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Clinical Relevance of Increased Endothelial and Mesothelial Expression of Proangiogenic Proteases and VEGFA in the Omentum of Patients with Metastatic Ovarian High-Grade Serous Carcinoma[1,2]

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Abstract

Epithelial ovarian cancer (EOC) metastasis to the omentum requires implantation and angiogenesis. We propose that prometastatic changes in the omental endothelium (for angiogenesis) and mesothelium (for implantation) are critical. We investigated the expression of angiogenic proteases [cathepsin D (CD), cathepsin L (CL), and matrix metalloproteinase 2 (MMP2) and MMP9] and vascular endothelial growth factor A (VEGFA) in the mesothelium and endothelium of omentum from patients with EOC with omental metastases and control patients with benign ovarian tumors. Endothelial expression of CL, VEGFA, and MMP9 and mesothelial expression of VEGFA, MMP9, and CD were significantly increased in patients with metastasized EOC. High expression of MMP9 and VEGFA in endothelium and mesothelium and CD in mesothelium was positively associated with poor disease-specific survival (DSS). High MMP9 expression in either endothelium or mesothelium and presence of ascites prospectively showed the greatest risk of shorter DSS [hazard ratio (HR)= 6.16, 95% confidence interval (CI) = 1.76-21.6, P = .0045; HR = 11.42, 95% CI = 2.59-50.35, P = .0013; and HR = 6.35, 95% CI = 2.01-20.1, P = .002, respectively]. High endothelial MMP9 expression and ascites were independent predictors of reduced DSS and overall survival, together resulting in worst patient prognosis. Our data show that omental metastasis of EOC is associated with increased proangiogenic protein expression in the omental endothelium and mesothelium.

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Introduction

Epithelial ovarian cancer (EOC) is associated with a high mortality rate due to the late stage of the disease and transperitoneal spread at the time of presentation [1]. EOC often spreads to the omentum where the rich vasculature promotes tumor invasion, angiogenesis, and subsequent metastatic growth. This process requires complex interactions between cancer cells and the surrounding omental tissue including the mesothelial, endothelial, stromal, and myeloid cells and the production of pro-metastatic and angiogenic stimuli [2–4].

Successful tumor angiogenesis requires the complex temporal and spatial integration of pro-angiogenic molecules including growth factors such as vascular endothelial growth factor A (VEGFA), cytokines, extracellular matrix (ECM) components, adhesion molecules, and also proteolytic enzymes [5,6]. These enzymes include the matrix metalloproteinases (MMPs) and cathepsins that degrade the ECM, aiding new vessel branching, and it is now clear that they play a

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critical role in cancer progression. For instance, cathepsin D (CD) releases pro-angiogenic basic fibroblast growth factor from the ECM in breast cancer cells, whereas cathepsin L (CL) plays a role in the angiogenic switching of hyperplastic and dysplastic progenitor lesions in a mouse model of cervical cancer, as well as in tumor growth and tumor vascularization [7,8].

Accumulating evidence suggests that proteases play an important role in EOC. Not only is there a complex interplay between VEGFA and MMPs during EOC metastasis [9], but also EOC expression of VEGFA, MMPs, and cathepsins is associated with advanced stage disease, poor prognosis, and clinicopathologic parameters in patients with EOC [10–12]. Additionally, high MMP2/9 expression in primary EOC was significantly associated with aggressive features such as high stage, high grade, ascites, and positive lymph node status [13]. Importantly, preoperative serum levels of CL and MMP9 correlated with the degree of differentiation, the International Federation of Gynecology and Obstetrics (FIGO) staging, and peritoneal metastasis in patients with EOC [14].

The above work has focused on primary EOC cells. However, given the unfavorable prognostic outcome associated with omental metastatic lesions, pro-angiogenic changes in the omentum during metastasis may also contribute to EOC patient outcome. For instance, vascular endothelial cells are critical to the angiogenic process, stimulating ECM remodeling and facilitating new vessel growth, whereas mesothelial cells may provide metastatic cancer cells with a microenvironment conducive to survival and growth [15]. For both cell types, the presence of metastatic EOC cells in the omentum may change their protease expression profile, shifting them toward a pro-angiogenic, cancer-inducing response. Therefore, this study aimed to 1) examine the expression of MMP2, MMP9, CD, CL, and VEGFA in EOC, endothelial, and mesothelial cells in the omentum of patients with metastatic ovarian high-grade serous carcinoma compared with control patients with benign ovarian cystadenoma and 2) investigate the relationship between their expression in each cell type and clinical outcome for patients with EOC. We show that the endothelium and mesothelium of omentum hosting EOC metastases express significantly increased levels of pro-angiogenic proteases and VEGFA and that high endothelial and mesothelial expression of MMP9 is associated with significantly reduced overall survival (OS) and disease-specific survival (DSS). Importantly, high endothelial MMP9 expression combined with the presence of ascites is predictive of poor prognosis.

Materials and Methods

Clinical Samples

This study was undertaken in the diagnostic/research laboratory of the Royal Devon and Exeter NHS Foundation Trust (RD&E NHS Trust). Thirty-nine omental samples taken during ovarian tumor surgery and previously used for diagnostic staging were retrieved from the histopathology archives with approval from the Caldicott Guardian of the RD&E NHS Trust and the Devon and Torbay Local Research Ethics Committee. Hematoxylin and eosin stained slides were reviewed by histopathologists (N.C. and M.A.) to confirm the histopathologic diagnosis and tumor grading. Clinical information was obtained from the patients' medical records. Two distinct groups were identified: 1) women with high-grade, serous ovarian carcinoma with omental metastases (malignant group) and 2) women with benign ovarian pathology, i.e., serous cystadenoma and normal omental biopsies (control group).

Immunohistochemistry

Four-micrometer slices of formalin-fixed (10%), paraffin-embedded tissues were collected onto positively charged microscope slides and immunostained using primary mouse monoclonal antibodies for CD, MMP2, MMP9 (Novocastra), CL (Santa Cruz Biotechnology, Dallas, TX), and VEGFA (Abcam, Cambridge, UK). Details of the antibodies used and the preliminary testing to determine optimum working antibody concentrations and antigen recovery are presented in Table W1. Representative images of positive controls are presented in Figure W1. Antigen recovery, deparaffinization, rehydration, and immunohistochemistry were carried out by the Bond-MAX autostainer supplied with BOND reagents (Leica Microsystems, Milton Keynes, UK) at room temperature, unless otherwise specified. The following immunohistochemical protocol was applied to each slide with washes with bond wash buffer between stages 1 and 4 and dH_2O for subsequent rinses: 1) if required, antigen retrieval was performed; 2) primary antibody (15 minutes); 3) peroxide block (5 minutes); 4) post primary polymer penetration enhancer (8 minutes) and then 3× wash, followed by polymer poly-HRP anti-mouse/rabbit IgG containing 10% (vol/vol) animal serum (8 minutes); 5) 3,3′-diaminobenzidine (DAB, parts 1 and 2) mixed by Bond-MAX (10 minutes) and then 6× wash followed by enhancer (5 minutes) and 3× wash; 6) counterstain with hematoxylin (0.02% wt/vol; 4 minutes), followed by a wash in alkaline buffer to "blue" the counterstain. The slides were then dehydrated and mounted using automated procedures (Leica Microsystems).

Table 1. Clinicopathologic Characteristics of Patients with Benign Ovarian Tumor and Metastatic Serous Ovarian Carcinoma [on the Basis of CA125 Level, Patients Were Grouped as Low (<35 IU/ml) or High (≥35 IU/ml)].

Variable	Number	
	Control	Malignant
Median age (range)	61.5 (44-90)	64 (38-83)
Tumor type		
Benign	20	–
Malignant	–	19
Histologic subtype		
Serous	20	19
FIGO stage		
3B	–	4
3C	–	15
Ascites		
Absent	20	4
Present (</>100 ml)	–	4/11
Preoperative CA125 (IU/ml)		
(1) Low	14	–
(2) High	6	19
Mean	32.8	820.6
Median (range)	19.5 (7-111)	427 (46-2461)
Relapse (based on months)		
Present (</>24 months)	1 (0/1)	17 (5/12)
Absent (>24 months)	19	2
Distant metastasis		
No	20	3
Yes	–	16
OS		
Alive	17	7
Deceased	3	12

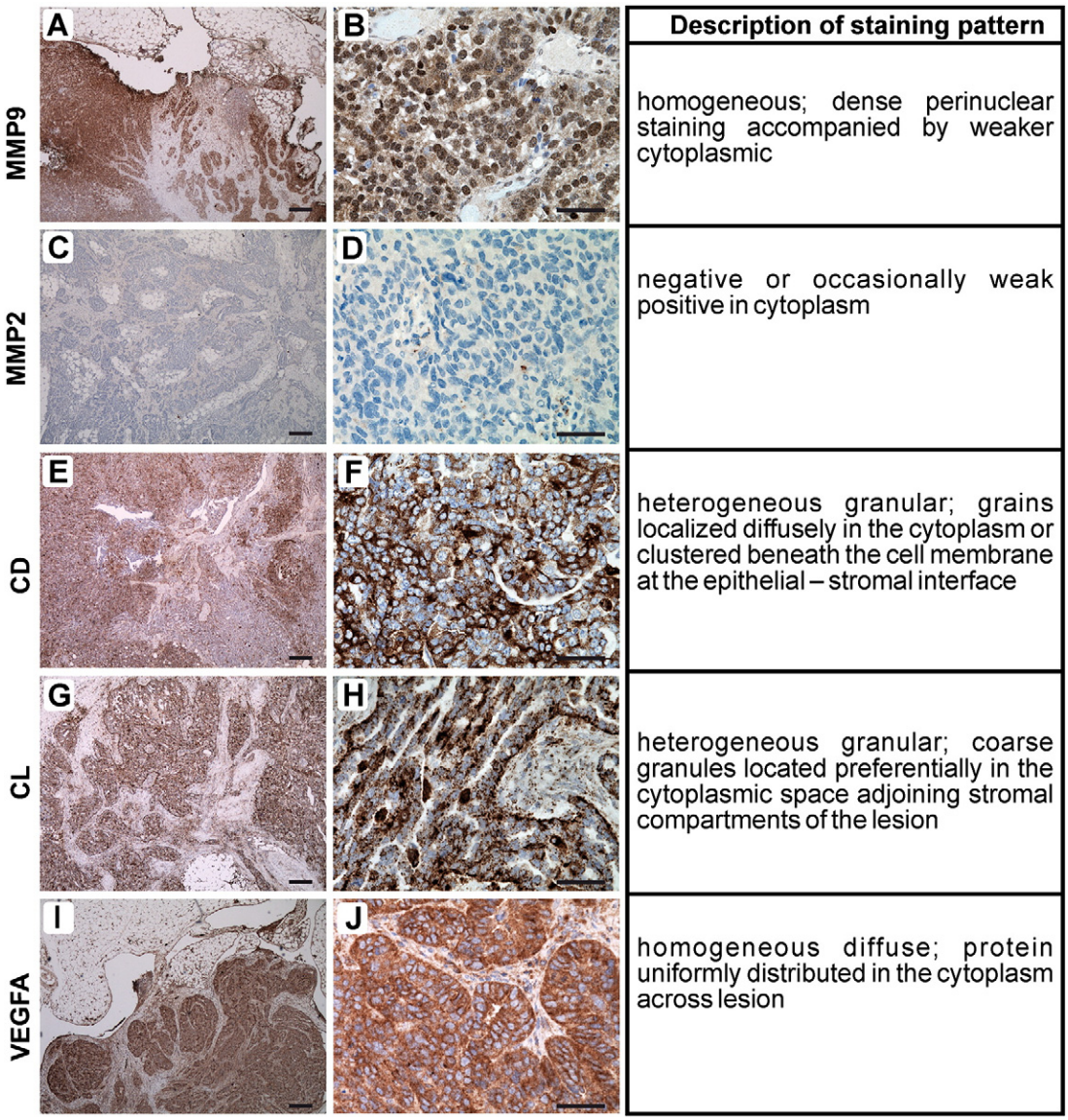


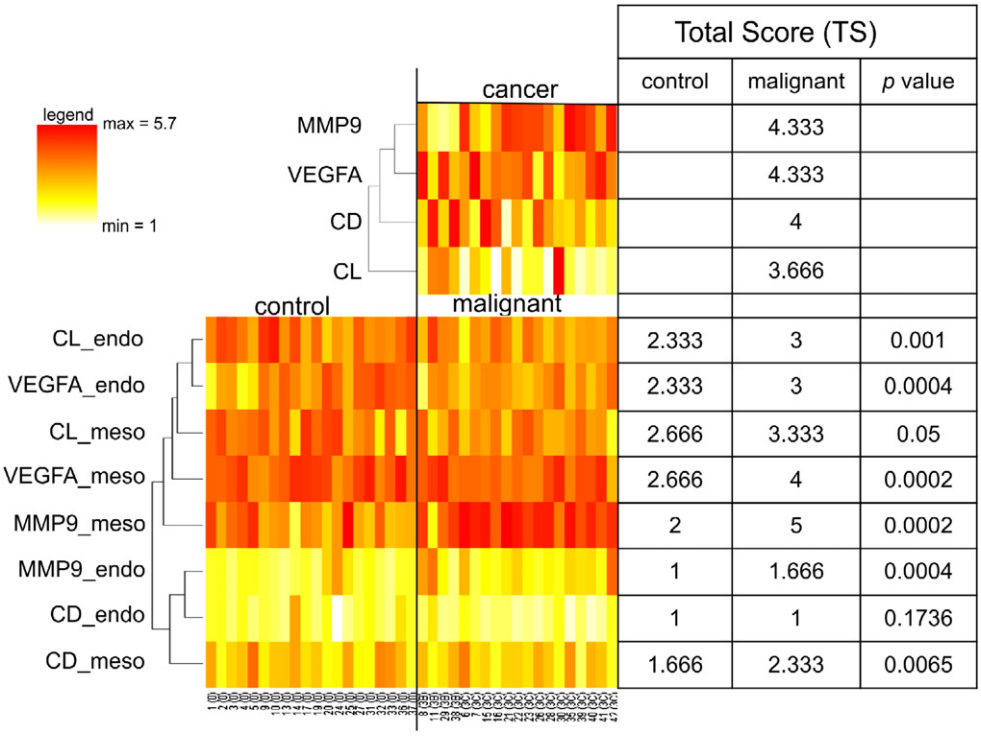
Figure 1. Pattern of expression of proteases and VEGFA in omental metastases of serous ovarian carcinoma. Four-micrometer sections were stained for the expression of MMP9 (A, B), MMP2 (C, D), CD (E, F), CL (G, H), and VEGFA (I, J) using the Bond Polymer Refine Detection System. Nuclei are stained blue, whereas brown staining indicates the presence of the assessed protein. Scale bars, 100 μm (A, C, E, G, I) and 50 μm (B, D, F, H, J). Representative images are shown.

Evaluation of Immunoreactivity

Immunoreactivity was evaluated over each whole slide in a semiquantitative way by three researchers blinded to the category of each section. Scoring criteria were determined during a preliminary evaluation using a multiheaded microscope to reach a consensus. The immunohistologic expression of each protein was scored for intensity of staining and percentage of positive cells over the whole slide, in three cell types: cancer, endothelial, and mesothelial cells, producing a total score (TS). The following scoring system was applied: intensity of staining scored (1) none or weak, (2) moderate, and (3) strong, and percentage of positive cells was scored (0) 0% to 5%, (1) 6% to 25%, (2) 26% to 75%, and (3) 76% to 100%. TS was calculated (for each protein in each cell type) as the mean sum (from the three researchers) of the intensity and percentage scores, i.e., between 1 and 6 with a score of 6 indicating strongest staining in the majority of cells. The interobserver variation was checked using weighted Kappa statistic for comparing three observers as described previously [16].

Clinicopathologic Data

Benign cysts (serous cystadenomas) were surgically removed, whereas the metastatic serous EOC patients' initial treatment was by surgical debulking. Adjuvant chemotherapy in the malignant group was a standard platinum-based regime directed by a gynecological oncologist. None of the subjects selected for the study had endometriosis or had received recent chemotherapy before surgery (within 10 months). FIGO staging, distant metastasis status, and ascites volume were defined by the operating gynecological surgeons. DSS was defined as survival without death due to ovarian cancer, and OS was defined as survival without death due to any cause. Relapse was defined as symptomatic disease based on physical examination, imaging studies, and CA125 levels, or for patients initially diagnosed



Total Score (TS)		
control	malignant	*p* value
	4.333	
	4.333	
	4	
	3.666	
2.333	3	0.001
2.333	3	0.0004
2.666	3.333	0.05
2.666	4	0.0002
2	5	0.0002
1	1.666	0.0004
1	1	0.1736
1.666	2.333	0.0065

Figure 2. Median overall expression levels, i.e., TSs and individual patient expression levels of MMP9, CL, CD, and VEGFA in cancer cells, endothelial cells (endo), and mesothelial cells (meso). A heat map of expression of each protein in all cell types studied in each patient was generated using R Statistical Computing software (v3.0.1). Protein TSs were calculated on the basis of the sum of staining intensity and percentage of positive cells. The median of the TS for all patients in each group is presented. For presentation, the expression of the proteins is clustered into two distinct modes: moderate-to-high (TSs ranging 2-5) and weak-to-moderate expression (TSs ranging 1-2.333). Differential expression of median TS for each protein in the mesothelium and endothelium in the two patient groups was assessed by Wilcoxon signed-rank test, and $P < .01$ was considered as significant, $n = 19$ or 20 (see Table 1) for omental endothelial and mesothelial tissues.

with benign disease, the subsequent development of malignant disease. On the basis of DSS, patients were divided into two relapse groups: recurrent disease (present) and no recurrent disease (absent). On the basis of CA125 level, patients were grouped as low (< 35 IU/ml) and high (≥ 35 IU/ml).

Statistical Analysis

For statistical analysis, medians of TS means were used. In all the subsequent analyses, the R Statistical Language [17] was used. All correlation coefficients presented in this manuscript are "rho" coefficients from Spearman rank test. Survival analyses, survival plots, and Cox proportional hazards regression models were generated by the package "survival" [18,19]. The *P* values presented in the plots are derived from the log-rank test. The survival function of DSS and OS was estimated using the Kaplan-Meier method. To determine the effect on likelihood of survival of combinations of proteins/clinicopathologic variables, the tree-structured survival analysis was used [20].

Results

Clinical Demographics and Pattern of Protein Expression

Patient clinical and pathologic data are summarized in Table 1. The follow-up for both groups was 60 months. Benign and metastatic ovarian tumors were both of serous type to exclude potential variations in protein expression between tumor subtypes. The interobserver variation was 0.8 (for all assessed proteins in all cell types).

Cancer Protein Expression. The expression patterns of all proteins were initially characterized in EOC cells. Representative EOC staining patterns for each protein are illustrated and discussed in Figure 1. Relatively high expression (median TS > 3.5) was observed for all proteins except MMP2 (Figure 2). The TS for MMP2 was 1 indicating minimal expression (data not shown), and thus, this protein was not included for further analysis. EOC cell TS of expression of each expressed protein in all individual patients studied and overall median TSs for each protein are shown in Figure 2.

Endothelium and Mesothelium Protein Expression. Next, we assessed expression of protein targets in the endothelium and mesothelium of both groups. Representative images, together with a description of the staining patterns, are presented in Figure 3. Endothelial and mesothelial cell TSs of expression of each protein in all individual patients studied and overall median TSs for each protein are shown in Figure 2. The malignant group mesothelium expressed the highest levels of MMP9, VEGFA, and CL, while the endothelium was particularly immunoreactive for VEGFA and CL. Mesothelial and endothelial MMP2 immunoreactivity was mainly negative or weakly positive in both groups. MMP9 immunoreactivity exhibited mainly diffuse, cytoplasmic staining, with stronger perinuclear pattern of staining observed in the mesothelium. This staining pattern suggests storage and processing of MMP9 in vesicles in these cell types before release at the cell surface. MMP9 expression was stronger in mesothelial cells close to the metastatic tumor and in mesothelial cells with a stratified, inflamed appearance than those remote from

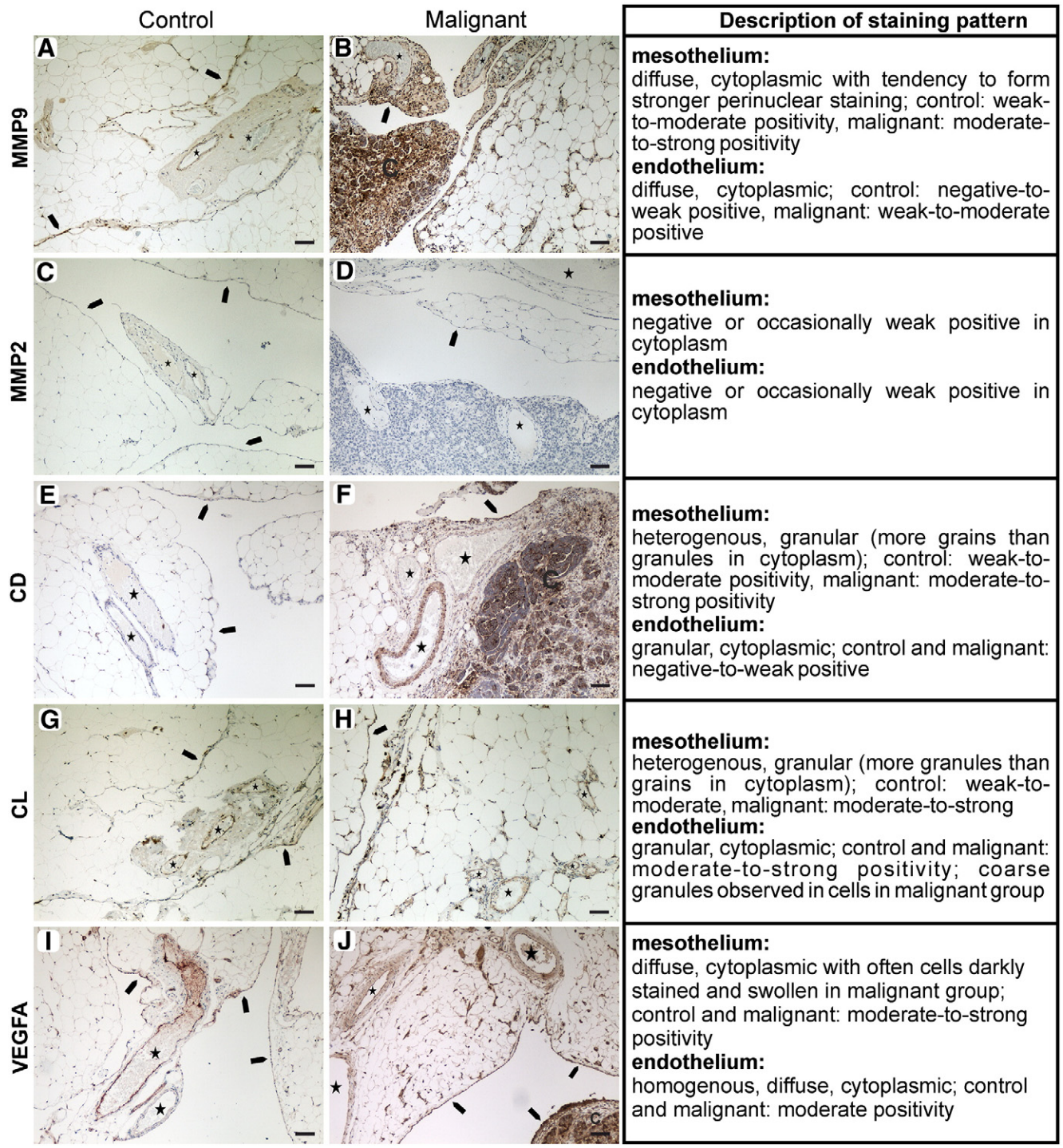


Figure 3. Pattern of expression of proteases and VEGFA in endothelium and mesothelium of the control and malignant omentum. Four-micrometer sections were stained for expression of MMP9 (A, B), MMP2 (C, D), CD (E, F), CL (G, H), and VEGFA (I, J) using the Bond Polymer Refine Detection System. Description of pattern of staining is presented in the enclosed table. A, C, E, G, I—control omentum; B, D, F, H, J—malignant omentum. Arrowheads indicate mesothelium, stars indicate blood vessels, and C indicates the presence of metastatic lesion of EOC. Nuclei are stained blue, whereas brown staining indicates the presence of the assessed protein. Scale bar, 100 μm.

the tumor. CL and CD expression displayed a granular cytoplasmic pattern, supporting their reported intracellular localization in lysosomal and secretory vesicles. CD expression was also stronger in inflamed, stratified mesothelium and mesothelium close to metastatic tumor. Lastly, VEGFA showed a diffuse, cytoplasmic localization (homogenous) in endothelium and mesothelium of both groups studied. Swollen, darkly stained VEGFA-positive mesothelial cells were often observed in the malignant group. Perivascular cells, e.g., vascular smooth muscle cells, exhibited various degrees of immunoreactivity for MMP9, CD, CL, and VEGFA in both groups.

A

variable	Spearman's rank correlation coefficients (r)					
	MMP9 endothelium	MMP9 mesothelium	CD mesothelium	CL endothelium	CL mesothelium	VEGFA endothelium
MMP9 endothelium	1.000	.	.	.	.	.
MMP9 mesothelium	0.551	1.000	.	.	.	.
CD mesothelium	0.381	0.710	1.000	.	.	.
CL endothelium	0.077	0.338	0.163	1.000	.	.
CL mesothelium	0.265	0.601	0.398	0.479	1.000	.
VEGFA endothelium	0.152	0.418	0.112	0.501	0.284	1.000
VEGFA mesothelium	0.338	0.653	0.516	0.480	0.542	0.569

variable	*p* values					
	MMP9 endothelium	MMP9 mesothelium	CD mesothelium	CL endothelium	CL mesothelium	VEGFA endothelium
MMP9 endothelium	1.000	.	.	.	.	.
MMP9 mesothelium	<0.001	1.000	.	.	.	.
CD mesothelium	0.017	<0.001	1.000	.	.	.
CL endothelium	0.643	0.036	0.321	1.000	.	.
CL mesothelium	0.102	<0.001	0.012	0.002	1.000	.
VEGFA endothelium	0.355	0.008	0.497	0.001	0.080	1.000
VEGFA mesothelium	0.035	<0.001	<0.001	0.002	<0.001	<0.001

B

varibale	MMP9		CD		CL		VEGFA	
	endothelium	mesothelium	endothelium	mesothelium	endothelium	mesothelium	endothelium	mesothelium
	r / *p*	r / *p*	r / *p*	r / *p*	r / *p*	r / *p*	r / *p*	r / *p*
CA125	0.54 / *0.0004*	0.63 / *<0.0001*	-0.03 / *0.8636*	0.49 / *0.0014*	0.33 / *0.0399*	0.24 / *0.1412*	0.45 / *0.0038*	0.49 / *0.0015*
Ascites	0.49 / *0.0016*	0.64 / *<0.0001*	0.03 / *0.8422*	0.32 / *0.0488*	0.30 / *0.0648*	0.19 / *0.2448*	0.34 / *0.0318*	0.30 / *0.0600*
Relapse	0.48 / *0.0020*	0.57 / *0.0002*	0.03 / *0.8488*	0.30 / *0.0670*	0.27 / *0.0924*	0.33 / *0.0420*	0.37 / *0.0222*	0.32 / *0.0480*
Distant metastasis	0.39 / *0.0130*	0.43 / *0.0066*	-0.13 / *0.4292*	0.16 / *0.3364*	0.09 / *0.5690*	0.16 / *0.3218*	0.27 / *0.0910*	0.24 / *0.1422*

Figure 4. Correlations in cell type–specific expression levels of MMP9, CD, CL, and VEGFA between (A) cell type and protein and (B) patient clinicopathologic variables. (A) Intercell and intracell type correlations between protein expression in omentum from all patients assessed by Spearman correlation, $n = 39$. (B) Spearman rank correlation test for protein expression and clinicopathologic variables for all patients. Patients were categorized as described in the Materials and Methods section and in Table 1, $n = 39$. r = Spearman's rank correlation coefficient (rho), P = Spearman rank correlation P value. $P < .01$ was considered as significant.

Protein Expression—Relationship and Association with Clinicopathologic Features

Previous work suggests that expression of proteases and VEGFA increases as tissue changes from a normal-to-benign-to-malignant phenotype, presumably associated with the induction of a "pro-angiogenic" state [8,21]. Initial analysis indicated that omental endothelial expression of MMP9, CL, and VEGFA and omental mesothelial expression of CD, MMP9, and VEGFA were significantly higher in the malignant group compared to the control group (Figure 2). We then investigated intercell and intracell type (endothelial and mesothelial) correlations in expression of all investigated proteins, because a complex interplay between proteases and VEGFA during tumor progression has been reported [9]. Numerous nominal significant associations were observed (complete data in Table W2). However, most of the highly significant associations ($P < .001$, $r > 0.5$) clustered with high mesothelial MMP9 and VEGFA expression (Figure 4*A*), indicating the development of a pro-metastatic phenotype in the mesothelium.

We next analyzed the relationship between clinicopathologic parameters and protein expression in the omental endothelium and mesothelium. Several significant correlations were evident (for r and P values, see Figure 4*B*). High endothelial and mesothelial expression of MMP9 correlated with an increase in all assessed clinicopathologic variables, whereas high mesothelial and endothelial expression of VEGFA associated with increased CA125 levels, as did high mesothelial CD expression.

The Impact of Omental VEGFA and Protease Expression on Clinical Outcome

Kaplan-Meier survival curves were plotted followed by log-rank tests for DSS and OS to determine the relationship between protein expression levels in endothelium and mesothelium and survival. High expression of MMP9 and VEGFA in endothelium and mesothelium and high mesothelial expression of CD were positively associated with EOC disease-specific death (DSS; $P = .0012$, $P < .0001$, $P = .0084$, $P = .021$, $P = .011$, respectively; Figure 5, *A*–*E*). However, significantly reduced OS was only observed in patients with high MMP9 expression in endothelium and mesothelium ($P = .0097$ and $P = .032$, respectively; Figure 5, *F* and *G*).

To explore the prognostic relevance of endothelial/mesothelial changes in protein expression on DSS, a univariate analysis of protein expression for clinical outcome was performed. Cox proportional hazards regression modeling revealed that patients with high expression of MMP9 in either the endothelium or mesothelium had the greatest risk of shorter median DSS [hazard ratio (HR) = 6.16, 95% confidence interval (CI) = 1.76-21.6, $P = .0045$; HR = 11.42, 95% CI = 2.59-50.35, $P = .0013$, respectively; Table 2*A*]. Other significant risks of reduced DSS were high mesothelial expression of CD and high mesothelial or endothelial expression of VEGFA; however, these risks were less pronounced (Table 2*A*). Among clinicopathologic variables, the presence of ascites was most strongly correlated with reduced DSS (HR = 6.35, 95% CI = 2.01-20.1, $P = .002$; Table 2*B*).

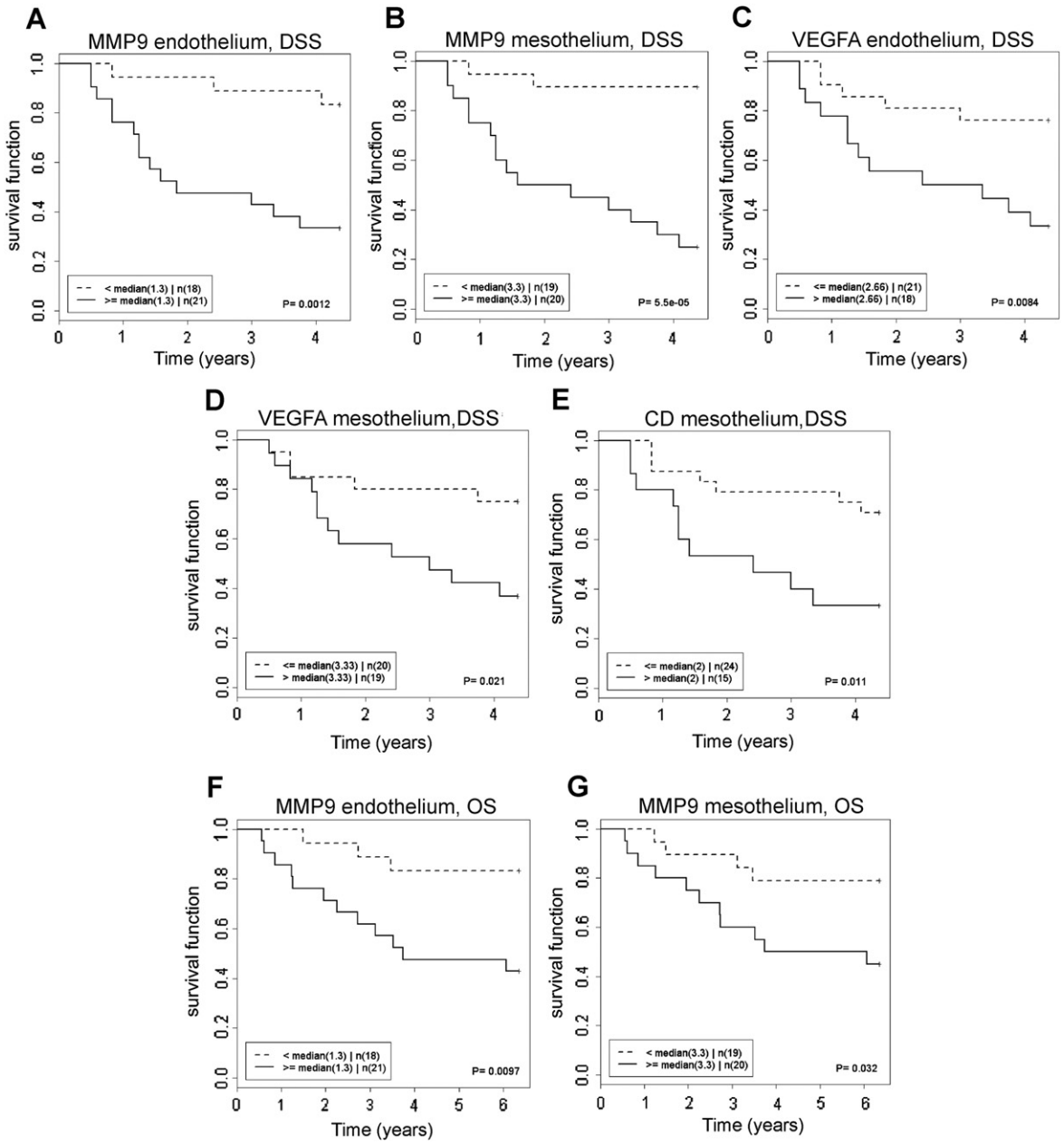


Figure 5. High expression of MMP9 in either endothelium or mesothelium associates with significantly reduced EOC DSS and OS. Kaplan-Meier survival curves were plotted for (A–E) DSS and (F, G) OS. $n = 39$, P values were calculated using the log-rank test. For analysis, protein expression was classified as either high or low based on the median expression value of each protein of all samples included in this study. High endothelial expression of VEGFA and high mesothelial expression of CD also associated with reduced DSS but not with OS.

Predictor(s) of Poor Clinical Outcome in Patients with EOC

To define the protein expression pattern associated with the worst clinical outcome, a tree-structured analysis for DSS and OS was performed with patients stratified by MMP9 expression in either mesothelium or endothelium, since MMP9 expression was the best predictor of survival/death. Reduced DSS was observed in patients with high endothelial or mesothelial MMP9 expression coupled with high endothelial VEGFA expression (condition 1), high mesothelial VEGFA expression (condition 2), and high mesothelial CD expression (condition 3; DSS for MMP9, endothelium: $P < .001$ for all three associations; DSS for MMP9, mesothelium: $P < .001$ for all three associations; see Figure 6, *A–C*, for endothelium and Figure W2, *A–C*, for mesothelium). However, only patients with high endothelial MMP9 expression had significantly reduced OS ($P = .049$, $P = .038$, and $P = .034$, respectively, for conditions 1, 2, and 3; Figure 6, *D* and *E*). Follow-up tree-structured HR analysis indicated that high endothelial MMP9 expression was the single best predictor of reduced DSS and OS (DSS, HR = 6.16, 95% CI = 1.76-21.6, $P = .005$; OS, HR = 4.59, 95% CI = 1.29-16.3, $P = .019$; for survival trees, see Figure W2, *D–F*). An additive effect of decreased OS was observed in patients with high expression of MMP9 in both endothelium and mesothelium; however, the HR for DSS was not further reduced compared to univariate analysis for MMP9 (OS, HR = 18.75, 95% CI = 2.43-144.75, $P = .005$; DSS, HR = 5.94, 95% CI =

Table 2. Association of Pro-Metastatic Protein Expression Pattern and Clinicopathologic Characteristics with Length of Ovarian Cancer DSS.

A

Variable	Expression	Median DSS (Years)	HR (95% CI)	*P*
MMP9				
Endothelium	High	1.83	6.16 (1.76-21.61)	.0045
	Low	4.36		
Mesothelium	High	1.99	11.42 (2.59-50.35)	.0013
	Low	4.36		
CD				
Endothelium	High	3.68	1.22 (0.35-4.25)	.75
	Low	4.36		
Mesothelium	High	2.41	3.25 (1.23-8.60)	.018
	Low	4.36		
CL				
Endothelium	High	3.17	2.69 (0.94-7.66)	.064
	Low	4.36		
Mesothelium	High	3.71	1.83 (0.70-4.83)	.22
	Low	4.36		
VEGFA				
Endothelium	High	2.88	3.70 (1.30-10.54)	.014
	Low	4.36		
Mesothelium	High	3	3.19 (1.12-9.1)	.03
	Low	4.36		

B

Variable	Expression	Median DSS (Years)	HR (95% CI)	*P*
CA125	High	2.42	4.61 (1.04-20.5)	.045
	Low	4.36		
Ascites	Present	1.42	6.35 (2.01-20.1)	.002
	Absent	4.36		
Distant metastasis	Present	1.21	3.33 (1.20-9.23)	.02
	Absent	4.36		

(A) Association of omental endothelial and mesothelial expression levels of MMP9, CL, CD, and VEGFA with DSS. Cox proportional hazard regressions of endothelial and mesothelial expression (medians) of each protein in all patients were calculated to test association with DSS. $P < .05$ was considered as significant. Patients were categorized on the basis of median protein TS for each protein in each cell type. Elevated MMP9 expression in omental endothelium and mesothelium was associated with the shortest length of DSS (HR = 6.16, 95% CI = 1.76-21.6, $P = .0045$; HR = 11.42, 95% CI = 2.59-50.35, $P = .0013$, respectively).

(B) Association of CA125 levels, presence of ascites, and presence of distant metastases with DSS. Cox proportional hazard regressions of CA125 levels, ascites, and distant metastasis of all patients were calculated to test association with DSS. $P < .05$ was considered as significant. Patients were categorized as described in the Materials and Methods section and in Table 1. The presence of ascites had highest impact on reduced DSS (HR = 6.35, 95% CI = 2.01-20.1, $P = .002$).

1.30-27.19, $P = .022$; survival plots not shown). Finally, to confirm the predictive significance of elevated endothelial MMP9 expression, we generated a tree-structured analysis of multivariable Cox proportional hazard regression models for DSS and OS where, initially, all clinicopathologic parameters were included. In our final model, both elevated endothelial MMP9 expression (DSS, HR = 6.16, 95% CI = 1.76-21.6, $P = .005$; OS, HR = 4.59, 95% CI = 1.29-16.3, $P = .019$) and the presence of ascites (DSS, HR = 9.92, 95% CI = 2.15-45.7, $P = .003$; OS, HR = 43.2, 95% CI = 5.33-350, $P = .0004$) were independent predictors of DSS and OS, together resulting in worst patient prognosis (Figure 6*G*).

Discussion

This study demonstrates an increase in expression of pro-angiogenic proteases and VEGFA in omental tissue with metastasized EOC compared to control omentum. Specifically, we show for the first time that omentum with metastatic disease has significantly increased endothelial expression of MMP9, CL, and VEGFA and mesothelial expression of CD, MMP9, and VEGFA. Further analysis indicated that high omental mesothelial and endothelial expression of MMP9 and VEGF and high mesothelial expression of CD is associated with decreased DSS and/or OS in EOC. Most importantly, high omental endothelial MMP9 expression together with the presence of ascites predicts poor prognosis.

MMPs and cathepsins have been implicated in tumor progression and have been widely investigated in cancers showing overexpression of these proteases, including ovarian cancer [22–25]. Similarly, altered expression of pro-angiogenic factors such as VEGFA has been investigated in ovarian cancer, since angiogenesis is known to correlate with prognosis [10,26]. Importantly, ovarian cancer–secreted CD, CL, and MMP9 have been shown to regulate a range of cellular responses of omental microvascular endothelial cells in *in vitro* studies, highlighting their role as key alternative angiogenic mediators during omental progression of EOC [27]. Our data support these previous studies since the metastasized EOC cells were strongly immunoreactive for MMP9 and VEGFA and moderately for CD and CL. However, little or no expression of MMP2 was observed, in contrast to a previous study by Schmalfeldt et al. [28].

In addition to the expected expression of pro-angiogenic factors in the metastasized EOC, our study is the first to specifically show overexpression of MMPs, cathepsins, and VEGFA in the endothelium and mesothelium of the omental tissue surrounding EOC metastases. These factors are likely to have a close pro-angiogenic relationship since protease degradation/remodeling of the ECM during angiogenesis can release pools of ECM-bound growth factors (i.e., VEGFA and basic fibroblast growth factor) that facilitate new vessel growth [7,29]. Importantly, our data suggest that the dissemination of EOC may engage a "cellular triangle" involving cancer cells (primary invaders and switchers of the microenvironment), endothelial cells (mediators of tumor-induced angiogenesis), and mesothelial cells (signal disseminators). Thus, invasion of the omentum by EOC is associated with pro-angiogenic protein expression in the surrounding omental tissue creating a microenvironment conducive to metastatic growth and disease progression. It is not possible to conclude from our data whether this is driven by the cancer cells, the endothelial/mesothelial cells, or a feedback loop between all three cell types "feeding" metastasis growth. However, our observation that both MMP9 and CD expression was stronger in mesothelial cells close to the metastatic tumor suggests that a paracrine effect of factors secreted from the tumor cells contributes to the increased MMP9 and CD expression. For MMP9, this is supported by the observation that the secretome of colorectal tumor cells induced increased expression of MMP9 in primary human omental mesothelial cells [30]. In contrast, Davidson and co-workers [31] showed that while MMP2/9 protein expression was detected in primary and omental metastases of EOC, higher expression was found in pleural and peritoneal effusions containing active mesothelial cells and concluded that the MMPs were predominantly synthesized by EOC cells in effusions, where cells acquired their metastatic potential from the local microenvironment, and by local native cells, i.e., mesothelial cells.

Importantly, high mesothelial and endothelial expression of MMP9 and VEGF, high mesothelial expression of CD, and the presence of ascites were associated with significantly reduced DSS in our study. Previously, Kamat and colleagues found that stromal expression of MMPs (particularly MMP9 and MT1-MMP in fibroblasts and endothelial cells) was an independent predictor of shorter DSS in patients with EOC [13]. In our investigation, both endothelium and mesothelium appeared to be involved in defining a

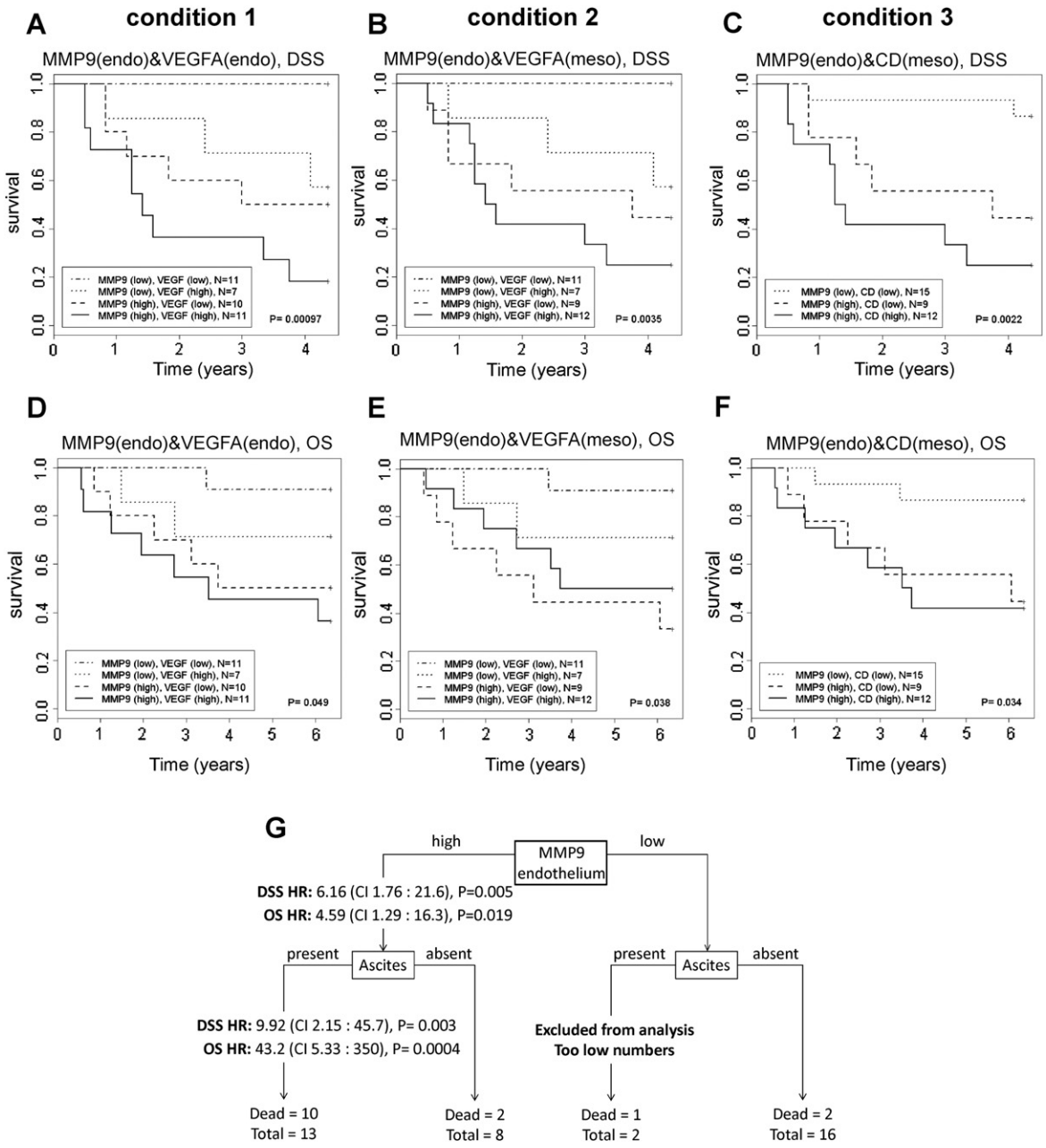


Figure 6. Elevated MMP9 expression in endothelial cells independently predicts reduced EOC DSS and OS and, coupled with the presence of malignant ascites, predicts worst patient outcome. Kaplan-Meier survival curves are shown for each "node" of the tree-structured analysis: (A–C) DSS and (D–F) OS; condition 1—high endothelial MMP9 expression coupled with high endothelial VEGFA expression, condition 2—high endothelial MMP9 expression coupled with high mesothelial VEGFA expression, condition 3—high endothelial MMP9 expression coupled with high mesothelial CD expression; $n = 39$; P values were calculated using the log-rank test. (G) Multivariable Cox proportional regression tree-structured analysis of endothelial MMP9 expression and clinicopathologic parameter (ascites), $n = 39$. For analysis, expression of each protein was characterized as high or low based on the median expression value of all samples included in this study.

"malignant omental" microenvironment through an increased expression of not only proteases (i.e., MMP9 and CD) but also VEGFA.

Interestingly, only patients with high endothelial expression of MMP9 coupled with high mesothelial VEGFA or CD or endothelial VEGFA expression had significantly reduced OS. This complements previous *in vitro* data indicating an upstream regulatory function of CD on MMP9 activity that translates to an enhanced endothelial pro-angiogenic potential [32]. Interestingly, CD has been postulated as a mitogenic factor acting on both cancer and endothelial cells independently of its catalytic activity, affecting cell proliferation, angiogenesis, and apoptosis [33]. We postulate that high cancer and mesothelial CD expression might contribute to EOC growth and facilitate a pro-angiogenic omental environment. However, confirmation would require further study.

In conclusion, we have shown increased expression of pro-angiogenic proteases and VEGF in the endothelium and mesothelium in omentum hosting metastatic EOC and that high endothelial

expression of MMP9 together with a presence of malignant ascites predicts poor clinical outcome. We suggest that there is a complex cross-talk between cancer, mesothelial, and endothelial compartments in the omentum with metastases contributing to disease progression and that targeting pro-angiogenic proteases and VEGF in both omental mesothelium and endothelium may be required for optimum treatment of EOC-induced angiogenesis and disease progression.

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Supplementary materials

Table W1. Details of the Primary Mouse Monoclonal Antibodies Used in This Study, Including Preliminary Dilutions Tested on Positive Controls and on Omental Samples Taken from Patients with EOC (cancer) and Control Cystadenoma, and Antigen Recovery Determination (no PT, no pretreatment; HIER ER2 10′/20′, heat-induced epitope retrieval using Bond Epitope Retrieval Solution 2 (EDTA, high pH) for 10 or 20 minutes at 100°C).

Antibody	Dilution and Pretreatment		Control Tissues	Omental Samples
MMP2 (clone 17B11)	1:50	HIER ER2 20′	Inflamed large bowel	Cancer/cystadenoma
	1:100	HIER ER2 20′	Inflamed large bowel	
	1:200	HIER ER2 20′	Inflamed large bowel	
MMP9 (clone 15W2)	1:50	No PT	Liver	Cancer/cystadenoma
	1:100	No PT	Liver	
	1:200	No PT	Liver	
CD (clone C5)	1:50	No PT	Liver	
	1:100	No PT	Liver	Cancer/cystadenoma
	1:200	No PT	Liver	
CL (clone 33/2)	1:3000	HIER ER2 10′	Liver	
	1:5000	HIER ER2 20′	Liver	Cancer/cystadenoma
	1:10000	HIER ER2 20′	Liver	
VEGFA (clone VG-l)	1:200	HIER ER2 20′	Ovary (corpus luteum)	Cancer/cystadenoma
	1:400	HIER ER2 20′	Ovary (corpus luteum)	
	1:800	HIER ER2 20′	Ovary (corpus luteum)	
	1:200	HIER ER2 20′	Angiosarcoma	
	1:400	HIER ER2 20′	Angiosarcoma	
	1:800	HIER ER2 20′	Angiosarcoma	

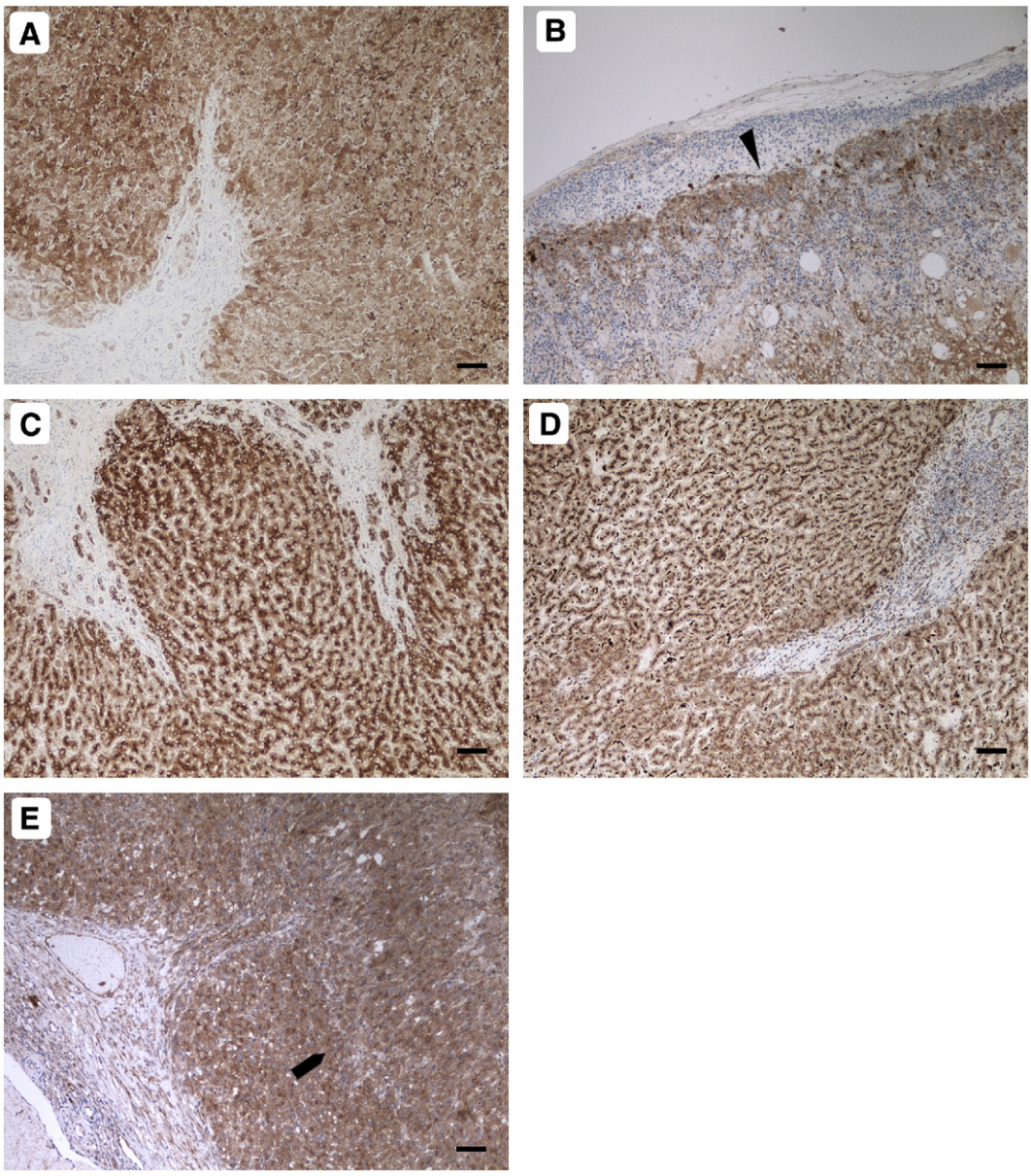

Figure W1. Positive control tissue staining. Four-micrometer sections were stained for expression of MMP9 (A), MMP2 (B), CD (C), CL (D), and VEGF (E) using the Bond Polymer Refine Detection System. Expression of antibodies was determined in the liver (A, C, and D), inflamed large bowel (B), and corpus luteum of the ovary. ▶, inflamed submucosal cells of the large bowel; ▬, corpus luteum. Scale bar, 100 μm.

Table W2. All investigated intercell and intracell type correlations between protein expression in the omentum of all patients studied assessed by Spearman rank correlation test, $n = 39$; r = Spearman rank correlation coefficient (rho), P = Spearman rank correlation P value; $P < .01$ was considered as significant.

Variable	Spearman Rank Correlation Coefficients (r)							
	MMP9 Endothelium	MMP9 Mesothelium	MMP2 Mesothelium	CD Endothelium	CD Mesothelium	CL Endothelium	CL Mesothelium	VEGFA Endothelium
MMP9 endothelium	1.000	–	–	–	–	–	–	–
MMP9 mesothelium	0.551	1.000	–	–	–	–	–	–
MMP2 mesothelium	0.160	0.195	1.000	–	–	–	–	–
CD endothelium	0.158	−0.258	−0.069	1.000	–	–	–	–
CD mesothelium	0.381	0.710	0.197	0.031	1.000	–	–	–
CL endothelium	0.077	0.338	−0.029	0.040	0.163	1.000	–	–
CL mesothelium	0.265	0.601	−0.029	−0.227	0.398	0.479	1.000	–
VEGFA endothelium	0.152	0.418	−0.022	−0.231	0.112	0.501	0.284	1.000
VEGFA mesothelium	0.338	0.653	0.066	0.029	0.516	0.480	0.542	0.569

Variable	P Values							
	MMP9 Endothelium	MMP9 Mesothelium	MMP2 Mesothelium	CD Endothelium	CD Mesothelium	CL Endothelium	CL Mesothelium	VEGFA Endothelium
MMP9 endothelium	1.000	–	–	–	–	–	–	–
MMP9 mesothelium	<.001	1.000	–	–	–	–	–	–
MMP2 mesothelium	.330	.233	1.000	–	–	–	–	–
CD endothelium	.336	.112	.677	1.000	–	–	–	–
CD mesothelium	.017	<.001	.230	.851	1.000	–	–	–
CL endothelium	.643	.036	.859	.810	.321	1.000	–	–
CL mesothelium	.102	<.001	.860	.164	.012	.002	1.000	–
VEGFA endothelium	.355	.008	.894	.157	.497	.001	.080	1.000
VEGFA mesothelium	.035	<.001	.692	.860	<.001	.002	<.001	<.001

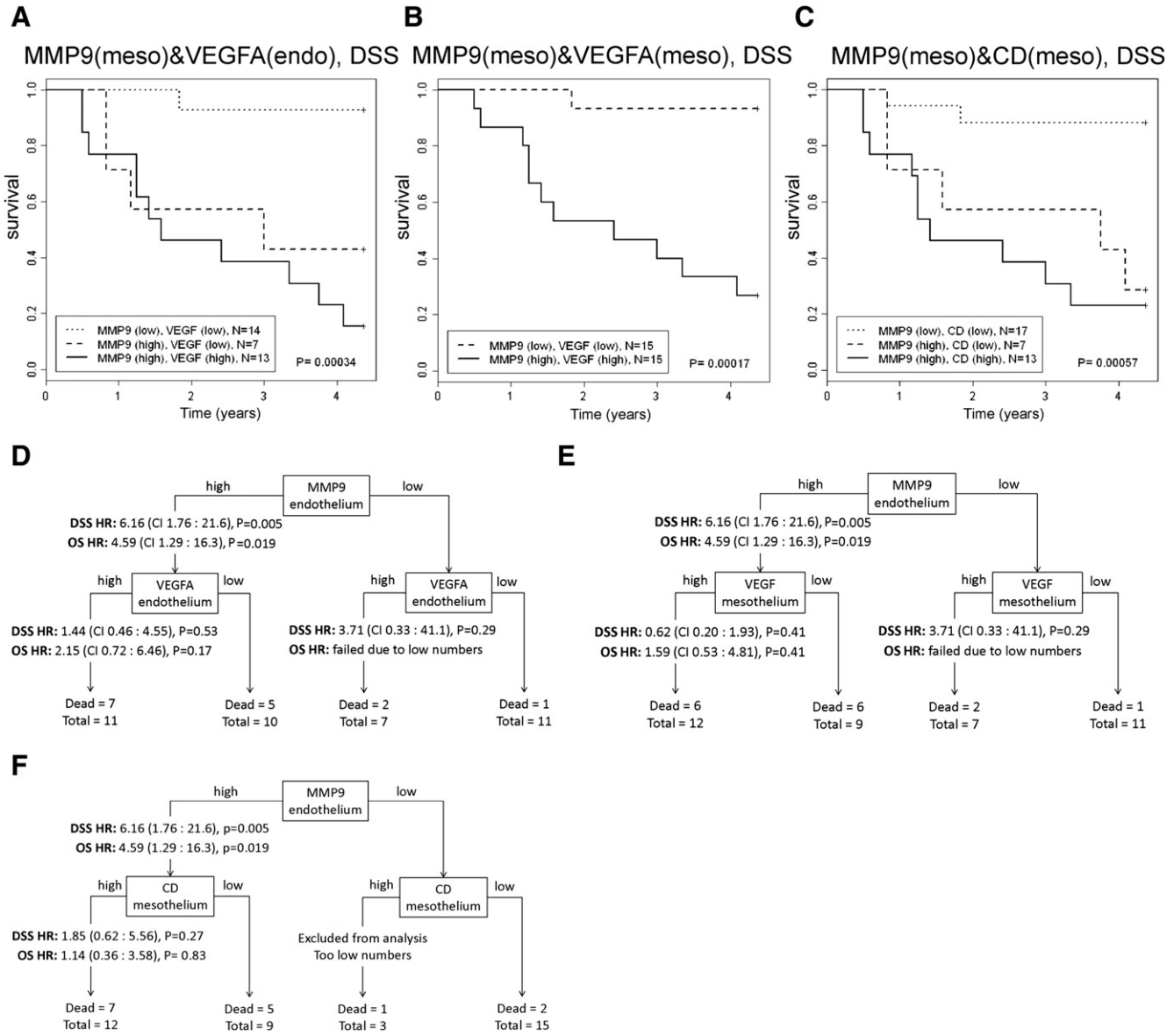

Figure W2. Kaplan-Meier survival curves of DSS for each "MMP9 mesothelial node" of the tree-structured analysis (A–C), $n = 39$; P values were calculated using the log-rank test. Tree-structured hazards ratio analysis of endothelial MMP9 expression with other proteins in endothelium or mesothelium (D–F). Protein expression was characterized as either high or low based on the median expression value of each protein in all samples included in this study.