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Expression levels of vascular endothelial growth factor, matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases 1 and 2 in the plasma of patients with ovarian carcinoma

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Abstract

We measured the levels of the vascular endothelial growth factor (VEGF), matrix metalloproteinases type 2 and type 9 (MMP-2 and MMP-9) and tissue inhibitors of matrix metalloproteinase 1 and 2 (TIMP-1 and TIMP-2) in the plasma of patients with ovarian carcinoma ($n=40$), in other gynaecological pathologies ($n=30$) and in the plasma of healthy volunteers ($n=26$). MMP-2 and MMP-9 (pro and active forms) gelatinolytic activity was measured by zymography. Enzyme-linked immunosorbent assays (ELISA) were used to assay soluble VEGF and TIMPs. Preoperative plasma VEGF levels were significantly higher in patients with ovarian cancer than in healthy volunteers ($P<0.0001$) or patients with a benign gynaecological pathology ($P<0.0001$). The expression of pro-MMP-9 was higher in the plasma of ovarian cancer patients than in the plasma of women with non-malignant disease ($P=0.01$) or healthy women ($P<0.0002$). Pro-MMP-2 was detected in the plasma of ovarian cancer patients, but levels did not differ from those in non-malignant disease or healthy donor samples. Plasma TIMP-1 and TIMP-2 levels were significantly higher in patients with ovarian carcinomas than in healthy volunteers ($P<0.0001$ and $P=0.006$, respectively) or in the patients with a non-malignant pathology ($P<0.0001$ and $P=0.002$, respectively). Sub-group analysis showed that VEGF and pro-MMP-9 were higher in the plasma of patients with serous carcinomas than other histological types. Furthermore, plasma VEGF and pro-MMP-9 levels were higher in the plasma of cancer patients with thrombocytosis. Throughout the study, and in the univariate analysis, no correlation was found between the VEGF, MMP and TIMP levels. Only TIMP-1 was associated with a poor survival and mortality risk.

Keywords: MMP-2; MMP-9; Ovarian carcinoma; TIMP-1; TIMP-2; VEGF

1. Introduction

Ovarian cancer is the most common gynaecological malignancy [1], with a prevalence of 40 cases per 100 000 women aged 50 years and over. Epithelial ovarian cancer has been described as a silent killer because when it is first diagnosed the disease has already spread outside the ovary and pelvis. Only 15% of ovarian cancers are diagnosed at stage I, when cure rates approach 90%. Cure rates at advanced stages are 30–35%. As a result, 50% of these patients die within 5 years [1,2].

The most common route of dissemination of epithelial ovarian cancer into the peritoneal cavity is by exfoliation of malignant cells through the surface of the ovary capsule. The circulation of peritoneal fluid facilitates the dissemination of these cells onto the intraperitoneal surfaces [2]. The early and extensive metastatic dissemination of ovarian cancer suggests that angiogenesis

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is an important event in the progression of this disease [3,4]. The production of growth factors and cytokines and the activation of proteolytic enzymes are responsible for ovarian cancer-associated angiogenesis and tumour dissemination [5]. Their identification, therefore, might have important implications for prognosis and therapy [6,7].

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors in solid tumours [8–10]. The expression of VEGF and its receptors (VEGFR) in ovarian carcinoma has been associated with growth and invasion [6]. High levels of VEGF have been found in serum or plasma and in ascitic fluid of ovarian cancer patients [9] and VEGF levels have been proposed as an additional prognostic factor [11,12]. Indeed VEGF, recognised as a vascular permeability factor, plays an important role in ascites formation. A correlation between ascites volume and VEGF levels has been reported in experimental models [13,14]. Inhibitors of VEGF activity reduce the formation of malignant ascites in human ovarian carcinoma xenograft models [15].

The invasion and metastatic capacity of ovarian cancer cells is related to their ability to degrade the extracellular matrix and components of the basement membrane [16]. The matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases able to degrade components of the extracellular matrix and basement membrane [17]. MMPs are secreted as inactive proenzymes and transformed into active forms after cleavage of a propeptide domain of the molecule [18]. Gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are believed to be vital in the invasion of malignant tumours and angiogenesis. Expression of MMP-2 and MMP-9 is elevated in several human tumours and is related to tumour aggressiveness and overall survival [19,20]. The use of secreted metalloproteases as a serum marker of malignant cancer has also been evaluated [21]. MMP-2 and MMP-9 are expressed in ovarian cancer tissue, ascites and cultured cells [22,23]. Lengyel and colleagues showed that pro-MMP-9, but not active MMP-9 or MMP-2, serves as an independent prognostic factor in FIGO stage III ovarian cancer. Davidson and colleagues suggested that MMP-2 and MMP-9 were predictors of poor survival in advanced ovarian carcinoma [24]. Experimental studies have shown that MMP-2 and MMP-9 are important in favouring the invasion and metastasis of ovarian cancer cells, and animals bearing ovarian carcinoma xenografts treated with MMP inhibitors had less tumour burden and ascites formation, and a longer survival [25].

MMPs work in concert with tissue inhibitors of the matrix metalloproteinase (TIMPs), a family of endogenous proteins that consists of four homologous members, TIMPs 1–4 [26]. By inhibiting active MMPs, TIMPs inhibit cell invasion *in vitro* and tumorigenesis and metastasis *in vivo* [27]. TIMPs can also associate with proMMPs. TIMP-1 selectively binds proMMP-9. TIMP-2 associates with proMMP-2 in a crucial step in the cell-mediated activation of MMP-2, through the formation of a TIMP-2/MMP-2/MT1-MMP complex (membrane type-1 MMP) [28]. TIMPs have been studied in the plasma and tumour tissue of patients with various cancers [29]. There appears to be a positive correlation between high TIMP levels and a poor prognosis in human malignancies [19,30].

Our study was designed to assay VEGF, MMP-2 and MMP-9 and TIMP-1 and TIMP-2 levels in plasma and ascitic fluid of patients with ovarian cancer. An initial analysis of the correlation with clinical parameters and outcome is described. MMP-2 and MMP-9, their inhibitors and VEGF, were chosen because they play an important role in tumour metastasis and angiogenesis.

2. Patients and methods

Peripheral venous blood samples were collected from 96 women: 26 healthy women, 30 with non-malignant gynecological disease, and 40 with ovarian carcinoma diagnosed at the Department of Gynecological Oncology, S. Gerardo Hospital, Monza, Italy, between January 1994 and June 2001. The local ethics committee approved peripheral venipuncture. Informed consent for the taking of venous blood was obtained from all of the patients. Healthy volunteers gave their permission verbally. The main clinical and pathological data, including age, histological type, grade and residual tumour mass are shown in Tables 1 and 2.

Criteria for inclusion in the study were: histological diagnosis of ovarian carcinoma, untreated patients,

Table 1
Main clinical–pathological characteristics and haematological parameters

Patients	Mean					
	Number of cases	Age in years (range)	Hb (g/l)	WBC (10^9/l)	RBC (10^{12}/l)	Plt (10^9/l)
Ovarian cancer	40	59 (29–82)	11.3	8.6	4.2	276.0
Non-malignant	30	41 (22–71)	13.1	6.5	4.4	199.5
Healthy individuals	26	44 (23–68)	13.2	7.0	4.5	228.5

Hb, haemoglobin; WBE, White Blood Cells; RBC, Red Blood Cells; Plt, platelets.

follow-up information and no history of other invasive cancer. Histological diagnosis and clinical stages were determined according to the International Federation of Gynecology and Obstetrics (FIGO) classification [31]. After surgery, patients were enrolled in different therapy protocols. The patients in the non-malignant group had ovarian cysts, leiomyoma, vagina prolapse and endometriosis (Table 2). The healthy volunteers had no concomitant illnesses.

2.1. Sample collection

Peripheral venous blood samples were collected on the day of surgery in sterile plastic tubes containing 3.8% tri-sodium citrate dihydrate, final volume 1/10, and immediately centrifuged at 3000 rotations per minute (rpm) 3000 rpm for 20 min. Plasma was aliquoted and stored at −80 °C until further processing.

2.2. ELISA

VEGF was measured by an enzyme-linked immunosorbent assay (ELISA) (Quantikine Human VEGF Immunoassay; R&D Systems, Minneapolis, USA) that detects soluble $VEGF_{121}$ and $VEGF_{165}$. The minimum detectable dose is 9.0 pg/ml (samples <9 pg/ml were considered to be 0 pg/ml). TIMP-1 was measured by ELISA (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), which recognises both TIMP-1 and MMP-complexed human TIMP-1. The sensitivity is 1.25 ng/ml. TIMP-2 was detected using the Biotrak TIMP-2 human ELISA (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Free and MMP-complexed TIMP-2 are detected in this ELISA. Sensitivity is 3.0 ng/ml.

Assays were conducted according to the manufacturer's directions. Standards and samples were tested in duplicate. Results were calculated from a standard curve, generated by a four-parameter logistic curve-fit, and expressed in ng/ml for TIMPs and pg/ml for VEGF.

Table 2
Histopathological characteristics of the patients

	Patients	
	N	(%)
Ovarian cancer[a]		
Histological grade		
G1-G2	6	(15)
G3	34	(85)
Histological type		
Serous	24	(60)
Endometrioid	10	(25)
Other	6	(15)
FIGO stage		
I,II	9	(23)
III,IV	31	(77)
Residual disease		
⩽2 cm	16	(40)
>2 cm	24	(60)
Platelets		
<400 (10^9/l)	31	(77)
⩾400 (10^9/l)	9	(23)
Non-malignant[b] gynaecological diseases		
Ovarian cyst	11	(37)
Endometriosis	5	(17)
Leiomyoma	7	(23)
Vaginal prolapse	7	(23)

G, grade; n, number; FIGO, International Federation of Gynecology and Obsterics.

[a] Histopathological variables of patients with ovarian carcinoma ($n=40$ patients).

[b] Histological type of non-malignant gynaecological diseases ($n=30$ patients).

2.3. Gelatin zymography

Gelatinolytic activity of MMPs was analysed using gelatin zymography. Briefly, 100 μg protein (determined by the Bio-Rad Protein Assay) were combined with 70 mM Tris–HCl pH 6.8, 10% glycerol (v/v), 2% sodium dodecyl sulphate (SDS) (w/v) and 0.01% bromophenol blue (w/v). The mixture was then applied to 10% polyacrylamide gels co-polymerised with 1 mg/ml gelatin. After electrophoresis, the gels were washed three times for 20 min with 2.5% Triton X-100 at room temperature and incubated overnight at 37 °C in 50 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij-35. The gels were then stained with 0.5% Coomassie blue (w/v) in 25% methanol (v/v) and 10% acetic acid (v/v), and de-stained in the same solution without the Coomassie blue.

The supernatant of WM983A melanoma cells activated with *p*-aminophenylmercuric acetate (APMA) was used as a reference standard for human pro-MMP-9, pro-MMP-2 and their activated forms. Plasma from a healthy volunteer was used in different zymographies as a standard to compare the amounts of MMP-2 and MMP-9 in the different gels. Gel images were acquired with a Duoscan T1200 scanner (AGFA), and the levels of MMPs (pro- and activated forms) were quantified by the Image-Pro Plus 4.1 program. The results are expressed in arbitrary units (IOD).

2.4. Statistical analysis

The relationships between the plasma values were assessed using Kendall τ–*b* partial correlation coefficients, adjusting for group (healthy, non-malignant and carcinomas). The distributions in each group and the clinical characteristics were compared with the Kruskall–Wallis test for continuous variables or the Mantel–Haenszel test for discrete ones. Only in cases of statistically sig-

nificant results, were pairwise comparisons done, using either the Mann–Whitney U test or the Mantel–Haenszel test, with the Bonferroni Method for multiple comparisons. Survival curves were calculated with the Kaplan–Meier method. The relationships between the factors analysed and survival were investigated by univariate analysis. Then variables found to be significantly associated with survival were included in a multivariate Cox proportional hazards regression model.

Unless otherwise specified, significance was set at 0.05, for a two-sided test. The Statistical Analysis System (SAS) software package (version 8.1, SAS Institute Cary, NC, USA) was used.

3. Results

3.1. VEGF

Plasma levels of VEGF were compared in patients with ovarian cancer, patients with non-malignant disease and healthy volunteers (Fig. 1). The median VEGF concentration was 109.1 pg/ml (range 0–2845.2 pg/ml) for ovarian carcinoma patients and 18.8 pg/ml (0–121.3 pg/ml) for non-malignant gynaecological patients. VEGF was also detectable in healthy individuals, but at lower levels (median 0 pg/ml; range 0–48.4 pg/ml). VEGF levels were significantly higher ($P<0.0001$) in the ovarian carcinoma patients than in the other two groups. VEGF levels were higher in plasma of non-malignant gynaecological patients than in the healthy donors ($P<0.0017$).

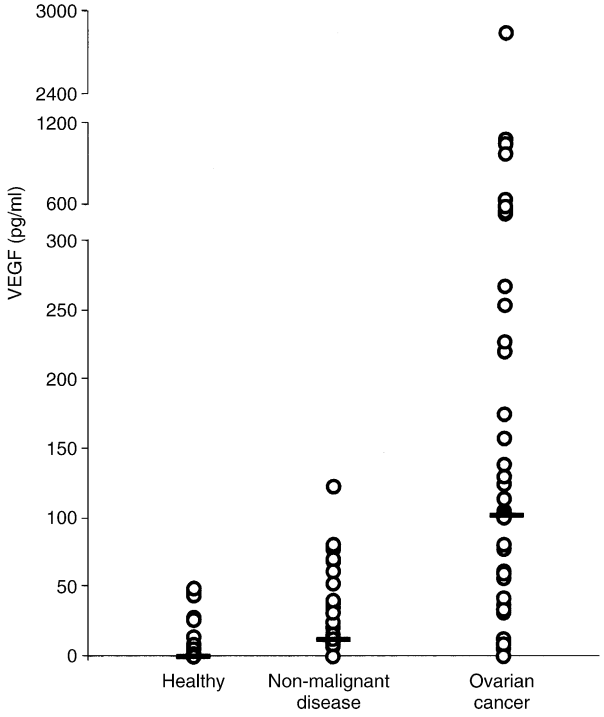

Fig. 1. Vascular endothelial growth factor (VEGF) levels. Preoperative plasma VEGF levels (pg/ml) in patients with ovarian carcinoma ($n=40$), non-malignant gynaecological disease ($n=30$) and healthy volunteers ($n=26$). Bars indicate the median.

3.2. MMP-2 and MMP-9

The expression of MMP-2 and MMP-9 (pro- and activated forms) was measured by zymography in the plasma of ovarian cancer patients, women with non-malignant disease and healthy volunteers (Fig. 2a and b). A representative gel is shown in Fig. 2c. The expression of pro-MMP-9 in the plasma of ovarian carcinoma patients (median 4.62 arbitrary units of IOD, range 0.15–34) was significantly higher than in healthy volunteers (median 1.2 arbitrary units of IOD, range 0.15–3.04, $P<0.0002$), and in women with non-malignant gynaecological disease (median 2.09 arbitrary units of IOD, range 0.101–5.71; $P=0.0102$) (Fig. 2a). The levels of MMP-9 in non-malignant disease were higher than in the healthy donors ($P<0.0024$). 45% of ovarian cancer patients and 33% of non-malignant disease had the activated form of MMP-9 in their plasma, but this was never found in healthy volunteers (healthy versus non-malignant $P<0.0013$; healthy versus ovarian cancer patients $P<0.0001$).

Pro-MMP-2 was detectable in the plasma of all the individuals, with no differences in the level of expression in the three groups (Fig. 2b). The activated form of MMP-2 was detectable only in 12.5% of the cancer patients.

3.3. TIMP-1 and TIMP-2

TIMP-1 and TIMP-2 levels in the plasma of the ovarian cancer patients were compared with women with non-malignant disease and healthy volunteers (Fig. 3). TIMP-1 levels were significantly higher ($P<0.0001$) in ovarian carcinoma patients (median 1343 ng/ml, range 524.3–2949 ng/ml) than in healthy volunteers (median 610 ng/ml, range 395–892 ng/ml) and women with non-malignant disease (median 488.8 ng/ml, range 294–2024 ng/ml) (Fig. 3a).

TIMP-2 levels were higher in the plasma of ovarian carcinoma patients (median 29.8 ng/ml, range 9.1–198 ng/ml) than in patients with non-malignant disease (median 20.9 ng/ml, range 9.2–222.8 ng/ml, $P<0.0022$) and healthy volunteers (median 22 ng/ml, range 2–245 ng/ml, $P=0.006$) (Fig. 3b).

3.4. Correlation with clinical and histological parameters

The τ–b Kendall model analysis showed no correlation between the plasma levels of VEGF, MMPs

(pro- and activated forms) and TIMPs in patients with ovarian carcinoma. Table 3 shows the levels of VEGF, MMPs and TIMPs in the plasma in relation to clinical parameters such as histological type, stage, platelets and residual disease.

3.4.1. VEGF, MMPs and TIMPs and histological type

VEGF levels in the plasma were higher in patients with serous cancer than other histological types, although the difference did not reach significance ($P = 0.060$). Preoperative plasma levels of pro-MMP-2

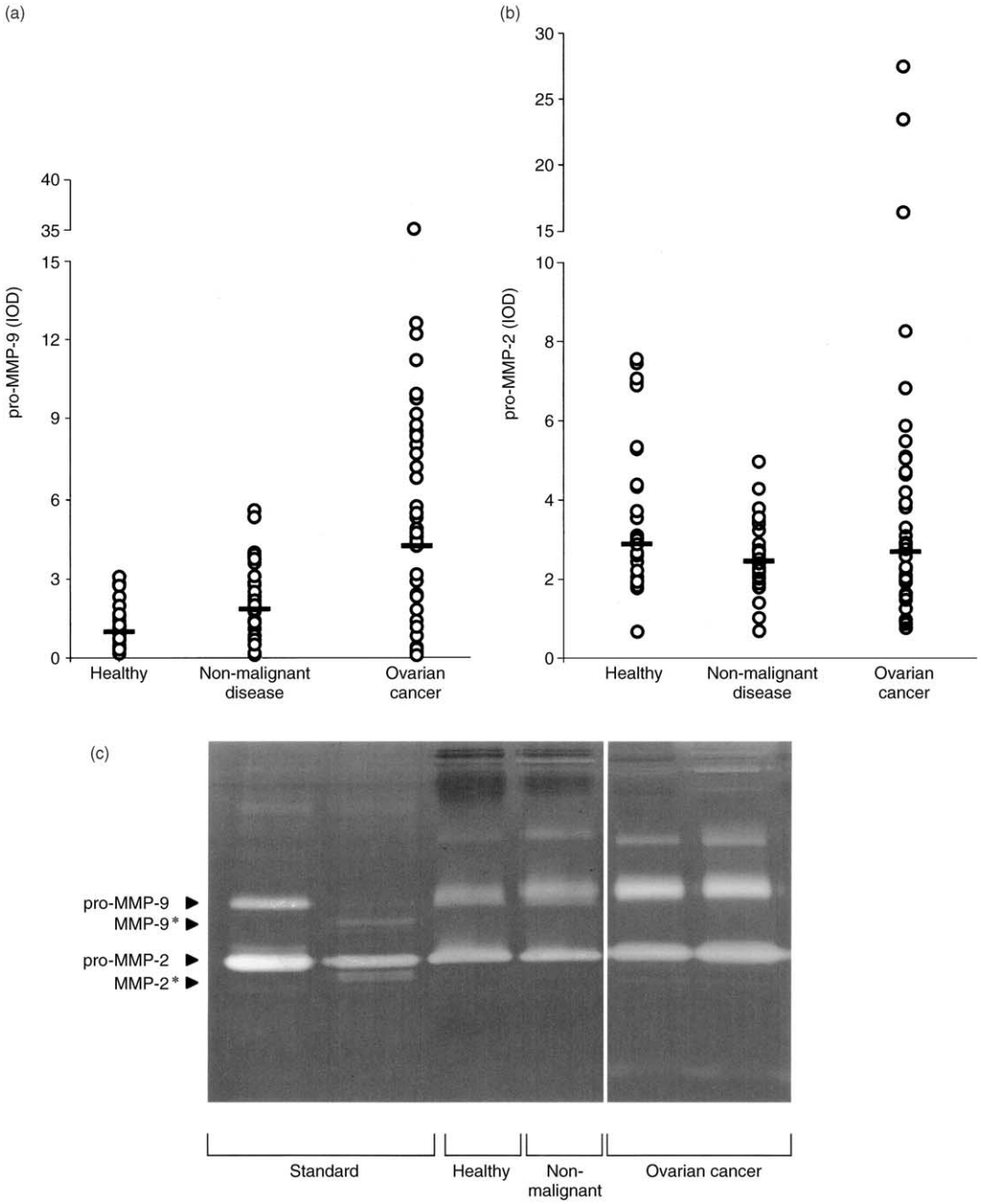

Fig. 2. pro-matrix metalloproteinases (MMP)-9 and pro-MMP-2 expression. Expression of pro-MMP-9 (a) and pro-MMP-2 (b) in plasma of patients with ovarian carcinoma ($n = 40$), women with non-malignant gynaecological disease ($n = 30$) and healthy volunteers ($n = 26$). Data are expressed as arbitrary units (IOD). Bars indicate the median. A representative gelatin zymography (c) of plasma from 2 patients with ovarian cancer and non-malignant gynaecological disease is shown. Supernatant of WM983A melanoma cells activated with *p*-aminophenylmercuric acetate (APMA) was used as a reference standard for human pro-MMP-9, pro-MMP-2 and their activated forms. Plasma from healthy volunteer is also shown. Pro-MMP-2 (matrix metalloproteinases-2); MMP-2* (matrix metalloproteinases-2 activated form); pro-MMP-9 (matrix metalloproteinases-9); MMP-9* (matrix metalloproteinases-9 activated form).

and pro-MMP-9 were significantly higher in serous ovarian carcinoma than in patients with other histological types ($P=0.007$ and $P=0.002$ for pro-MMP-2 and pro-MMP-9, respectively). No correlation was found with the levels of TIMPs (Table 3).

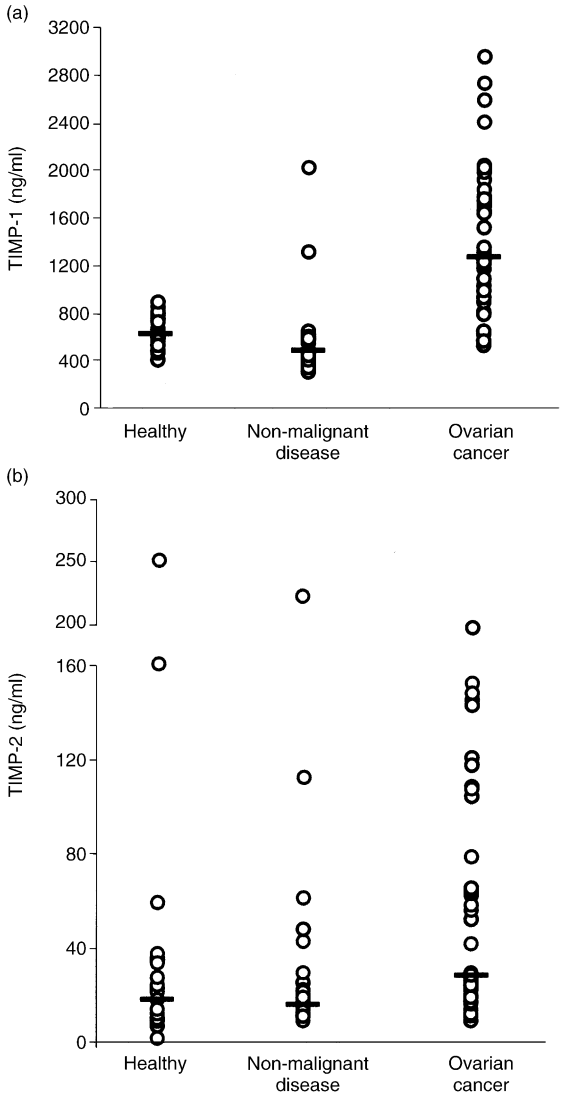

Fig. 3. Tissue inhibitors of matrix metalloproteinases (TIMP)-1 and TIMP-2 levels. Preoperative plasma TIMP-1 (a) and TIMP-2 (b) levels in patients with ovarian carcinoma ($n=40$), non-malignant gynaecological disease ($n=30$) and healthy volunteers ($n=26$). Bars indicate the median.

3.4.2. VEGF, MMPs and TIMPs and FIGO stage

The expression of active MMP-9 in plasma was higher in patients with advanced disease (stage III-IV) than in stages I or II, although the difference did not reach significance ($P=0.08$). No association was found between VEGF or TIMPs levels and FIGO stage (Table 3).

3.4.3. VEGF, MMPs and TIMPs and residual disease

Residual disease did not correlate with the level of VEGF, MMPs or TIMPs, with the exception of total MMP-9, namely pro-MMP-9 + active MMP-9 (data not shown), which was higher ($P=0.09$) in patients with residual disease greater than 2 cm.

3.4.4. VEGF, MMPs and TIMPs and platelets

Plasma levels of VEGF and pro-MMP-9 were higher in cancer patients with thrombocytosis than patients with a low platelet count ($P=0.046$ and $P=0.012$, respectively). No association was observed with the expression of MMP-2 or the level of TIMPs (Table 3).

3.5. Correlation with survival

At a median follow-up time of 19.3 months, 17 patients have died. At univariate analysis, only TIMP-1 levels in the plasma were prognostic for survival. Poor survival and a high mortality risk were associated with increasing plasma levels of TIMP-1 (hazard ratio 2.6, 95% CI 1.2–5.8 for a difference of 1000 ng/ml of TIMP-1, $P=0.0173$). No association was found between VEGF, MMP-9, MMP-2 and TIMP-2 and survival.

4. Discussion

Ovarian cancer almost inevitably presents with an extension of disease outside of the pelvis, with ascites and omental tumour implants. Pleural effusion or swelling of the inguinal or axillary lymph nodes may be a later event. Malignant ascitic fluid accumulates because of the hyperpermeability of microvessels lining the peritoneal cavity, the angiogenesis associated with marked peritoneal neovascularisation, and a weakened lymphatic recovery system [13,14].

Table 3
VEGF, TIMPs and MMPs and clinical parameters in plasma

Parameter	VEGF [a]	TIMP-1[a]	TIMP-2[a]	pro-MMP-9[a]	MMP-9[a] (active)	pro-MMP-2[a]	MMP-2[a] (active)
Histological type (serous versus others)	0.06	0.312	0.658	0.002[b]	0.848	0.007 [b]	0.829
Stage of disease (I-II versus III-IV)	0.837	0.955	0.445	0.268	0.08	0.588	0.256
Residual disease ($>$ 2 cm versus $\leqslant$2 cm)	0.956	0.692	0.898	0.111	0.115	0.536	0.237
Platelets ($\times 10^9$/l) ($\geqslant$400 versus $\leqslant$400)	0.046	0.603	0.334	0.012[c]	0.406	0.119	0.275

[a] Pairwise comparisons were done using the Cochran–Mantel–Haenszel test; $P<0.0167$ was significant.
[b] Significant correlation between pro-MMP-9 and pro-MMP-2 and serous histological type.
[c] Significant correlation between pro-MMP-9 and thrombocytosis.

One of the emerging clinical applications of research is the use of angiogenesis-related parameters in prognosis [32]. Measuring circulating proteins is a convenient and non-invasive method that is potentially applicable to any patient.

In this study, we measured the levels of VEGF, MMP-2 and MMP-9 and TIMP-1 and TIMP-2 in the plasma of ovarian cancer patients, in comparison with healthy volunteers and women with non-malignant gynaecological disease and evaluated the correlation between the levels of these molecules and some clinical parameters.

Preoperative plasma VEGF levels were significantly higher in ovarian carcinoma patients than in healthy volunteers or in patients with non-malignant gynaecological diseases. This is in agreement with previous reports of higher VEGF levels in the blood of ovarian cancer patients than in the blood of those with tumours of a low malignant potential, or benign neoplasms [33]. Prognostic correlations between a high serum VEGF level and survival in patients with ovarian carcinoma have given contrasting results [7,34]. We did not found any relationship between VEGF levels in the plasma and the stage of the disease, or with survival, but the limited number of patients might account for these results.

VEGF plays an important role in the formation of ascites [14]. VEGF concentrations were high in the ascitic fluid of patients with epithelial ovarian carcinoma, and preoperative serum VEGF levels were always significantly elevated in the presence of ascites [8,33]. We too found elevated levels of VEGF in ovarian carcinoma patients' ascites (data not shown). These findings suggest that VEGF could be useful as a therapeutic target for this type of tumour. Ascites was reduced in mice bearing ovarian carcinomas treated with monoclonal antibodies against VEGF or inhibitors of the VEGFR tyrosine kinase [15].

Levels of VEGF in plasma of healthy individuals were detectable and quite stable. They were higher in patients with non-malignant gynaecological disease. We did not find that the VEGF level was related to age or haematological parameters. The differences in individual VEGF levels may depend on various factors, including the host response and hormonal status. Host cells such as lymphocytes, macrophages and platelets express VEGF [35] and its expression is increased in patients with inflammatory disease [4]. It is possible that in healthy individuals and patients with non-malignant disease, VEGF in the circulation derives from these cells.

We found high levels of VEGF in the plasma of patients with thrombocytosis, although the rise was not significant. Correlation studies between VEGF in blood, plasma or serum, and platelet numbers have given contrasting results [10,36,37]. Patients with cancer have an increased platelet turnover and patients with thrombocytosis have a poor prognosis [38,39]. Platelets are activated in the tumour vasculature as a consequence of 'blood stasis' and platelet–tumour vasculature interactions. Their degranulation induces the release of potent angiogenesis stimulators in the circulation [40,41]. Therefore, high levels of VEGF and a high platelet count could contribute to the progression of cancer and tumour-associated angiogenesis in thrombocytotic patients.

Expression of MMP-2 and MMP-9 is elevated in various cancer tissues. Their expression has also been detected in specimens of ovarian carcinoma [16]. In this study, we found elevated levels of MMP-9 (pro- and active forms), but not MMP-2, in the plasma of ovarian cancer patients.

The expression of pro-MMP-9 correlated strongly with the presence of tumour as its level was significantly higher than in patients with non-malignant disease. This is in line with the report by Lengyel and colleagues that pro-MMP-9, but not active MMP-2 or MMP-9, is a prognostic factor in ovarian cancer [23]. However, our analysis failed to find a significant correlation between the plasma levels of either form of MMP-9 and advanced disease or residual disease, with the exception of a significant correlation between plasma pro-MMP-9 and the serous histological type. Active MMP-9 was elevated in 45% of the patients, but 35% of those with non-malignant disease showed the activated form as well.

MMP-9 activation may reflect the presence of host-infiltrating cells. Several studies have suggested that host cells may be responsible for the increase of MMP-9 and the tumour environment may contribute to their stimulation through the production of regulatory factors, including cytokines [42]. A recent article proposed that MMP-9, derived from tumour infiltrating macrophages, promotes angiogenesis and growth of ovarian cancer xenografted in nude mice [43].

We also found a significant correlation between plasma pro-MMP-9 and thrombocytosis, meaning that platelets themselves might act as a source of MMP-9 in the blood. This is supported by other studies showing that MMP-9 is secreted by megakaryocytes and released by thrombin stimulation [44]. Considered in the light of the correlation between VEGF levels and thrombocytosis, this shows the importance of platelet activation and degranulation in releasing molecules involved in angiogenesis and matrix degradation.

We did not find particularly high levels of MMP-2 in the plasma, although MMP-2 levels were high in the ascitic fluid (data not shown) of the same patients. Ovarian cancer cells might use MMPs to detach from surface epithelia and invade the organs peritoneal cavity [45]. The contribution of MMP-2 and MMP-9 to the invasive phenotype appears to be very complex and

differs in carcinomas of different origin [46]. Further studies are needed to clarify their role in the malignant phenotype of ovarian tumours.

We found higher levels of TIMP-1, and to a lesser extent TIMP-2, in the plasma of patients with ovarian carcinoma than in controls. Cox model analysis indicated that high TIMP-1 levels in the plasma were associated with a poor survival ($P < 0.017$). Our findings are in agreement with recent clinical studies that found high levels of TIMPs correlated with a poor prognosis in different types of carcinomas [19,47,48]. We also found high levels of TIMP-1 in the ascitic fluid of these patients (data not shown). We could not find any correlation between TIMPs levels and advanced stage, histological type or residual disease. Although we are not able to identify the source of TIMP-1, our data suggest that this protein released in the plasma might serve as a prognostic factor, but further investigations with more patients are needed to confirm this. All together these observations suggest a complex role for these inhibitors in tumour progression [28].

To conclude, we found levels of VEGF, TIMP-1 and TIMP-2 and MMP-9 and MMP-2 (pro- and active forms) in the plasma of ovarian carcinoma patients. Only VEGF, pro-MMP-9 and TIMP-1 strongly correlated with the presence of tumour as their levels were significantly higher than in patients with non-malignant gynaecological disease or healthy women. No correlation was found between the levels of VEGF, gelatinase and their inhibitors, or with tumour stage and grading. Larger numbers of patients will be necessary to establish whether high levels of these molecules are predictive of a more aggressive tumour. VEGF and MMPs/TIMPs are highly expressed in ovarian cancer and their presence suggests that targeting MMP or VEGF might be useful approach to control angiogenesis, tumour dissemination and fluid accumulation in these cancers.

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