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Prognostic significance of tumour vascularisation on survival of patients with advanced ovarian carcinoma

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Summary. Objective. The prognostic significance of microvessel density in ovarian cancer is still a matter of debate. Classically, the degree of vascularisation is assessed in areas of high vascular density (hot spots), considered as regions of increased probability of metastasis. Since ovarian tumours have a particular progression and dissemination behaviour, vascularisation outside hot spots may also contribute to their evolution. Methods. In the present study, the degree of tumour vascularisation was estimated both in whole histogical sections and in hot spots, in 235 patients with ovarian carcinoma, using fully automatic image analysis methods. Six parameters were estimated: mean microvessel density (MVD) and mean microvessel surface fraction (MSP) on the whole section, mean and maximum values of MVD and MSP inside hot spots (MVDHS1, MSPHS1 and MVDHS2, MSPHS2). Relationships between vascular parameters and clinicopathologic features were analysed. Results. In stage III-IV patients multivariate analysis showed that stage IV disease (hazards ratio (HR)=1.72, p=0.001), post-surgical residual disease 1cm (HR=2.86, p<0.001), upper MVD tercile (HR=1.45, p<0.022) and medial MVDHS1 tercile (HR=1.36, p=0.060) retained an independent prognostic value upon overall survival. Conclusion. Our results suggest that quantification of blood vessels, both on the whole histological section and in hot spots might be helpful in evaluating prognosis in advanced ovarian carcinomas.

Key words: Ovarian cancer, Prognosis, Vascularisation, Angiogenesis, Microvessel density

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Introduction

Ovarian cancer is the sixth most common cancer in females and the most lethal gynecological malignancy in western countries (Parkin et al., 2005). Almost 70% of patients are diagnosed with advanced and metastatic disease. Despite surgical cytoreduction and chemotherapy, its prognosis is generally poor due to persistent residual recurrent disease associated with chemoresistance (Vasey, 2003). The global 5-year overall survival rate is approximately 40%, ranging from more than 90% in stage I to less than 30% in stage IV disease (Jemal et al., 2005). The two main prognostic indicators are FIGO (International Federation of Gynecology and Obstetrics) stage and size of residual disease after initial surgery (McCluggage et al., 2002). Despite numerous published studies, the number of biological markers recognized as prognostic is limited. Neovascularisation has been established as a crucial factor in carcinogenesis influencing tumour growth, invasion and metastasis development. Accumulating experimental and clinical evidence suggests that ovarian cancer critically depends on neovascularisation for expansive growth (Bamberger and Perrett, 2002; Martin and Schilder, 2007; Schumacher et al., 2007). VEGF seems to have a crucial role in angiogenesis in ovarian cancer, and targeting tumour angiogenesis by blocking VEGF pathway has produced tangible clinical results (Monk et al., 2005; Kaye, 2007). A recent study with

Abbreviations: CDDP, cisplatine; CL, confidence limits; MSP, mean microvessel surface proportion, in %; MSPHS1, mean microvessel surface proportion within hot spot; MSPHS2, maximum microvessel surface proportion within hot spot; MVD, mean microvessel density; MVDHS1, mean microvessel density within hot spot; MVDHS2, maximum microvessel density within hot spot

metronomic chemotherapy on ovarian carcinoma showed a high reduction of microvessel density and a significant prolongation in survival, suggesting that vascular development is central to ovarian cancer biology (Kamat et al., 2007). The activity of angiogenesis, as assessed by microvessel density (MVD) in selected areas of high vascularity (hot spots), has been reported as a powerful prognostic indicator in numerous human solid tumours, including breast and prostate cancers (Nico et al., 2008). In ovarian cancer, several studies analysed the association of angiogenesis with patient outcome. Early reports indicated that a high MVD in hot spots was an independent predictor of poor survival (Hollingsworth et al., 1995; Gasparini et al., 1996). A series of following articles confirmed this result (Alvarez et al., 1999; Heimburg et al., 1999; Obermair et al., 1999; Goodheart et al., 2002; Raspollini et al., 2004b). On the contrary, one study reported that increased vascularisation predicted improved survival, while three others failed to show any association between vascularisation and survival (Birner et al., 2001; Gadducci et al., 2003; Ferrero et al., 2004; Losch et al., 2004). In the study by Ogawa et al. MVD in hot spots was not associated with progression-free survival in advanced stages. For patients with stages I-II, only in clear cell carcinomas was a high MVD in hot spots significantly correlated with better survival (Ogawa et al., 2002). The role of angiogenesis in the progression of ovarian carcinoma was also challenged based on a comparative analysis between breast and ovarian cancers (Nakayama et al., 2001). In a homogeneous series of stage III patients with ovarian serous carcinomas, Raspollini et al. found that MVD in hot spots was an independent predictor of survival and of brief disease relapse (Raspollini et al., 2004a). In a recent study, Suhonen et al. reported that high angiogenesis measured by the Chalkley method predicted poor overall survival in their whole series of 175 patients, as well as in patients with advanced stage diseases (Suhonen et al., 2007). They found no association between chemotherapy response and vascularisation, contradictory to other reports (Hollingsworth et al., 1995; Gadducci et al., 2003).

Quantitating blood vessels in hot spot areas implies several observer-dependent steps, a situation that may partly explain discrepancies between studies (Vermeulen et al., 2002). The Chalkley method provides a quicker and more objective procedure for evaluating tumour vascularisation (Vermeulen et al., 2002; Suhonen et al., 2007). The use of automated image analysis algorithms, in combination with microscopic scanning of whole tumour section, allows more objective hot spot detection and microvessel counting, but has not yet been applied to large series of patients (Belien et al., 1999; Vermeulen et al., 2002; Kim et al., 2003). One may wonder whether limiting vascularisation assessment to areas of high vascular density is fully justified for ovarian tumours, based on their particular biological behaviour. In other solid tumours, the rationale of counting microvessels in such areas is that they correspond to highly angiogenic tumour cell clones which predominantly enter the circulation and give rise to vascularized metastasis (Vermeulen et al., 1996, 2002). Ovarian carcinoma, however, preferentially spread by direct extension to adjacent organs and seeding of the peritoneal cavity by exfoliated tumour cells. Hence, their dissemination might not primarily involve evasion of tumour cells from highly vascularized areas (Naora and Montell, 2005). On the other hand, the growth of primary ovarian tumour, which may favour passive peritoneal dissemination through contact with the omentum and/or increased probability of cell shedding, likely depends on neovascularisation throughout the whole tumour mass and not only in hot spots. Furthermore, as shown in experimental models, the overall extent and the blood vessel distribution may influence the kinetics of growth, as well as the capacity of tumour progression. These angiogenic characteristics depend on the level and pattern of expression of crucial factors, primarily VEGF, which in turn might contribute to progression by stimulating proliferation, survival and migration of neoplastic cells (Yoneda et al., 1998). Taken together, these data suggest that an overall evaluation of vascularisation might be of interest in determining prognosis of ovarian carcinoma. For this purpose, and given the intra-tumoural heterogeneity, identification of microvessels has to be done in whole tumour sections, in a reproducible manner. To date, studies of this kind remain unreported. In the present retrospective study on a large series of 235 women with ovarian carcinoma, tumour vasculature detected by von Willebrand factor immunostaining was automatically estimated on whole histological sections and in hot spots, using a dedicated image analysis software. Relationships between angiogenesis parameters and clinicopathologic features were studied, and their prognostic significance investigated in patients with advanced disease.

Materials and methods

Selection of cases

Two hundred and thirty-five patients suffering from epithelial ovarian cancer diagnosed from 1982 to 2000 were selected from the data base of the Comprehensive Cancer Center François Baclesse, Caen, France. Cases were selected because they all underwent a surgical resection at our institution and were treated with a platinum-based chemotherapy. The material corresponding to primary tumours was collected for all cases. Specimens were fixed in formalin and embedded in paraffin.

Histological classification was done according to the World Health Organization (WHO) system and tumours were graded as well differentiated (grade 1), moderately differentiated (grade 2), or poorly differentiated / undifferentiated (grade 3). Clinical stage assessment was based on the FIGO classification.

Treatment consisted in post-operative platinum-based chemotherapy with (n=50) or without (n=185) taxol chemotherapy. Up to six cycles of chemotherapy were administered. Residual disease after initial surgery was evaluated. A detailed description of patient characteristics is given in Table 1.

Blood vessel quantification

Immunohistochemical labelling of tumour blood vessels was performed on formalin-fixed paraffin-embedded samples of human ovarian tumours, using the ABC technique, as described previously in full detail (Kim et al., 2003). Briefly, tissue blocks were cut into 5 µm thick sections, heat enhanced retrieval of antigen was then performed at 95°C, in a microwave oven, using 10 mM pH 6 citrate buffer (ChemMate TM Dako) for 30 minutes. Sections were immunolabeled at room temperature for 1 hour, with a polyclonal anti-Von Willebrand Factor antibody (dilution 1:500, clone F8/86, Dako, France), using the Optimax automatic machine (Biogenex) to guarantee staining reproducibility. For negative controls, primary antibody step was omitted. The staining technique with an anti-Von Willebrand Factor antibody allowed having a high specificity and a strong contrast vessel staining (Palmer et al., 2007). These advantages are adequate to perform an automatic detection of vessels. The use of an anti-Von Willebrand Factor antibody may cause some regions of background staining on some samples. In order to avoid a potential bias in blood vessel estimation, a step of our automatic procedure suppresses the regions of background staining as previously described in full detail (Kim et al., 2003).

After digitization of the whole histologic slide at a resolution of 9.4 µm using a dedicated slide scanner (SprintScan 35Plus, Polaroid, France), images were processed using Pixcyt® image analysis software (Kim et al., 2003; Francoise et al., 2005; Elie et al., 2007). The automatic procedure quantification has been checked thanks to quality control and senior pathologist supervision. The inter-observer concordance and the comparison between "gold standard" (senior pathologist) and automatic quantification have been published in a previous study and were r=0.93 and r=0.94, respectively (Francoise et al., 2005). One of the advantages of an automatic image analysis procedure is the absence of intra-observer variation.

Before any analysis, normal tissue surrounding the tumour was discarded by drawing regions of interest. In order to determine a hot spot area a first step consists of an aggregation of all neighbouring microvessels with a distance under 100 µm. This procedure gives many tissue areas on histological section where the vessel borders are distant from at most 100 µm. In a second step, only regions were considered as hot spots when their surfaces were up to 900 μm^2 (equivalent surface to a microscope field at x10). In a last step, blood vessels were quantified in the limit of each hot spot.

Six vascular parameters were estimated from analysis of the whole section. Global microvessel density (MVD) was obtained as the ratio between the total number of microvessels and the total surface of the section. Global microvessel surface fraction (MSP) was obtained as the ratio between the total surface of microvessels and the total surface of the section. For hot spot analysis, the mean value of microvessel density (MVDHS1) and mean value of microvessel surface fraction (MSPHS1) were calculated, taking into account each hot spot value: Sum of individual values divided by number of hot spots. The maximum value of microvessel density (MVDHS2), and the maximum value of microvessel surface fraction (MSPHS2) result from the hot spot with the highest values. Using this procedure, an image of a section of approximately 5 cm^2 was recorded and analysed in less than six minutes.

Statistical analysis

The study had two aims. The first one was to analyse the relationships between patient and tumour characteristics and vascular parameters. The second was to evaluate the relative influence of blood vessel quantification parameters on overall survival of patients treated with a platinum-based chemotherapy.

In view of the fact that study patients had been treated over a 20-year period, prolonged tissue block storage may have resulted in altered immuno-histochemical staining. Also, parameter distributions were not normal. As an example, the MVD distribution by year of diagnosis is given in Figure 1A, which clearly shows that median MVD value increased with time. In Figure 1B, cases were grouped into those diagnosed before 1990, between 1990 and 1995, and in 1996 or later. Therefore, semi-quantitative variables (coded 1 to 3) were used that represented terciles of the corresponding vascular parameter in each diagnosis period, then the three periods were combined.

Relationships between patient or tumour characteristics (post-surgical residual disease, disease stage, histologic type, histologic grade) and semi-quantitative vascular parameters were analysed using the Fisher exact test. Relationships between patient or tumour characteristics and quantitative vascular parameters were analysed using the Kruskal-Wallis test. Two-by-two correlations between vascular parameters were analysed using the Spearman's correlation.

Overall survival was calculated from the date of initial surgery to the date of death, the date of last examination or January 1, 2005. Overall survival was estimated according to the Kaplan-Meier method and the rates between the groups were compared using the log-rank test. Ninety-five percent confidence limits (95% CL) were estimated using the method of Rothman and Boice (Rothman and Boice, 1982). Two-sided tests were used in reporting the results. Statistical significance was defined as a P-value of less than 0.05. In patients with stage III-IV disease, an analysis of prognostic factors was performed using the log-rank test (univariate

analysis) and the proportional hazards regression model with survival as dependent variable. In the latter, results were expressed using hazard ratios (HR) and corresponding 95% CL. Independent variables analysed were: age at diagnosis (< 50 vs 50 years), histologic type (serous vs endometrioid vs other subtypes), histologic grade (1+2 vs 3), disease stage (III vs IV), post surgical residual disease (< 1 cm vs 1 cm), type of chemotherapy (cisplatinum-based alone vs cisplatinum and taxol) and transformed vascular parameters, such as MVD (terciles 1+2 vs tercile 3), MSP (terciles 1+2 vs tercile 3), MVDHS1 (terciles 1+3 vs tercile 2), MSPHS1 (terciles 1+2 vs tercile 3), MVDHS2 (terciles 1+3 vs tercile 2), and MSPHS2 (tercile 1 vs terciles 2+3). Combinations of terciles were performed based on the results of univariate analyses. Results were reported according to REMARK recommendations (McShane et al., 2005). Stata statistical software (release 8.2) was used to analyse data. Data were updated on January 1, 2005. The median follow-up time was 32 months (range 1 month to 15 years).

Results

Patient characteristics

Patient characteristics and survival data are listed in Table 1, for the overall series (n=235) and for stages I-II (n=31) and III-IV (n=204, 87%) separately. The proportion of serous tumours was 50%; it was 54% in stage III-IV disease and 23% in stage I-II disease (p<0.001). Endometrioid histologic type represented 20% of all cases, undifferentiated type 11%, mixed type 10%, mucinous type 6% and clear cell type 3%.

Table 1. Patient characteristics.

		All patients		Stage I-II		Stage III-IV	
Patients at risk		235		31	13%	204	87%
Age at diagnosis, mean in years (range)		59.6	(32 to 84)	54.6	(38 to 76)	60.3	(32 to 84)
Diagnosis period	1982-1989	64	27%	7	22%	57	28%
	1990-1995	78	33%	16	52%	62	30%
	1996-2000	93	40%	8	26%	85	42%
Histologic type	serous	118	50%	7	23%	111	54%
	mucinous	13	6%	5	16%	8	4%
	endometrioid	47	20%	13	42%	34	17%
	undifferentiated	27	11%	1	3%	26	13%
	mixed	24	10%	4	13%	20	10%
	clear cell	6	3%	1	3%	5	2%
Histologic grade	1	37	16%	9	29%	28	14%
	2	91	39%	16	52%	75	37%
	3	107	45%	6	19%	101	49%
Disease stage	I	14	6%	14	45%	-	-
	II	17	7%	17	55%	-	-
	III	135	58%	-	-	135	66%
	IV	69	29%	-	-	69	34%
Post-surgical residual disease	none	89	38%	31	100%	58	28%
	[0 to 1 cm]	49	21%	-	-	49	24%
	[1 to 2 cm]	61	26%	-	-	61	30%
	[2+ cm]	36	15%	-	-	36	18%
Chemotherapy regimen	CDDP	185	79%	29	94%	156	76%
	CDDP plus taxol	50	21%	2	6%	48	24%
Number of cycles administered	1	14	6%	-	-	14	7%
	2	7	3%	-	-	7	3%
	3	10	4%	2	6%	8	4%
	4	34	14%	12	39%	22	11%
	5	16	7%	1	3%	15	7%
	6	154	66%	16	52%	138	68%
Mean follow-up time, months (range)		46	(1 to 190)	82	(8 to 169)	41	(1 to 190)
Last vital status	Alive	51	22%	19	61%	32	16%
	Dead	184	78%	12	39%	172	84%
3-year overall survival rate (95% CL)		43%	(37 to 50%)	81%	(62 to 91%)	38%	(31 to 44%)
5-year overall survival rate (95% CL)		32%	(26 to 38%)	67%	(48 to 81%)	27%	(21 to 33%)

CDDP denotes cisplatine; CL denotes confidence limits.

Histologic grade 3 represented 19% of stage I-II tumours and 49% of stage III-IV tumours (p=0.002). Patients with no residual disease after surgery represented 28% of patients with stage III-IV disease. All patients received cisplatin for a median of six courses. Cisplatin was associated with taxol in 21% of patients. At time of analysis, 184 (78%) patients had died leading to a 5-year overall survival rate of 32% (95% CL, 26 to 38%). Overall survival was 67% (95% CL, 48 to 81%) in patients with stage I-II disease and 27% (95% CL, 21 to 33%) in those with stage III-IV disease (p<0.001).

Vascular parameters

The global density of blood vessels (MVD) as well as the ratio of their surface to the overall tumour area (MSP) were highly variable, as shown in Table 2 and Figure 1A. MVD and MSP distributions were borderline significant (p=0.0731 and p=0.0595, respectively) when comparing stage I-II and stage III-IV tumours. Two-by-two correlations applied to vascular parameters showed that most of them strongly correlated (data not shown). However, no correlations were found between MVD and mean MVD in hot spots (MVDHS1) (p=0.81) or maximum MVD in hot spots (MVDHS2) (p=0.66) although the latter parameters (MVDHS1 and MVDHS2) correlated each other (p<0.001).

Relationships between vascular parameters and other clinicopathologic features

On the overall population, or when stage I-II and stage III-IV patients were considered separately, no association was found between global MVD and histologic subtype. A different situation was observed for MVDHS1, indeed in the overall population median value of this parameter was higher in mucinous (103.4±28.2 vessels per mm^2) and clear cell histology (136.4±30.7 vessels per mm^2) compared to serous (88.7±32.9 vessels per mm^2), endometrioid (94.8±35.6

Table 2. Vascular parameters: distribution of characteristics used.

Vascular parameters		All patients		Stage I-II		Stage III-IV	
		Median	(range)	Median	(range)	Median	(range)
MVD	mean microvessel profile density per mm^2	38.6	(2.1 to 166.2)	31.1	(8.2 to 81.6)	40.1	(2.1 to 166.2)
MSP	mean microvessel surface proportion, in %	8.3	(0.6 to 98.6)	6.2	(1.5 to 29.5)	8.5	(0.6 to 98.6)
MVDHS1	mean MVD within hot spot per mm^2	88.3	(1.7 to 194.3)	91.8	(35.7 to 194.3)	88.2	(1.7 to 180.2)
MSPHS1	mean MSP within hot spot, in %	20.3	(7.7 to 85.8)	19.6	(9 to 31.4)	20.4	(7.7 to 85.8)
MVDHS2	maximum MVD within hot spot per mm^2	171.8	(2.3 to 494.6)	165.6	(53 to 494.6)	173.0	(2.3 to 481.3)
MSPHS2	maximum MSP within hot spot, in %	37.1	(11.8 to 98.3)	35.9	(14.2 to 58.8)	37.3	(11.8 to 98.3)

MVD denotes mean microvessel profile density per mm^2; MSP denotes mean microvessel surface proportion, in %; MVDHS1 denotes mean MVD within hot spot per mm^2; MSPHS1 denotes mean MSP within hot spot, in %; MVDHS2 denotes maximum MVD within hot spot per mm^2; MSPHS2 denotes maximum MSP within hot spot, in %.

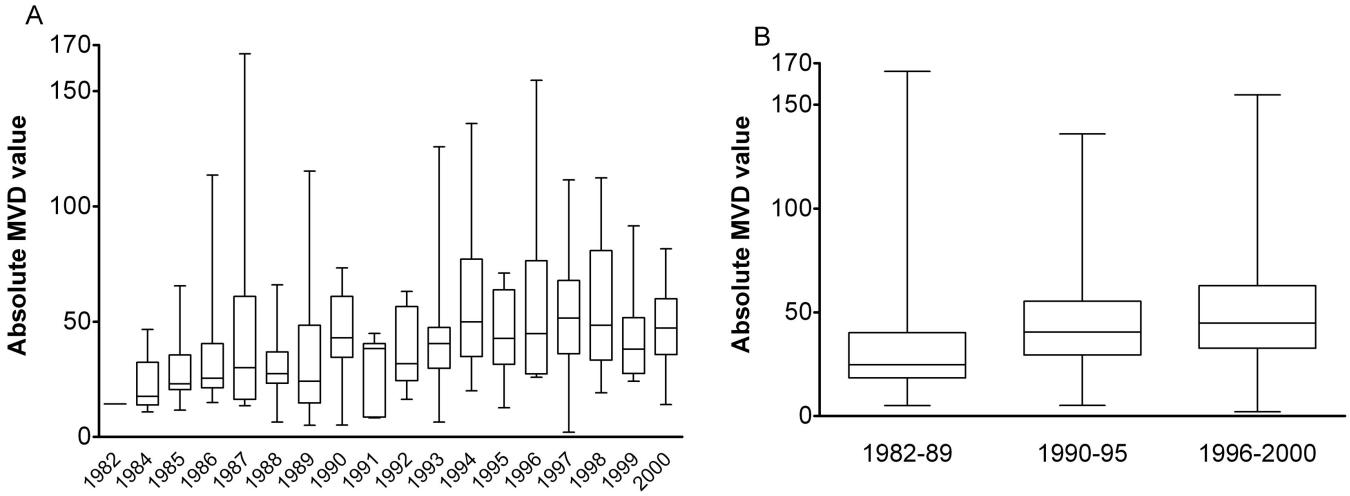

Fig. 1. A. Vascular parameter (MVD) distribution (box plot) by year of diagnosis. **B.** Same distribution with cases grouped by period of diagnosis: 1982-1989, 1990-1995, and 1996-2000.

vessels per mm^2), undifferentiated (89±34.1 vessels per mm^2) and in mixed histological subtype (95.6±31.6 vessels per mm^2) (p=0.038). The same order was observed in stage III-IV (p=0.0538), but not in stage I-II (p=0.14). No significant relationships were observed between tumour stage and any vascular parameters. In stage III-IV tumours, only median MVD inversely correlated (p<0.001) with histologic grade, i.e. 50.4 vessels per mm^2 in grade 1, 40.9 in grade 2 and 34.2 in grade 3 tumours. No significant relationships were observed between chemotherapy regimen or post-surgical residual disease and other vascular parameters.

Overall survival analysis in stage III-IV

In patients with stage III-IV disease, 5 of 12 parameters correlated with worse overall survival in univariate analysis: stage IV disease (p<0.001), post-surgical residual disease 1 cm (p<0.001) and three vascular parameters, i.e. medial MVDHS1 tercile (p=0.021), lower and medial MSPHS1 terciles (p=0.046) and medial and upper MSPHS2 terciles (p=0.007) (Table 3). Introduction of taxol in the cisplatinum-based chemotherapy regimen did not significantly influence survival rate (p=0.855). Results of the proportional hazards regression model considering all 12 factors simultaneously are given in Table 3 and show that two clinical factors (disease stage and post-surgical residual disease) expressed an independent impact on survival at the 0.05 level. Using a backward likelihood ratio elimination procedure a final model was elaborated in which stage IV disease (HR=1.72, p=0.001), post-surgical residual disease 1 cm (HR=2.86, p<0.001), upper MVD tercile (HR=1.45, p<0.022) and medial MVDHS1 tercile (HR=1.36, p=0.060) retained an independent statistically significant prognostic value upon overall survival (Table 4). In patients with stage III-IV disease and tumour of serous histologic type, however, the two vascular parameters (MVD and MVDHS1) no longer correlated with survival. The impact of global MVD (p=0.0213) and MVD in hot spots (MVDHS1) (p=0.0509) on overall survival after adjustment on disease stage and post-surgical residual disease is shown in Figures 2A and 2B, respectively. The value of the HRs associated with these two vascular parameters (MVD and MVDHS1) being similar, one can

Table 3. Prognostic value of clinical and biological factors of vascular parameters on overall survival (univariate analysis) in stage III-IV patients. Result for a model considering all factors simultaneously in stage III-IV patients (n=204).

		Univariate analysis			Regression model		
		5-yr rate	(95% CL)	P value	HR	(95% CL)	P value
Age at diagnosis	< 50	41%	(24 to 57%)		1.0		
	≥ 50	24%	(18 to 30%)	0.166	1.17	(0.76 to 1.81)	0.482
Histologic type	endometrioid	41%	(17 to 33%)		1		
	serous	25%	(25 to 57%)		1.32	(0.86 to 2.04)	0.208
	other types	21%	(12 to 33%)	0.310	0.98	(0.61 to 1.58)	0.934
Histologic grade	1-2	33%	(25 to 41%)		1		
	3	20%	(7 to 24%)	0.225	1.18	(0.86 to 1.64)	0.309
Disease stage	III	33%	(25 to 41%)		1		
	IV	14%	(7 to 24%)	< 0.001	1.59	(1.11 to 2.26)	0.011
Post-surgical residual disease	[0 to 1 cm]	42%	(33 to 52%)		1		
	[≥ 1 cm]	9%	(5 to 16%)	< 0.001	2.87	(2.05 to 4.02)	< 0.001
Chemotherapy	CDDP	24%	(17 to 31%)		1		
	CDDP plus taxol	37%	(23 to 50%)	0.153	1.04	(0.70 to 1.74)	0.855
MVD	terciles 1-2	31%	(23 to 39%)		1		
	tercile 3	19%	(11 to 29%)	0.513	1.32	(0.92 to 1.88)	0.131
MSP	terciles 1-2	30%	(22 to 38%)		1		
	tercile 3	20%	(12 to 30%)	0.365	1.15	(0.80 to 1.66)	0.440
MVDHS1	terciles 1-3	30%	(22 to 38%)		1		
	tercile 2	19%	(11 to 30%)	0.021	1.34	(0.95 to 1.87)	0.093
MSPHS1	tercile 3	35%	(24 to 46%)		1		
	terciles 1-2	22%	(16 to 30%)	0.046	1.31	(0.89 to 1.92)	0.168
MVDHS2	terciles 1-3	28%	(21 to 36%)		1		
	tercile 2	23%	(14 to 34%)	0.255	1.18	(0.84 to 1.68)	0.340
MSPHS2	tercile 1	31%	(23 to 39%)		1		
	terciles 2-3	18%	(10 to 28%)	0.007	1.15	(0.75 to 1.67)	0.464

MVD denotes mean microvessel profile density per mm^2; MSP denotes mean microvessel surface proportion, in %; MVDHS1 denotes mean MVD within hot spot per mm^2; MSPHS1 denotes mean MSP within hot spot, in %; MVDHS2 denotes maximum MVD within hot spot per mm^2; MSPHS2 denotes maximum MSP within hot spot, in %; HR denotes hazards ratio; CL denotes confidence limits; CDDP denotes cisplatine.

use the number of parameters present as surrogate index. The corresponding survival curves adjusted on post-surgical residual disease and disease stage are shown in Figure 2C, where patients with no vascular factors present (n=90) had significantly (p=0.0029) better survival than the others (n=113). The presence of one of the two vascular factors being sufficient to select patients at high risk of death, a prognostic index was built using the combination of post-surgical residual disease (< 1 cm vs 1cm) and the presence (no vs yes) of adverse vascular parameters. There were 52 patients with post-surgical residual disease < 1 cm and no adverse vascular parameters (Group A), 54 patients with post-surgical residual disease < 1 cm and adverse vascular parameters present (Group B), 38 patients with post-surgical residual disease 1 cm and no adverse vascular parameters (Group C), and 59 patients with post-surgical residual disease 1 cm and adverse vascular parameters present (Group D). The corresponding overall survival curves adjusted on disease stage are displayed in Figure 2D which shows that patients with post-surgical residual disease < 1 cm whatever the absence or presence of adverse vascular parameters (Group A + Group B) had significantly (p<0.0001) better survival than the other two subgroups of patients. Two-by-two comparisons demonstrated that: i) survival of patients of Group A did not differ from that of patients of Group B (p=0.59); ii) survival of patients of

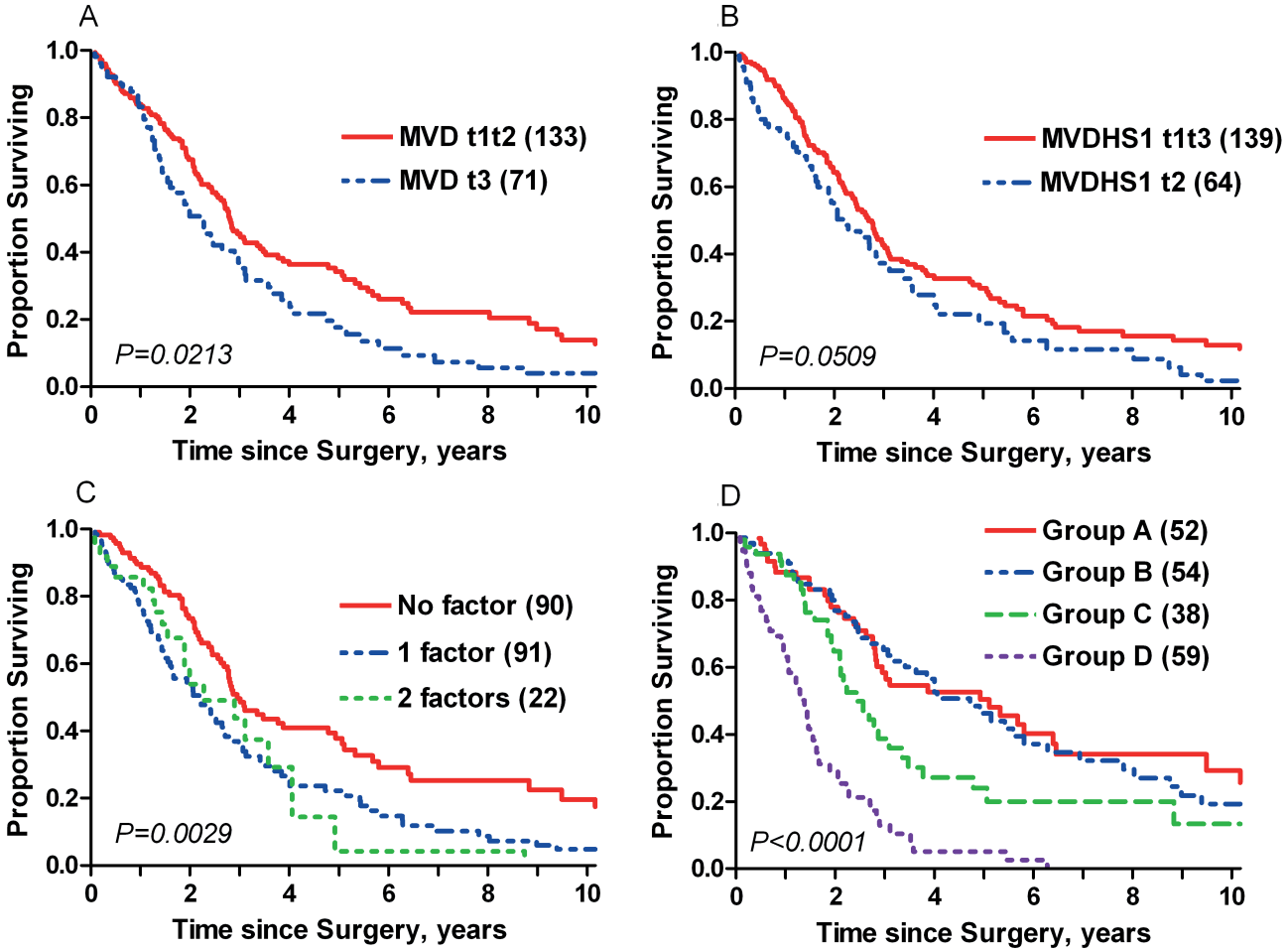

Fig. 2. Overall survival by **(A)** MVD and **(B)** MVDHS1 in stage III-IV patients, adjusted on post-surgical residual disease (< 1 cm vs 1 cm) and disease stage (III vs IV). **C.** Overall survival by number of adverse factors present (none vs MVD tercile 3 or MVDHS1 tercile 2 vs MVD tercile 3 and MVDHS1 tercile 2) in stage III-IV patients, adjusted on post-surgical residual disease and disease stage. **D.** Overall survival by number of adverse factors present (previous graph) and post-surgical residual disease: Group A corresponds to patients with post-surgical residual disease < 1 cm and no adverse vascular parameters, Group B to patients with post-surgical residual disease < 1 cm and adverse vascular parameters present, Group C to patients with post-surgical residual disease 1 cm and no adverse vascular parameters, and Group D to patients with post-surgical residual disease 1 cm and adverse vascular parameters present. MVD denotes mean microvessel profile density per mm^2 and MVDHS1 mean MVD within hot spot per mm^2. t1 denotes lower tercile, t2 medial tercile and t3 upper tercile of the distribution by period of diagnosis (1982-1989, 1990-1995, and 1996-2000).

Table 4. Final model (multivariate analysis) in stage III-IV patients (n=204): vascular parameters that independently correlate with overall survival.

		HR	(95% CL)	P value
Disease stage	III	1.0		
	IV	1.72	(1.25 to 2.38)	0.001
Post-surgical residual disease	[0 to 1 cm]	1.0		
	[≥ 1 cm]	2.86	(2.07 to 3.94)	< 0.001
MVD	terciles 1-2	1.0		
	tercile 3	1.45	(1.06 to 1.99)	0.022
MVDHS1	terciles 1-3	1.0		
	tercile 2	1.36	(0.99 to 1.87)	0.060

MVD denotes mean microvessel profile density per mm^2; MVDHS1 denotes mean MVD within hot spot per mm^2; HR denotes hazards ratio; CL denotes confidence limits.

Group A and B (3-year overall survival rate 62%; 95% CI, 52-70%) significantly ($p<0.0001$) differed from that of patients of Group C (3-year overall survival rate 39%; 95% CI, 25-53%); and survival of patients of Group C significantly ($p<0.0001$) differed from that of patients of Group D (3-year overall survival rate 13%; 95% CI, 8-22%). The impact of taxol chemotherapy on survival of Group C and D patients could not be assessed since the number of patients who were administered cisplatin and taxol chemotherapy were only 5 and 7, respectively.

Discussion

This study analyses for the first time the clinical significance of tumour vascularisation throughout whole tissue section (global MVD), and in automatically-found hot spots, in patients with ovarian cancer. In stage III-IV patients (n=204), high global MVD (upper tercile), as a variable of increased overall tumour vascularisation, is an independent predictor of poor overall survival. Interestingly, global MVD and MVD in hot spots (MVDHS1) are not correlated with each other, and the latter parameter also reaches significance in predicting overall survival. In patients with post-surgical residual disease 1 cm, the two parameters (MVD and MVDHS1) help in selecting a subset of patients with worse prognosis.

In a previous study, we had shown that the computer-assisted method we used in this study gives a reliable estimate of tumour-associated vasculature compared to classical visual estimation at microscopical level (Francoise et al., 2005). Automatic microvessel detection after low resolution scanner imaging, coupled to fully automated image analysis, gives simultaneous access to vessel density in the entire tissue section and in automatically-found hot spots, and ensures objective and reproducible quantification of blood vessels (Kim et al., 2003). Since histological section is analysed as a whole from a single image, the problem of microscopic field sampling is no more encountered, an important observer-dependent step when Weidner or Chalkley methods are used for microvessel quantification in hot spots (Vermeulen et al., 1996; Suhonen et al., 2007). Long term storage of paraffin blocks and/or changes in fixation process could account for partial degradation of antigenic sites of Von Willebrand Factor (measured using microvessel density) with time. This potential drawback was hardly avoidable since our aim is to enrol a large and homogeneous retrospective series of patients with long follow-up. Three inclusion periods are defined based on the distribution of microvessel density and semi-quantitative vascular parameters, taking into account variation of antigenicity expression with time. To our knowledge, the eventuality of a time-dependent decrease in immunohistochemical staining has not been considered in previous retrospective studies, which focused on the impact of tumour vasculature on clinical outcome of patients with ovarian carcinoma, including series with inclusion periods longer than ten years (Gasparini et al., 1996; Schoell et al., 1997; Darai et al., 1998; Ogawa et al., 2002; Raspollini et al., 2004b). This is a matter of concern since evidence exists indicating that loss of immunohistochemical staining intensity can modify the results of prognostic studies (Mirlacher et al., 2004). The high inter-tumour variability of global MVD suggests that each individual ovarian tumour displays a different angiogenic phenotype. This is not unexpected, since the extent of angiogenesis is determined by multiple molecular and cellular factors in tumour microenvironment, the presence of which may depend on the developmental history of each tumour. Remarkably, no strong association is found between global vascularisation and classical clinico-pathological parameters in this series of ovarian tumours, a reminder of what is regularly reported when highly vascularized areas are considered (Bamberger, for a review) (Bamberger and Perrett, 2002). The highest values of MVD are found among stage III-IV tumours compared to stage I-II, but there is considerable overlap between the distributions. In stage III-IV tumours, a statistically significant decrease in global vascularisation with histological grade is found, but considerable heterogeneity remains even in grade 3 tumours. Future studies on the relationships between the density and patterns of microvessels in whole tumour section and the presence of cellular and molecular factors known to regulate angiogenesis will be useful in identifying the determinants of the angiogenic phenotype of ovarian tumours, and will help to understand the origin of inter-tumour variability. Our results show for the first time that high MVD in whole tumour section compromises survival of stage III-IV ovarian cancer patients undergoing surgery and platinum-based chemotherapy. It is noticeable that the impact of tumour vascularisation on prognosis is limited to patients with sub-optimal surgery. The most straightforward interpretation is that

tumour residues have an average angiogenic potential similar to that of resected primary tumour, and that post-treatment evolution of the disease is partly dependent on the level of this potential. Indeed, in the course of intraperitoneal spreading from the primary tumour, neoplastic cells may keep unchanged their ability to promote angiogenesis, for example in term of expression of pro-angiogenic factors such as VEGF (Naora and Montell, 2005; Suhonen et al., 2007). Potent neovascularisation should provide better conditions for growth and progression of residual peritoneal metastases, accelerating the development of bowel obstruction and/or favouring resistance to drug-induced cell death (Alvarez et al., 1999). In addition, increased expression of pro-angiogenic factors associated with active angiogenic phenotypes could also act directly on cancer cells to stimulate their proliferation, survival and migration, as shown for VEGF (Wong et al., 2003). Another possibility would be that the degree of vascularisation of primary tumour, operating as a surrogate marker in prognostic analysis, may reflect a more general property of the whole tumour cell population.

In our series of stage III-IV patients, MVD in hot spots (MVDHS1) displayed a significant impact on patient outcome, although no association was found between this parameter, or other hot spot-associated parameters, and clinicopathologic features. Interestingly, whereas there was no association between global MVD and histological subtypes, however, mucinous and clear cell histology was associated with a higher value of MVDHS1, especially in stage III-IV patients. This is in keeping with other studies showing that angiogenesis seems to be dependant on histological subtypes in epithelial ovarian cancer. Due to the small number of patients in these subtypes (n=19 for the overall population and n=13 for stage III-IV patients), we did not attempt to assess separately the prognostic evaluation of vascularisation. Areas of higher microvessel density may represent particular tumour compartments, the biological behaviour of which influences disease evolution. The absence of correlation between global MVD and MVDHS1 is in keeping with this assumption, and suggests that highly vascularized tumour areas can emerge from backgrounds of variable angiogenic potential. Previous studies have found that high MVD in hot spots predicts either for poor or improved survival, or that vascularisation in hot spots has no prognostic value in patients with ovarian carcinoma. Nakayama et al. have suggested that the degree of variation of angiogenesis (MVD in hot spots) in each ovarian carcinoma may not be large enough for a prognostic indicator (Nakayama et al., 2001). Our results, however, do not confirm this statement. Differences in methodology may partly explain contradictory results and make any comparison difficult. In the present study, MVDHS1 refers to the average density of microvessels in areas of high vascular density automatically searched throughout the whole tissue section, and may not be exactly assimilated to parameters used in most of the above-mentioned studies.

Medial MVDHS1 tercile is associated with poor overall survival in univariate analysis and in the final Cox model. This result is not incompatible with the interpretation proposed above since hot spots microvessel densities (MVDHS1) in this tercile are roughly in the same range as those in the third tercile of global densities (MVD). Conversely, a majority of hot spots from tumours in the third MVDHS1 tercile exhibit microvessel densities superior to the highest observed values of global density. The very high angiogenic potential of tumour cells derived from these particular areas may increase the probability for residual tumour to be more sensitive to chemotherapy, due to better drug accessibility or efficiency. In patients treated by a paclitaxel / platinum-based regimen, Gadduchi et al. reported a positive association between MVD in hot spots of primary tumours, response to chemotherapy and clinical outcome. This was attributed to the direct antiangiogenic activity of paclitaxel (Gadducci et al., 2003). In our study, cisplatinum was associated with taxol in twenty-four percent of patients with advanced stage disease. That this may have influenced the relationship between MVD in hotspots and survival is unlikely because i) there was no statistical differences in treatment distribution between MVDHS1 terciles, and ii) introduction of taxol in the cisplatinum-based chemotherapy regimen did not significantly influence survival rate. Alternatively, a more chaotic vessel organization associated with excessively active angiogenic phenotype could be less favourable to unlimited tumour growth (Hlatky et al., 2002). To strengthen our findings, future studies should investigate the impact of tumour vascularization on survival for patients that receive both platinum and taxol.

In conclusion, we found that microvessel density in whole histological section and in hot spot areas are independently associated with overall survival in a large series of patients with advanced stage ovarian cancer, reinforcing the major role of neovascularisation in this malignancy. The fact that regions other than vascular hot spots could have an impact on prognosis is plausible, owing to the particular mode of growth and dissemination of epithelial ovarian tumours. Recent advances in virtual microscopy and availability of image analysis software henceforth allow the objective and reproducible estimation of global vascularisation, and their implementation should be recommended in future investigations. Our results suggest that such an approach could help in selecting out subsets of patients who may benefit from more tailored chemotherapy, associated or not with new targeted therapies. Further studies should also aim at better evaluating the importance of microvessel density and other angiogenesis descriptors, measured in whole tumour section and in hot spots, as predictive markers of tumour response to antiangiogenic

drugs, a subject currently a matter of debate.

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