

Matrix Metalloproteinase-2 (MMP-2) and -9 (MMP-9) and their Tissue Inhibitors (TIMP-1 and TIMP-2) in Differential Diagnosis Between Low Malignant Potential (LMP) and Malignant Ovarian Tumours

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Abstract. *Background:* Matrix metalloproteinase-2 (MMP-2) (gelatinase A) and MMP-9 (gelatinase B) have the ability to degrade several extracellular matrix components. This study aimed to evaluate whether matrix metalloproteinases (MMP-2, MMP-9, MMP-2-TIMP-2 complex) or their tissue inhibitors (TIMP-1, TIMP-2) could be used as preoperative serum markers in differentiating between low malignant potential (LMP) and malignant ovarian tumours. *Patients and Methods:* The study population consisted of 61 patients with ovarian neoplasms (28 benign, 11 LMP and 22 malignant). MMP-2, MMP-9, MMP-2-TIMP-2 complex, TIMP-1 and TIMP-2 were analysed from serum samples using enzyme-linked immunoassay (ELISA). *Results:* Serum TIMP-1 values significantly increased from benign (median 250 µg/l, range 137-616 µg/l) to LMP (median 357 µg/l, range 63-587 µg/l) and further to malignant (median 443 µg/l, range 199-983 µg/l) ovarian neoplasms ($p < 0.001$). There was a significant difference in the ratios of TIMP-1 to MMP-2 and TIMP-1 to MMP-2-TIMP-2 complex between the patients with benign vs. malignant and an LMP vs. malignant tumour. *Conclusion:* The value of circulating TIMP-1 and the ratios of TIMP-1 to MMP-2 and TIMP-1 to MMP-2-TIMP-2 complex may be valuable for differentiating between LMP and malignant ovarian tumours.

Ovarian tumours of low malignant potential (LMP), also called borderline ovarian tumours, account for about 10% to

15% of all epithelial ovarian malignancies (1). The most important criterion for LMP ovarian tumour is the lack of invasion. The overall survival of patients with an LMP tumour is significantly better compared to those with a malignant ovarian tumour. Preoperative differential diagnosis between LMP and malignant ovarian tumours is often difficult. At least in advanced ovarian cancer, the most significant factor that predicts survival is the presence of a gynaecological oncologist at the operation (2). Therefore, it would be useful to differentiate between LMP and malignant ovarian tumours before surgery in order to choose the hospital where the surgery is performed. LMP should also be operated on by a gynaecological oncologist since the presence of implants is relevant for correct staining.

Greatly enhanced synthesis of fibril collagens is a characteristic of ovarian malignancy, as is the simultaneously enhanced breakdown of soft tissue and its collagenous framework (3, 4). Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent enzymes, which have the ability to degrade these components. MMP-2 (gelatinase A, 72 kDa type IV collagenase) and MMP-9 (gelatinase B, 92 kDa type IV collagenase) have the ability to degrade several extracellular matrix components, such as type IV collagen, a major component of the basement membrane (5). Four members of the tissue inhibitor of metalloproteinase (TIMP) family have been characterised so far (TIMPs 1-4). TIMPs participate in cancer progression and have been suggested to have a multifunctional role in cancer growth (6).

Our previous study (7) showed that the tissue expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 increased from benign to LMP and further to malignant ovarian tumours. This study aimed to determine whether circulating metalloproteinases (MMP-2, MMP-2-TIMP-2 complex and MMP-9) or their inhibitors (TIMP-1 and TIMP-2) could be potential preoperative serum markers in differentiating between LMP and malignant ovarian tumours.

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Patients and Methods

The study population consisted of 61 patients with ovarian neoplasms (Table I). All the patients were treated in the Department of Obstetrics and Gynaecology, University of Oulu, Finland, during the years 1988-1998. Venous blood samples were collected before surgery and stored at -20°C until assayed. The local ethics committee approved the study.

All patient groups included both pre- and postmenopausal women. The LMP and malignant tumours were staged according to the International Federation of Gynaecology and Obstetrics (FIGO) classification. In the malignant group, 8 of the tumours were grade I, 4 were grade II and 10 were grade III. FIGO staging was: stage I: 8, stage II: 3, stage III: 9 and stage IV: 2 patients. Surgery was performed through a vertical midline incision. Total abdominal hysterectomy, bilateral salpingo-oophorectomy and omental resection were performed on all patients with a malignant ovarian tumour. Lymph node biopsies were performed on 19 patients. Cisplatin-based chemotherapy was used as the main postoperative treatment in the malignant cases.

Assay for the immunoreactive protein of MMP-2, MMP-2-TIMP-2 complex, MMP-9, TIMP-1 and TIMP-2. The immunoreactive proteins for MMP-2, MMP-2-TIMP-2 complex, MMP-9, TIMP-1 and TIMP-2 were assayed from the sera of the patients with benign, LMP and malignant ovarian tumours using enzyme-linked immunoassay (ELISA). The quantification of the total protein of MMP-2, MMP-2-TIMP-2 complex, MMP-9, TIMP-1 or TIMP-2 was performed using standard protocols (8). Briefly, the ELISAs were performed on EIA/RIA 8-well stripes (Corning Incorporated, Corning, New York, USA). A polyclonal antibody produced in chicken against each of the analytes was used as a secondary antibody. O-phenylenediamine dihydrochloride (OPD) (Sigma, Steinheim, Germany) was used to visualize the peroxidase label. Colour formation was measured on 450 nm (Anthos 2001 microplate reader), and calculations were done using the Windows-based control and evaluation software for Rosys Anthos microplate readers (Anthos labtec instruments, Wals, Austria).

An ELISA system (9), which measures both the free form of MMP-2 and the complex form of MMP-2-TIMP-2, was used for serum MMP-2 (code RPN2617, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) measurements. The monoclonal antibody against MMP-2-TIMP-2 recognises the soluble MMP-2-TIMP-2 complex. The monoclonal antibody against MMP-9 (code Ge-213) recognises both free MMP-9 and that bound to its inhibitor TIMP-1. The monoclonal antibody against TIMP-1 (code DB-102D1) recognises both free TIMP-1 and that complexed with MMP-9. It does not cross-react with TIMP-2. The monoclonal antibody against TIMP-2 (code T2-101) recognises both free TIMP-2 and that complexed with MMP-2.

Statistical analysis. Statistical analysis was performed with an SPSS (v. 11.5) for Windows (SPSS Inc, Chicago, Ill. USA) software package. The Kruskal-Wallis test and the Mann-Whitney *U*-test were used to investigate the significance of the differences between medians. A box plot and whiskers technique was used to present the distribution of the markers. Receiver operator characteristic curves (ROCs) were used to determine the accuracy of TIMP-1 concentration and the ratios of TIMP-1 to MMP-2, and TIMP-1 to MMP-2-TIMP-2 complex as discriminators between LMP and

Table I. Patient characteristics.

Characteristics	Benign tumour (n=11)	LMP tumour (n=22)	Malignant tumour (n=28)
Mean age, years ¹	50 (20-79)	55 (20-79)	53 (43-69)
BMI, kg/m ² , ¹	25 (20-34)	25 (20-34)	25 (21-33)
Histology			
Serous	9	4	14
Mucinous	9	6	5
Others ²	10	1	3

LMP=low malignant potential; n=number of patients; ¹*p*-value for the difference between the groups was not significant; ²benign: simple cysts 4, corpus luteum cysts 3, dermoid cysts 3; LMP: Brenner tumour 1; malignant: endometrioid adenocarcinomas 3.

malignant ovarian tumours over a range of cut-off points. A *p*-value <0.05 was considered statistically significant.

Results

The median levels of serum MMP-2 and MMP-2-TIMP-2 complex were higher in the patients with a LMP ovarian tumour compared to those with a malignant tumour (Table II, Figure 1). No such difference was seen in the serum MMP-9 concentrations between the groups (Table II).

Serum TIMP-1 seemed to discriminate between benign, LMP and malignant tumours better than the other markers evaluated here. The serum TIMP-1 level was highest in the patients with a malignant ovarian tumour and lowest in the patients with benign ovarian neoplasms (Table II, Figure 2). There was a significant difference in the serum TIMP-1 concentrations between the groups of benign and malignant and LMP and malignant tumours but not between the benign and LMP groups. In contrast to serum TIMP-1, there was no significant difference in the serum TIMP-2 levels between the patient groups (Table II). None of the markers correlated with the stage or grade of the malignant tumours.

Due to the differences in behaviour of serum TIMP-1, MMP-2 and MMP-2-TIMP-2 complex between benign, LMP and malignant tumours, we decided to find out whether the ratio of TIMP-1 to MMP-2 or TIMP-1 to MMP-2-TIMP-2 complex could discriminate even better between the LMP patients and those with malignant tumours. The ratio of TIMP-1 to MMP-2 and TIMP-1 to MMP-2-TIMP-2 complex was shown to be significantly higher in the patients with a malignant tumour than in those with a LMP tumour (Figure 3). A significant difference was also found in the ratio of TIMP-1 to MMP-2 and TIMP-2 to MMP-2-TIMP-2 complex when benign and malignant tumours were compared (Figure 3).

ROC curves were used to calculate the area under the curve (AUC) values. When LMP and malignant ovarian tumours

Table II. Serum levels of MMP-2, MMP-2-TIMP-2 complex, MMP-9, TIMP-1 and TIMP-2 in patients with benign, LMP and malignant ovarian tumours.

Serum marker	Benign tumour	LMP tumour	Malignant tumour	P-value ¹
MMP-2 ² (µg/l)	822 (381-2140)	1080 (778-1360)	716 (175-1660)	0.009
MMP-2-TIMP-2 ² (µg/l)	629 (366-1210)	894 (414-1410)	666 (344-1050)	0.083
MMP-9 (µg/l)	177 (55-661)	177 (58-387)	287 (42-1010)	0.124
TIMP-1 ³ (µg/l)	250 (137-616)	357 (63-587)	443 (199-983)	<0.001
TIMP-2 (µg/l)	139 (62-276)	130 (108-201)	147 (81-205)	0.320

The results are expressed as median (range). ¹Kruskal-Wallis test; ²LMP vs. malignant, $p=0.036$ (Mann-Whitney *U*-test); ³benign vs. LMP, $p=0.045$; benign vs. malignant, $p<0.001$; LMP vs. malignant $p=0.032$ (Mann-Whitney *U*-test).

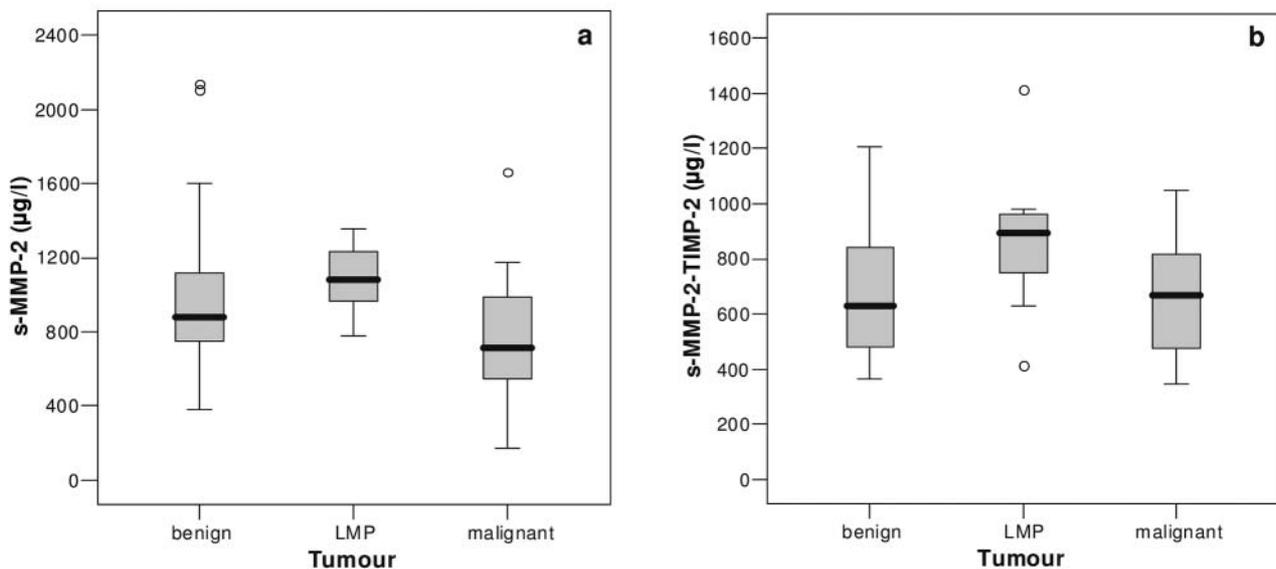


Figure 1. Box plot and whiskers of serum MMP-2 (a) and MMP-2-TIMP-2 complex (b). The boxes include 50% of the values, and the horizontal lines indicate the median. The whiskers are extended to the 10th and 90th percentiles, and the dots represent the outliers. The p -values for the differences in the serum MMP-2 (a) and MMP-2-TIMP-2 complex (b) levels between benign, LMP and malignant ovarian tumours were 0.009 and 0.083, respectively (Kruskal-Wallis test).

were compared with regard to TIMP-1, the AUC value for TIMP-1 was 0.73. With a cut-off value of 417 µg/l, sensitivity was 0.54 and specificity 0.82 (Figure 4a). As regards the ratio of TIMP-1 to MMP-2, the AUC value was 0.87. With a cut-off value of 0.41, sensitivity was 0.82 and specificity 0.90 (Figure 4b). In terms of the ratio of TIMP-1 to MMP-2-TIMP-2 complex, the AUC value was 0.89. With a cut-off value of 0.44, sensitivity and specificity were 0.90 and 0.91, respectively (Figure 4c).

Discussion

There are neither specific early symptoms nor reliable tests available to differentiate between LMP and malignant ovarian neoplasms. CA125, the golden standard as a tumour

marker in ovarian cancer, has been used most commonly. Though 80-90% of the patients with clinically recognisable ovarian cancer show elevated serum levels of CA125, over 50% of the patients with stage I have normal values. Another frequently used method is ultrasound. Its efficacy, however, is not good enough to differentiate between LMP and malignant ovarian tumours.

Rising serum TIMP-1 levels from benign to LMP and further to malignant ovarian tumours were found in the present study. It is interesting that TIMP-1, which was originally assumed to counteract the effect of metallo-proteinase activity, actually seems to be associated with tumour progression rather than with an indolent clinical course of an ovarian tumour. In some tumours, the relative

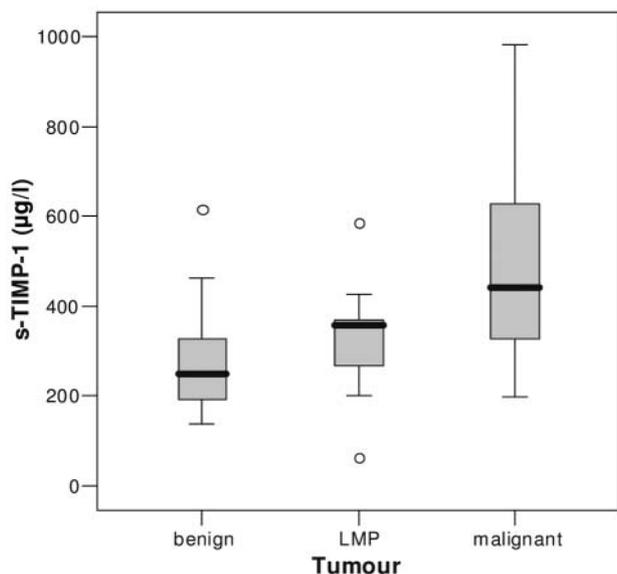


Figure 2. Box plot and whiskers of serum TIMP-1. The boxes include 50% of the values, and the horizontal lines indicate the median. The whiskers are extended to the 10th and 90th percentiles, and the dots represent the outliers. The p-value for the difference in the serum TIMP-1 levels between benign, LMP and malignant ovarian tumours was <0.001 (Kruskal-Wallis test).

amount of MMP and its inhibitor is associated with aggressive behaviour of the disease (10-12). Rauvala *et al.* reported that an elevated preoperative serum TIMP-1 concentration correlated to the aggressive behaviour of ovarian cancer (13). TIMP-1 has been shown to participate in tissue remodelling during embryonic growth and tumour progression, ovulation, pregnancy, angiogenesis, tumour cell invasion and metastasis (14). TIMP-1 also had mitogenic activity on a number of cell types, but overexpression of this inhibitor reduced tumour cell growth (15). In experimental models, TIMP-1 could inhibit tumour growth, invasion and metastasis (15).

For the first time, the usefulness of the ratios of TIMP-1 to MMP-2 and TIMP-1 to MMP-2-TIMP-2 complex in discriminating between benign, LMP and malignant ovarian tumours has been evaluated using ROC. The present study revealed a significant difference in these ratios between benign and malignant and LMP and malignant ovarian tumours. The values for the AUC confirmed that the ratios of TIMP-1 to MMP-2 and TIMP-1 to MMP-2-TIMP-2 complex were more useful than the serum TIMP-1 concentration in differentiating between LMP and malignant ovarian tumours. This result is due to the serum TIMP-1 level being highest in the patients with malignant tumours, whereas the MMP-2 and MMP-2-TIMP-2 complex levels are highest in the LMP group.

Serum MMP-2 or MMP-9 were not useful in differentiating between benign, LMP and malignant ovarian tumours. This finding is consistent with that reported by De Nictolis *et al.*, who evaluated the levels of MMP-2 in

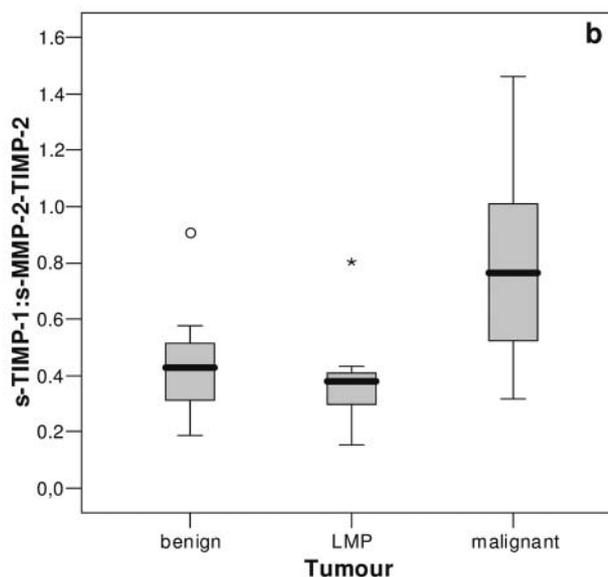
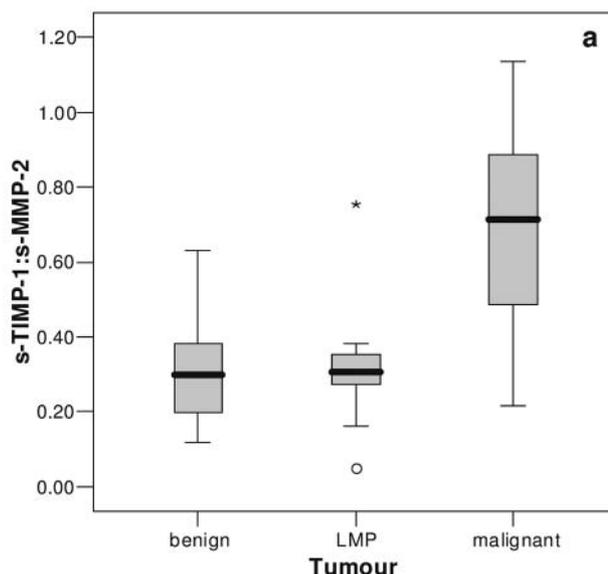


Figure 3. Box plot and whiskers of the ratios of TIMP-1 to MMP-2 (a) and TIMP-1 to MMP-2-TIMP-2 complex (b). The boxes include 50% of the values, and the horizontal line indicates the median. The whiskers are extended to the 10th and 90th percentiles, and the dots and asterisks represent the outliers and extreme values, respectively. The differences in the ratios TIMP-1 to MMP-2 (a) and TIMP-1 to MMP-2-TIMP-2 complex (b) were significant when benign and malignant ($p<0.001$) and LMP and malignant tumours ($p<0.001$) were compared (Mann-Whitney U-test).

patients with ovarian cystadenocarcinoma (16). In our previous study (7), however, an increasing tendency in positive immunostaining of MMP-2 and MMP-9 from benign to LMP and, further, to malignant ovarian tumours was found. Some preclinical *in vitro* studies have also linked the activity of the MMPs with aggressive behaviour of

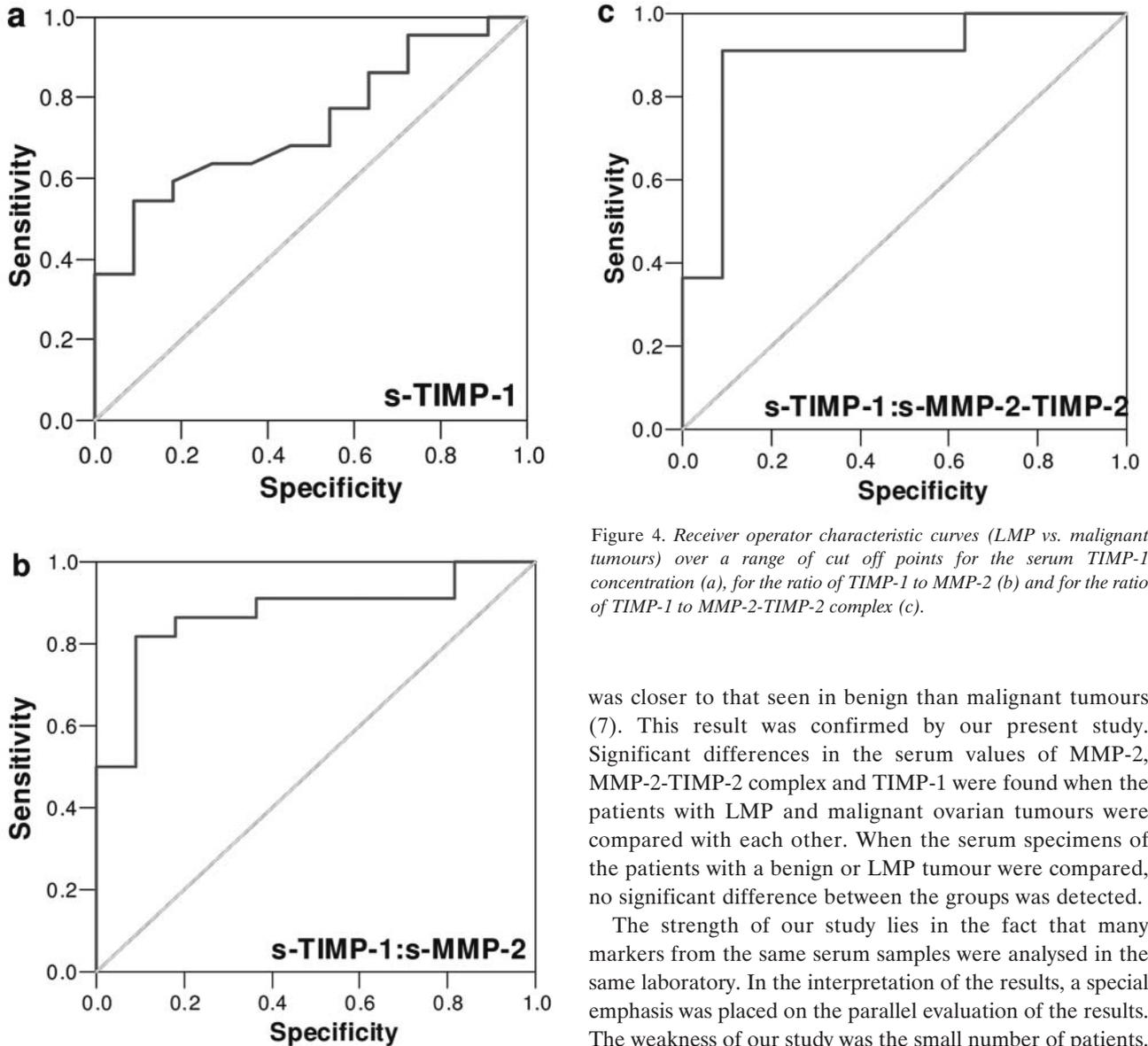


Figure 4. Receiver operator characteristic curves (LMP vs. malignant tumours) over a range of cut off points for the serum TIMP-1 concentration (a), for the ratio of TIMP-1 to MMP-2 (b) and for the ratio of TIMP-1 to MMP-2-TIMP-2 complex (c).

ovarian cancer (17-19). Cultured ovarian cancer cells are able to maintain MMP-2 expression after primary cultures, while MMP-9 expression decreases over the passage of cells (19). It is possible that ovarian cancer cells are dependent on stromal interactions for their MMP expression (17, 19). In this study, the association between the expression of MMPs in tumour tissue and the corresponding circulating levels of MMPs was not evaluated. In one study, where the tissue and circulating MMP-2 concentrations were analysed in malignant and benign ovarian tumours, serum MMP-2 levels were found to correlate with tissue staining (20).

Our previous study showed that the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in LMP ovarian tumours

was closer to that seen in benign than malignant tumours (7). This result was confirmed by our present study. Significant differences in the serum values of MMP-2, MMP-2-TIMP-2 complex and TIMP-1 were found when the patients with LMP and malignant ovarian tumours were compared with each other. When the serum specimens of the patients with a benign or LMP tumour were compared, no significant difference between the groups was detected.

The strength of our study lies in the fact that many markers from the same serum samples were analysed in the same laboratory. In the interpretation of the results, a special emphasis was placed on the parallel evaluation of the results. The weakness of our study was the small number of patients. Moreover, the study groups were heterogeneous, consisting mostly of serous and mucinous ovarian tumours, but also including simple, corpus luteal and dermoid cysts, one borderline Brenner tumour and endometrioid adenocarcinomas. Our results should be evaluated cautiously and taken as encouragement for further investigation. A study with more patients is needed to define more accurate cut-off values for clinical purposes for the ratios of TIMP-1 to MMP-2 and TIMP-1 to MMP-2-TIMP-2 complex.

We conclude that serum TIMP-1 and especially the ratios of TIMP-1 to MMP-2 and TIMP-1 to MMP-2-TIMP-2 complex could be potential tools in the preoperative differential diagnosis between LMP and malignant ovarian tumours in the future. Our data also suggest that LMP ovarian tumours are more similar to benign than malignant ovarian neoplasms.

References

- 1 Trope C and Kaern J: Management of borderline tumours of the ovary: state of the art. *Semin Oncol* 25: 372-380, 1998.
- 2 Eisenkop SM, Spirtos NM, Montag TW, Nalick RH and Wang HJ: The impact of subspecialty training on the management of advanced ovarian cancer. *Gynecol Oncol* 47: 203-209, 1992.
- 3 Santala M, Risteli J, Risteli L, Puistola U, Kacinski BM, Stanley ER and Kauppila A: Synthesis and breakdown of fibrillar collagens; concomitant phenomena in ovarian cancer. *Br J Cancer* 77: 1825-1831, 1998.
- 4 Santala M, Simojoki M, Risteli J, Risteli L and Kauppila A: Type I and type III collagen metabolites as predictors of clinical outcome in epithelial ovarian cancer. *Clin Cancer Res* 5: 4091-4096, 1999.
- 5 Nelson AR, Fingleton B, Rothenberg ML and Matrisian LM: Matrix metalloproteinases: biological activity and clinical implications. *J Clin Oncol* 18: 1135-1149, 2000.
- 6 Gomez DE, Alonso DF, Yoshiji H and Thorgeirsson UP: Tissue inhibitors of metalloproteinases: structure, regulation and biological function. *Eur J Cell Biol* 74: 111-122, 1997.
- 7 Määttä M, Santala M, Soini Y, Talvensaaari-Mattila A and Turpeenniemi-Hujanen T: Matrix metalloproteinases (MMP-2, MMP-9) and their inhibitors (TIMP-1, TIMP-2) in low malignant potential ovarian tumours. *Tumor Biol* 25: 188-192, 2004.
- 8 Ylisirniö S, Höyhty M and Turpeenniemi-Hujanen T: Serum matrix metalloproteinases -2, -9 and tissue inhibitors of metalloproteinases -1, -2 in lung cancer – TIMP as a prognostic marker. *Anticancer Res* 20: 1311-1316, 2000.
- 9 Fujimoto N, Mouri N, Iwata K, Ohuchi E, Okada Y and Hoyakawa T: A one-step sandwich enzyme immunoassay for human matrix metalloproteinase-2 (72-kDa gelatinase/type IV collagenase) using monoclonal antibodies. *Clin Chim Acta* 221: 91-103, 1993.
- 10 Turpeenniemi-Hujanen T: Gelatinases (MMP-2 and -9) and their natural inhibitors as prognostic indicators in solid cancers. *Biochimie* 87: 287-297, 2005.
- 11 Hoikkala S, Pääkkö P, Soini Y, Makitaro R, Kinnula V and Turpeenniemi-Hujanen T: Tissue MMP-2/TIMP-2-complex are better prognostic factors than serum MMP-2, MMP-9 or TIMP-1 in stage I-III lung carcinoma. *Cancer Lett* 236: 125-132, 2006.
- 12 Talvensaaari-Mattila A, Santala M, Soini Y and Turpeenniemi-Hujanen T: Prognostic value of matrix metalloproteinase-2 (MMP-2) expression in endometrial endometrioid adenocarcinoma. *Anticancer Res* 25: 4101-4105, 2005.
- 13 Rauvala M, Puistola U and Turpeenniemi-Hujanen T: Gelatinases and their inhibitors in ovarian tumours; TIMP-1 is a predictive as well as a prognostic factor. *Gynecol Oncol* 99: 656-663, 2005.
- 14 Reunanen N and Kähäri VM: Matrix metalloproteinase in cancer cell invasion. *In: Cell Invasion*. Heino J and Kähäri VM (eds.). Georgetown, Eureka.com/Landes Bioscience, pp. 1-19, 2002.
- 15 Wurtz SO, Schrol AS, Sorensen NM, Lademann U, Christensen IJ, Mouridsen H and Brunner N: Tissue inhibitor of metalloproteinase-1 in breast cancer. *Endocr Relat Cancer* 12: 215-227, 2005.
- 16 De Nictolis M, Garbisa S, Lucarini G, Goteri G, Masiero L, Ciavatti A, Garzetti GG, Stetler-Stevenson WG, Fabris G, Biagini G and Prat J: 72-Kilodalton type IV collagenase, type IV collagen, and Ki 67 antigen in serous tumors of the ovary: a clinicopathologic, immunohistochemical, and serological study. *Int J Gynecol Pathol* 15: 102-109, 1996.
- 17 Miyagi E, Yasumitsu H, Hirahara F, Nagashima Y, Minaguchi H, Miyazaki K and Umeda M: Marked induction of gelatinases, especially type B, in host fibroblasts by human ovarian cancer cells in athymic mice. *Clin Exp Metastasis* 13: 89-96, 1995.
- 18 Westerlund A, Hujanen E, Puistola U and Turpeenniemi-Hujanen T: Fibroblasts stimulate human ovarian cancer cell invasion and expression of 72-kDa gelatinase A (MMP-2). *Gynaecol Oncol* 67: 76-82, 1997.
- 19 Fishman DA, Bafetti LM, Banionis S, Kearns AS, Chilukuri K and Stack MS: Production of extracellular matrix-degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells. *Cancer* 80: 1457-1463, 1997.
- 20 Garzetti GG, Ciavattini A, Lucarini G, Goteri G, de Nictolis M, Garbisa S, Masiero L, Romani C and Graziella B: Tissue and serum metalloproteinase (MMP-2) expression in advanced ovarian cystadenocarcinomas: clinical and prognostic implications. *Anticancer Res* 15: 2799-2804, 1995.

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