Intratumoral microvessel density in advanced epithelial ovarian cancer and its use as a prognostic variable

Intratumoral Microvessel Density in Advanced Epithelial Ovarian Cancer and its Use as a Prognostic Variable

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Abstract. *Background: The aim of this retrospective study was to assess whether the intratumoral microvessel density (IMD) in primary tumour specimens had any impact on the clinical outcome of patients with advanced epithelial ovarian cancer treated in two Italian departments of gynaecological oncology. Materials and Methods: The study was conducted on 101 patients who underwent initial surgery followed by platinum-based chemotherapy (37) or paclitaxel/platinum- based chemotherapy (64) for International Federation of Gynecology and Obstetrics (FIGO) stage III-IV epithelial ovarian cancer. The median follow-up of survivors from initial surgery was 65 months (range, 27 to 132 months). Paraffin-embedded sections of primary tumour specimens were analysed for IMD by immunohistochemistry using anti-CD34 antibodies. Results: Progression-free survival and overall survival were significantly better in patients with IMD ≥40 microvessels/field compared with those with lower IMD ($p=0.0105$ and $p=0.0065$, respectively). Cox model showed that IMD was the strongest independent prognostic variable for both progression-free survival ($p=0.0267$) and overall survival ($p=0.0189$). Conclusion: An elevated IMD was associated with a significantly better progression-free survival and overall survival in patients with stage III-IV epithelial ovarian cancer who underwent initial surgery followed by chemotherapy, mainly consisting of a paclitaxel/platinum-based regimen.*

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Neoangiogenesis, *i.e.,* the development of new blood vessels from the existing vasculature, is an essential step of solid tumour growth and spread, and several experimental investigations have revealed that tumour diameters, when angiogenesis is blocked or absent, range approximately between 0.2 mm and 2 mm (1-3). Tumour angiogenesis can be microscopically assessed as intratumoral microvessel density (IMD), *i.e.,* mean value of microvessel count obtained using a specific objective magnification with known field diameter in three or four fields subjectively selected from the so-called "hot spots" (4). Antibodies against a variety of endothelial markers have been tested to stain microvessels (4-7).

The prognostic relevance of IMD has been long assessed in different human malignancies, with contrasting results. Several papers have reported that a high IMD was related to a poor survival in patients with breast cancer (6, 8), lung cancer (7, 9), prostate cancer (10, 11), gastric cancer (12), colon cancer (13), endometrial cancer (14, 15) and cervical cancer (16-19). Conversely, the multivariate analysis of other studies has shown no relationship or a positive relationship between IMD and the clinical outcome of patients with breast cancer (5, 20), renal cancer (21), brain cancer (22), endometrial cancer (23) and cervical cancer (24). Conflicting data have been also reported for epithelial ovarian cancer. Some authors detected that a high IMD was a poor prognostic variable (25-33), others observed that this parameter had no impact on survival (34-39), others again reported that a high IMD was associated with a better prognosis (40-43). In this paper the prognostic relevance of IMD on primary tumour specimens from 101 patients with advanced epithelial ovarian cancer treated in two Italian departments of gynaecological oncology, has been investigated.

Materials and Methods

This retrospective study was conducted on 101 patients who underwent initial surgery followed by platinum-based or paclitaxel/platinum-based chemotherapy for advanced epithelial ovarian cancer at the Departments of Gynecology and Obstetrics of the Universities of Pisa and Torino, between March 1993 and November 2002.

The tumour stage and histological diagnosis of each patient were determined according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO) and histological typing system of the World Health Organization (WHO), respectively. Tumours were graded as well- (G_1), moderately- (G_2), or poorly-differentiated (G_3).

The evaluation of the clinical course of disease was based on clinical examination, serum CA 125 assay, chest X-ray, abdominal-pelvic ultrasound and computed tomography scan. Additional investigations were performed when appropriate. After the sixth cycle of chemotherapy the patients with no evidence of disease at clinical, serologic, sonographic and radiologic examinations were defined as being in clinical complete response. Three to 5 weeks after the end of chemotherapy a second-look surgery was usually proposed to clinically complete responders, mostly to patients enrolled onto clinical trials. A pathological complete response at second-look surgery was defined as the disappearance of all macroscopic tumour deposits with negative peritoneal washing and negative multiple random biopsies. All patients with clinically or surgically detectable persistent disease, as well as some pathologically complete responders, received additional chemotherapy.

All patients were periodically observed until they died or until May 2005. The median follow-up of survivors from initial surgery was 65 months (range, 27 to 132 months).

Formalin-fixed paraffin-embedded sections of primary tumour specimens obtained from all patients were independently analyzed for histopathological and immunohistochemical examination by all pathologists and inconsistencies were solved by a simultaneous review.

Immunohistochemistry

Antibodies. Sections were incubated with the primary mouse anti-human CD34 antibody. The incubation time was 12 h at 4°C. Sections were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxide activity was blocked by 10 min incubation in hydrogen peroxide solution (1% hydrogen peroxide in methanol). In order to unmask the antigens, the slides were microwaved for 10 min in citrate buffer (10 mM, pH 6). After blocking non-specific staining with serum, the sections were first incubated with the primary antibody, and then with biotin-labelled secondary antibody (dilution 1:500; 30') and avidin-biotin-complex (30'). 3-3'Diaminobenzidine tetrahydrochloride was used as chromogen. The sections were counterstained with haematoxylin, dehydrated and mounted.

Vascular endothelial cells were used as positive controls for CD34. Negative controls were obtained by omitting the primary antibody.

IMD determination was performed with the methodology described by the International Consensus in 1996 (44). The vessels were counted in the three most vascularized areas ("hot spots") of the tumour and a mean value was then considered. Microvessel counts were performed at x200 (x20 objective lens and x10 ocular lens; 0.74 mm^2 per field). A single microvessel was defined as discrete clusters or single cells stained for CD34 and the presence of a lumen was not required for scoring as a microvessel. The antibodies used for tissue section immunostaining and the methodology employed for IMD determination were the same for both centers.

Statistical methods. The SAS statistical package (release 8.2; SAS Institute, Cary, NC, USA) was used for computations.

IMD expression was compared to the clinico-pathological variables using median exact test and Pearson's χ^2 test (or two-tailed Fisher's exact test when appropriate).

The cumulative probability of progression-free survival and overall survival from the time of initial surgery were estimated by the product-limit method. The log-rank test was used to compare the homogeneity of progression-free survival and overall survival functions across strata defined by categories of prognostic variables.

A multiple regression analysis based on the Cox proportional hazards model was used to test jointly the relative importance of variables as predictors of progression-free survival and overall survival times.

Results

The median age of the patients was 63 years (range, 25 to 80 years). The tumour stage was III in 90 patients and IV in 11 patients. Histologically, 75 carcinomas were serous, 10 endometrioid, 11 undifferentiated and 5 mucinous. Tumour grade was G_1 in 5 patients, G_2 in 15 patients and G_3 in 81 patients. Residual disease after initial surgery was ≤1 cm in 23 patients and >1 cm in 78 patients. The planned combination chemotherapy consisted of six cycles of paclitaxel plus carboplatin in 55 patients, epidoxorubicin plus paclitaxel plus carboplatin in 9 patients, cyclophosphamide plus epidoxorubicin or doxorubicin plus cisplatin in 15 patients, cyclophosphamide plus cisplatin in 2 patients, single-agent carboplatin in 19 patients, and single-agent cisplatin in one patient.

The IMD in primary tumour specimens ranged from 4 to 114 microvessels/field, and, the 25%, 50% and 75% quantiles were 10, 18 and 39 microvessels/field, respectively.

No relationship was detected between IMD and the common clinico-pathological variables (Table I).

By log-rank test progression-free survival was significantly related to age, histological type, residual disease and IMD (Table II). Progression-free survival was significantly better in patients with IMD ≥40 microvessels/field compared with those with lower IMD ($p=0.0105$) (Figure 1).

By log-rank test overall survival was significantly related to age, histological type and IMD (Table II). Overall survival was significantly better in patients with IMD ≥40 microvessels/field compared with those with lower IMD ($p=0.0065$) (Figure 2).

Cox model showed that IMD was the strongest independent prognostic variable for both progression-free survival ($p=0.0267$) (Table III) and overall survival ($p=0.0189$) (Table IV).

Table I. *Relationship between IMD and the common clinico-pathological variables in patients with advanced epithelial ovarian cancer.*

		IMD (microvessels/field)				
Variable	No. of patients	≤10	11-18	19-39	≥40	*p*-value
Age (years)						
≤63	51	16 (31.4%)	13 (25.5%)	7 (13.7%)	15 (29.4%)	ns
>63	50	16 (32.0%)	8 (16.0%)	16 (32.0%)	10 (20.0%)	
FIGO stage						
III	90	28 (31.1%)	20 (22.2%)	20 (22.2%)	22 (24.4%)	ns
IV	11	4 (36.4%)	1 (9.1%)	3 (27.3%)	3 (27.3%)	
Histology						
serous	75	24 (32.0%)	13 (17.3%)	16 (21.3%)	22 (25.3%)	ns
not serous	26	8 (30.8%)	8 (30.8%)	7 (26.9%)	3 (11.5%)	
Tumour grade						
G_1-G_2	20	8 (40.0%)	4 (20.0%)	4 (20.0%)	4 (20.0%)	ns
G_3	81	24 (29.6%)	17 (21.0%)	19 (23.5%)	21 (25.9%)	
RD residual disease						
≤1 cm	23	9 (29.1%)	3 (13.0%)	4 (17.4%)	7 (30.4%)	ns
>1 cm	78	23 (29.5%)	18 (23.1%)	19 (24.4%)	18 (23.1%)	

IMD, intratumoral microvessel density; G_1, well-differentiated; G_2, moderately-differentiated; G_3, poorly-differentiated ; RD, residual disease; ns, not significant.

Table II. *Variables predictive of progression-free survival and overall survival in patients with advanced epithelial ovarian cancer (univariate analysis).*

		Progression-free survival				Overall survival		
		5-year %	median (months)	*p*-value		5-year %	median (months)	*p*-value
Age (years)								
≤63	51	25	22	0.0363	47	56	0.0221	
>63	50	13	16			28	40	
FIGO stage								
III	90	19	20.5	0.7572		38	45	0.7146
IV	11	23	15			40	51	
Histology								
serous	75	24.5	22	0.0301		45	51	0.0242
not serous	26	0	17			18	36	
Tumor grade								
G_1-G_2	20	15	17	0.2632		30	35	0.0880
G_3	81	20	21			40	46	
RD								
≤1 cm	23	36	27	0.0151		1	71	0.1086
>1 cm	78	14	17			33	40	
IMD (microvessels/field)								
≤10	32	15	16.5	0.0105		25	35	0.0065
11-19	21	11	18			21	43	
20-39	23	11	15			32	40	
≥40	25	36	36			67		not reached

G_1, well-differentiated; G_2, moderately-differentiated; G_3, poorly-differentiated; RD, residual disease; IMD, intratumoral microvessel density.

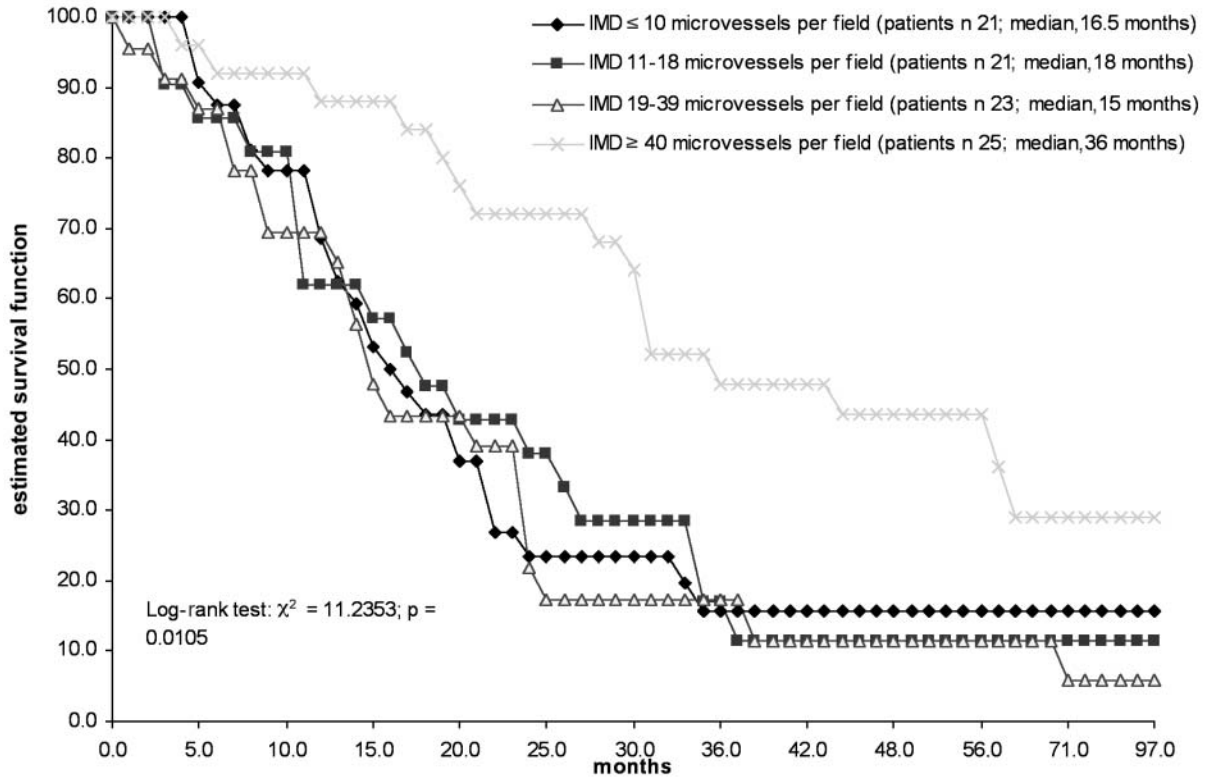

Figure 1. *Progression-free survival in advanced ovarian cancer patients by intratumoral microvessel density (IMD).*

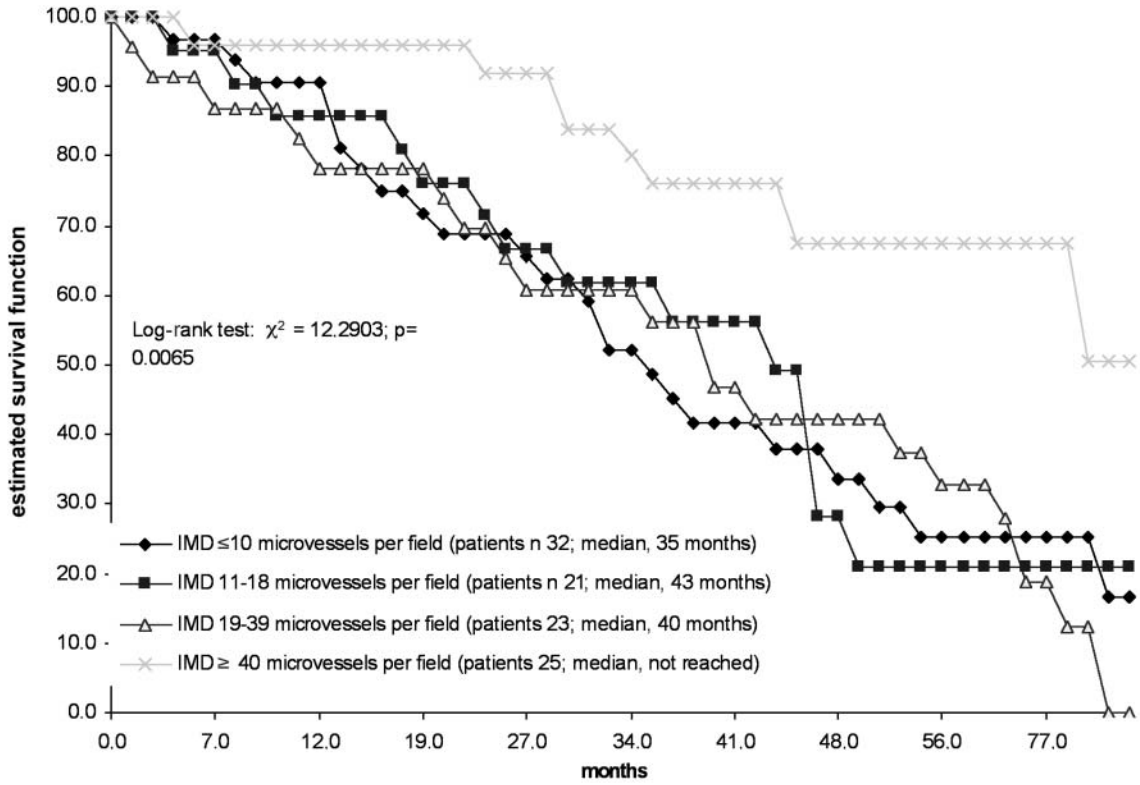

Figure 2. *Overall survival in advanced ovarian cancer patients by intratumoral microvessel density (IMD).*

Table III. *Variables predictive of progression-free survival in patients with advanced epithelial ovarian cancer (Cox model).*

Variable	Parameter estimate	Standard error	Wald χ^2	*p*-value	OR	95%CI
IMD	–0.01165	0.00526	4.9087	0.0267	0.988	0.978-0.999
Age	0.50821	0.23358	4.7340	0.0296	1.662	1.052-2.627
RD	0.57650	0.29557	3.8044	0.0511	1.780	0.997-3.177
Histotype	0.33397	0.26086	1.6392	0.2004	1.397	0.838-2.329
Tumour grade	–0.22221	0.27976	0.6309	0.4270	0.801	0.463-1.386
Stage	0.04845	0.39803	0.0148	0.9031	1.050	0.481-2.290

OR, odds ratio; 95%CI, 95% confidence internal; IMD, intratumoral microvessel density; RD, residual disease.

Table IV. *Variables predictive of overall survival in patients with advanced epithelial ovarian cancer (Cox model).*

Variable	Parameter estimate	Standard error	Wald χ^2	*p*-value	OR	95%CI
IMD	–0.01488	0.00634	5.5067	0.0189	0.985	0.973-0.998
Age	0.53605	0.25264	4.5019	0.0339	1.709	1.042-2.804
RD	0.41384	0.32108	1.6613	0.1974	1.513	0.806-2.838
Histotype	0.43549	0.27809	2.4524	0.1173	1.546	0.896- 2.666
Tumour grade	–0.37682	0.29029	1.6850	0.1943	0.686	0.388-1.212
Stage	–0.06123	0.46029	0.0177	0.8942	0.941	0.382-2.319

OR, odds ratio; 95%CI, 95% confidence internal; IMD, intratumoral microvessel density; RD, residual disease.

Table V. *Prognostic relevance of IMD in epithelial ovarian cancer.*

Authors	Ref.	No. of patients	FIGO stage	Endothelial marker	Correlation between high IMD and survival
Hollingsworth et al.	(25)	43	III-IV	CD34	worse disease-free survival at multivariate analysis
Gasparini et al.	(26)	60	III-IV	CD31	worse survival at univariate, not at multivariate
Alvarez et al.	(28)	88	I-IV	vWF	worse survival at univariate, not at multivariate
Obermaier et al.	(30)	63	I-III	CD34	worse survival at univariate, not at multivariate
Raspollini et al.	(31)	83	III	CD34	worse survival at multivariate analysis
Goodheart et al.	(32)	33	Ic	CD31	worse survival at multivariate analysis
van Diest et al.	(34)	49	III-IV	Ulex	no correlation with survival
Terai et al.	(36)	54	I-IV	CD34	no correlation with survival
Nakajama et al.	(37)	42	I-IV	CD34	no correlation with survival
Ali-Fehmi et al.	(40)	118	III-IV	CD34	no correlation with survival
Chan et al.	(43)	46	III-IV	CD34	better survival at multivariate analysis
Present series		101	III-IV	CD34	better survival at multivariate analysis

IMD, intratumoral microvessel density; vWF, von Willebrand Factor; Ulex, Ulex europeas lectin.

Discussion

Several authors reported that the risk of progression and death of patients with epithelial ovarian cancer increased with raising IMD (Table V) (25-33). Hollingsworth *et al.* (25) detected that higher stage, higher average IMD at 200x and 400x magnification and highest IMD at 400x magnification conferred worse disease-free survival in patients with advanced epithelial ovarian cancer, and that the lowest average IMD at 400x was the strongest predictor of disease-free survival at multivariate analysis. An elevated IMD (>48 microvessels/field) was a poor prognostic indicator for overall survival at univariate analysis ($p=0.034$) but not at multivariate analysis in a series of patients with advanced disease who received a platinum-based chemotherapy (26). By using a computer-aided image analysis system to quantify IMD, Schoell *et al.* (27) found that the endothelial area in tumour specimens of the 14 patients who survived for ≥6 years was smaller compared with that of the 14 patients matched for stage

and treatment who died of disease (0.038 ± 0.026 mm^2 *versus* 0.110 ± 0.034 mm^2, $p<0.0001$). Alvarez *et al.* (28) reported a median survival of 2.7 years for women with IMD >10 microvessels/field *versus* 7.9 years for those with lower microvessel count ($p=0.03$), but IMD did not retain statistical significance after adjusting for stage at multivariate analysis. Similarly, Obermair *et al.* (30) found a better 5-year overall survival for the 18 patients with a low IMD (<10 microvessels/field) compared with the 45 patients with high IMD (55% *versus* 24%, $p=0.03$), but this angiogenic parameter failed to attain a significant value after adjustment for the common prognostic factors, such as patient age, tumour stage and histological grade. Raspollini *et al.* (31) evaluated IMD in tissue sections from patients with stage III, serous, poorly-differentiated epithelial ovarian cancer who underwent primary debulking surgery and platinum-based or paclitaxel/platinum-based chemotherapy. An IMD >70 microvessels/ high power field (HPF) was an independent poor prognostic variable for both disease-free ($p=0.001$) and overall survival ($p<0.0005$). In a further investigation, the same authors (33) compared the IMD assessed with a computer-aided image analysis system in 23 patients with FIGO stage III, serous, poorly-differentiated epithelial ovarian cancer, who were disease-free 5 years after primary surgery and platinum-based chemotherapy, and in 10 patients matched for stage, histological type, tumour grade and treatment, who died of disease within one year after primary therapy. Once again, this angiogenic parameter correlated with survival at multivariate analysis ($p=0.05$).

As for early epithelial ovarian cancer, an elevated IMD (>12 microvessels/HPF) was an independent poor prognostic variable for disease-specific survival (hazard ratio, 4.8; 95% confidence interval, 1.1-22; $p=0.04$) in 33 patients with stage Ic disease (32).

Conversely, other authors failed to detect a negative impact of an elevated IMD on the clinical outcome of patients with epithelial ovarian cancer (34-39) (Table V). Van Diest *et al.* (34) found no correlation between IMD in primary tumour sections and survival at either univariate or multivariate analysis in patients with advanced disease who received platinum-based chemotherapy. Orre *et al.* (35) found no differences in IMD, determined with anti-CD31 and anti-CD34 antibodies, among patients with different clinical outcomes. Similarly, no relationship between IMD and survival was observed in the series of Terai *et al.* (36), Nakayama *et al.* (37) and Ali-Fehmi *et al.* (39).

Some studies have even shown a better prognosis for patients with higher microvessel count (40-43) (Table V). An immunohistochemical study on surgical specimens from 105 primary ovarian cancers showed that, among women with early disease, an elevated IMD (≥70 microvessels/HPF) was related to a better progression-free survival (40). This association was significant only for the patients with clear cell carcinoma. Chan *et al.* (42) found that, among women with advanced disease, the median survival was better in the 24 patients with IMD >11 microvessels/field compared with the 22 with lower microvessel count (59.7 *versus* 23.9 months, $p=0.001$), and that this angiogenic variable retained a significant value at multivariate analysis. The same authors (43) reported that patients with high IMD (>11 microvessels/field) and high peri-tumoral mast cell infiltration had a better mean survival than those with low IMD or low mast cell density (80.3 *versus* 37.8 months, $p=0.015$). According to the authors the formation of new vessels could allow the accumulation of mast cells and release of mast cell tryptase that is a potent fibroblast growth factor, thus, enhancing fibroblast recruitment and fibrosis development that limits tumour growth and spread (43, 45).

In the present study an assessment was made of the clinical relevance of IMD in primary tumour specimens from 101 patients with stage III-IV disease who underwent initial surgery followed by platinum-based chemotherapy, mainly consisting of a paclitaxel/platinum-based regimen, in two Italian departments. Patients with highly vascularized tumours had a significantly better progression-free and overall survival at both univariate and multivariate analysis, in agreement with the results of a previous investigation of Gadducci *et al.* on a smaller number of cases (41). The positive association between an elevated IMD and a better clinical outcome might be due to the fact that high tumour vascularity could allow a major drug availability and greater sensitivity to paclitaxel (41, 42). Besides a direct cytotoxic effect on tumour cells, paclitaxel exhibits potent anti-angiogenic activity, even at very low concentrations, by an increase in microtubule dynamics in endothelial cells (46, 47). This anti-angiogenic activity might be particularly relevant in highly vascularized tumours.

The determination of IMD shows remarkable methodological variations among different papers, especially as for the number of hot spots counted, the definition of the minimum criteria of a countable microvessel, the areas and fields of magnification, and staining markers and methods used (4, 8). These methodological variations, as well as differences in patient characteristics and chemotherapy regimens used, may partly explain the contrasting results reported in the literature as for prognostic relevance of IMD in several malignancies including epithelial ovarian cancer.

Large studies with well standardized pathology laboratory procedures and with homogenous groups of patients as for stage, histological type and treatment, are warranted to better elucidate the prognostic relevance of IMD in epithelial ovarian cancer.

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