0.61 | 0.004-87.1 | 0.8 |

**Table 2**
MVD AND RESIDUAL DISEASE

menopausal status (p = 0.6), nor with the histopathological type of ovarian cancer (p = 0.33). MVD was associated with decreased TSP-1 expression in cases presenting MVD values that were greater or less than 21.7/HPF. An intense TSP-1 expression was evident in cases presenting mean MVD values below 9.

VEGF reaction was negative in the normal ovarian tissue surrounding the tumors, with the specification that most patients were postmenopausal females, and ovarian follicles were no longer identified.

For all mucinous tumor types (n=5), Brenner tumors (n=2), Sertoli cells tumor and yolk sack tumors included in our study, VEGF reaction was negative. However, we noticed a positive reaction in 18 out of 62 studied cases (29.03%). In the group of positive cases, 11 cases were scored 3- 4, and 7 were scored 5- 6. No correlation was found between the tumor histopathologycal type and VEGF expression. The relationship between VEGF expression and tumor stage ($p<0.2$) and between VEGF expression and tumor grade ($p=0.12$) showed a variable pattern of positive reactions. A constant positive reaction pattern was noticed in 5 out of 6 cases of clear cells carcinomas. The staining intensity was strong and was scored +3 (fig. 1). Clear cells

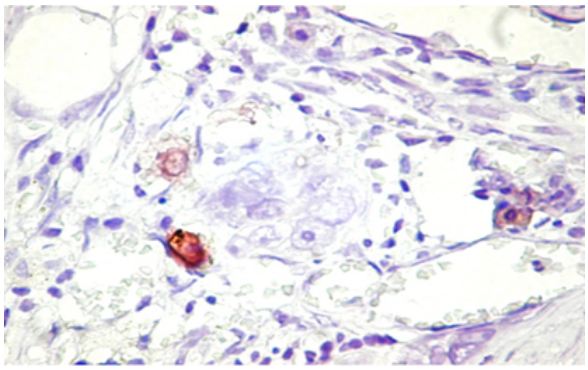

Fig. 1. VEGF positive reaction. Note the VEGF patterns of distribution and the staining intensity found in clear cells carcinomas. x400

were occasionally noticed within the lumen of small vessels.

Most serous adenocarcinomas included in our study showed a weak to moderate cytoplasmic VEGF reaction. We found the same expression pattern in the invasive areas (fig. 2), only the staining was characterized by a linear pattern that outlined the tumors. Surprisingly, VEGF was

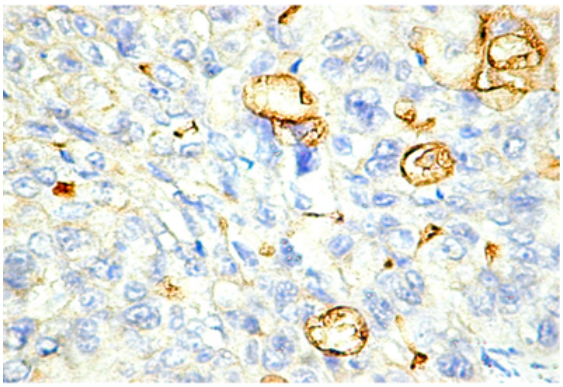

Fig. 2. VEGF positive reaction in the invasion area of ovarian carcinomas. Note the linear pattern of the staining outlining the tumors. x400

intensely expressed and exhibited a diffuse cytoplasmic pattern in 4 poorly differentiated cases of ovarian cancer.

MVD values and correlations with clinicopathological parameters shown by our results are similar to those already published in the literature. Szubert et al. investigated the associations between serum VEGF, bFGF and endoglin levels with microvessel density and expression of pro-angiogenic factors in benign and malignant ovarian tumors and found that MVD values were increased in epithelial ovarian cancer compared to benign ovarian tumors [14]. It seems that serum VEGF levels are a useful predictive marker for ovarian cancer MVD and tumor VEGF expression [14]. Several studies focused on the correlations between microvessel density (MVD) and clinicopathological parameters and found that an increased microvessel density CD34 expression is an independent mortality risk factor in ovarian cancer [15].

Ovarian cancer is usually diagnosed in advanced stages, thus remaining the most lethal gynecological cancer [16-18]. Currently, several pathogenic steps in ovarian carcinogenesis had been revealed, but the complexity of ovarian carcinomas and the numerous mechanisms that lead to malignant transformations in the ovary are still poorly understood. More data is needed in order to completely elucidate these issues along with exhaustive patient selection based on a great range of clearly defined criteria when attempting to improve the efficiency of antiangiogenic treatment strategies in ovarian cancer patients. In 2016, Li et al. performed a meta-analysis that aimed to investigate the effects of angiogenesis inhibitors in the treatment of patients with advanced or recurrent ovarian cancer and found that antiangiogenic therapy showed a clear progression survival free benefit but with the cost of an increased toxicity [19]. The impact of antiangiogenic drugs in overall survival was undefined for ovarian cancer patients [19]. These results support our findings regarding the necessity of proper patient selection when applying antiangiogenic therapeutic strategies in ovarian carcinoma cases.

Despite being the most important antiangiogenic treatment, anti-VEGF based therapy is followed by numerous side effects and is not the only efficient treatment in ovarian cancer. Novel scientific trials have recently pinpointed the direct correlation between SEMA4D and the degree of differentiation in epithelial ovarian cancer [20]. Chen Y et al. have concluded that VEGF along with SEMA4D possess synergistic effects in stimulating angiogenesis in ovarian carcinomas and the SEMA4D signaling pathway may become a potential target in the complex therapeutic management of patients diagnosed with epithelial ovarian cancer [20]. However, further experimental and clinical trials are needed in order to determine whether anti-SEMA4D alone could be sufficient in order to reduce MVD in ovarian cancer or a combined anti-VEGF/anti-SEMA4D would be more beneficial. Also, a proper patient selection based on firmly defined eligibility criteria is needed in order to reduce the degree of toxicity after combined antiangiogenic therapy. Advanced stages of ovarian carcinomas are known to be followed by chemotherapy resistance [21] depending on the cancer associated genetic abnormalities and biological behavior. Besides VEGF, other growth factor molecules are implicated either in promoting or inhibiting tumor angiogenesis in ovarian cancers. A recent study conducted by Pazos et al. shows that PDGF-B exerts an indirect inhibitory effect on the ovarian cancer vasculature [22]. It appears that PDGF-B normalizes the tumor vessels following single administration and favors gamma-secretase inhibitor (DAPT) anticancer action when being co-administrated [22]. However, the scientific data regarding PDGF implications in ovarian cancer remain controversial. Also, the exact interaction, if existent, between VEGF and PDGF, must be further investigated. In this regard, PDGFR-beta and VEGFR-2 are implicated in

promoting resistance to platinum-based chemotherapy in ovarian cancer patients [23]. Also, both PDGFR-beta and VEGFR-2 may become novel predictive biomarkers for therapy resistance and for overall and progression-free survival [23]. Moreover, despite the benefits on antiangiogenic therapy, a complex and properly defined therapeutic management in ovarian cancers may also include AXL receptor tyrosine kinase (AXL-RTK) inhibitors that detain a certified role in suppressing tumor growth and progression [24].

In the past year, a great range of therapeutic substances have emerged following both clinical and experimental trials focused on the different types of ovarian cancers. Besides bevacizumab, paclitaxel and carboplatin based chemotherapy, PARP (poly-ADP ribose polymerase) platinum and even immunotherapy are currently being taken into consideration for the management of patients diagnosed with ovarian cancer [25-28]. Antiangiogenic therapy and immunotherapy seem to become one of the major focuses of future scientific studies concerning ovarian cancers. Lyons et al. have demonstrated that ovarian tumor assoiated macrophages act as pro-angiogenic factors and that macrophage inhibition using CSF1R inhibitors determines the reduction of tumor growth [29]. VEGF is thus not the only factor that promotes ovarian cancer associated angiogenesis, several other molecules and even cells of the imune system being implicated either as independent factors or in association with VEGF. Considering these aspects, vascular endothelial cadherin (VEC) has gained interest in the research field of ovarian cancers due to its role in activating endothelial genes and triggering stability-related genes thus exerting a direct influence on the ovarian carcinoma vasculature [30]. Also, glycodelin is an important promoter of tumor angiogenesis in ovarian cancer and influences the differentiation and function of immune cells such as T and B cells, dendritic cells, macrophages and NK cells [31]. Under these circumstances, glycodelin may become an effective target in antiangiogenic therapy and immunotherapy in ovarian cancers. A potential combined antiangiogenic and immunotherapeutic strategy for patients diagnosed with malignant lesions of the ovary seems promising but is in need of further investigations.

## Conclusions

We found a statistically significant correlation between MVD, tumor stage ($p < 0.00021$) and degree of differentiation ($p < 0.0032$). We noticed no statistically significant correlation neither with the patients' age ($p = 0.33$), nor with the histopathological type of ovarian cancer ($p < 0.24$). 43 cases (69.35%) out of the total number of 62 cases were positive for Thrombospondin-1, but the results could not be correlated with MVD values when comparing serous with non-serous tumors. According to these results we conclude that MVD may be regarded as a useful indicator for local tumor progression and may al least partially explain the angiogenic behavior and distant dissemination of ovarian carcinomas cells. However, ovarian cancers are extremely heterogeneous diseases that require the discovery of well defined prognostic and therapeutic biomarkers. As stated above, ovarian tumor associated angiogenesis is a complex and poorly understood phenomenon that needs to be fully comprehended in order to ensure a proper patient management. MVD and VEGF represent only a small part of the numerous factors that influence tumor growth, angiogenesis, local dissemination and distant metastases.

Based on the discrepancies between VEGF expression, microvessel density and their correlation with clinical parameters, our results suggest a more complex angiogenic mechanism in ovarian cancer. The conflicting data arising from this study supports a more elaborate angiogenic process in ovarian cancer, involving other factors than VEGF. This aspect is sustained by the lack of correlation between VEGF and clinical parameters, and a significant correlation between microvessel density and clinicopathological parameters. Also, antiangiogenic treatment in ovarian cancers depending on their angiogenic profile is applicable to primary tumors. As far as we know, no scientific data is available in literature regarding the angiogenic profile of ovarian cancer metastases. Whether the angiogenic profile of the metastasis is different from that of the primary tumor is a controversial issue to be solved through further experimental and clinical trials. From this point of view, further studies may be able o ensure a complete evaluation of angiogenesis in ovarian cancer based on the current data regarding early and advanced stages of this disease. Ovarian cancers are heterogeneous pathological entities that include a wide range of genetic abnormalities and a variable clinical and biological behavior, thus being subjects to individualized and targeted therapies. Also, we support the refinement of the patient selection process in order to reduce the risk of false negative results following the application of novel therapeutic strategies such as antiangiogenic treatment and immunotherapy.

In another papers were studied the correlation between histopatological form and the degree og neuroendocrine differentiations in prostate cancer [32] and the reticular network contributes to the staging of idiopathic lung fibrosis [33].

*Acknowledgments. We would like to thank the Victor Babes University of Medicine and Pharmacy, Department of Microscopic Morphology/ Histology Timisoara, Romania and the Angiogenesis Research Center Timi'oara, Romania for supporting this study.*

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Manuscript received: 22.12.2017