Interactive CardioVascular and Thoracic Surgery 22 (2016) 411–418
doi:10.1093/icvts/ivv378 Advance Access publication 13 January 2016

ORIGINAL ARTICLE – THORACIC

Cite this article as: Fiorelli A, Ricci S, Feola A, Mazzella A, D'Angelo L, Santini M *et al*. Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in diagnosis of pleural effusion of malignant origin. Interact CardioVasc Thorac Surg 2016;22:411–18.

Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in diagnosis of pleural effusion of malignant origin

Alfonso Fiorelli[a,*], Serena Ricci[b], Antonia Feola[c], Antonio Mazzella[a], Luigi D'Angelo[d], Mario Santini[a], Marina Di Domenico[c,e] and Angelina Di Carlo[f]

[a] Thoracic Surgery Unit, Second University of Naples, Naples, Italy
[b] Department of Translational Medical Science, University of Naples "Federico II", Naples, Italy
[c] Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Naples, Italy
[d] Multidisciplinary Department of Medical-Surgical and Odontostomatological Specialties, Second University of Naples, Naples, Italy
[e] Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, Philadelphia, PA, USA
[f] Department of Medico-Surgical Sciences and Biotechnologies, "La Sapienza" University of Rome, Rome, Italy

* Corresponding author. Thoracic Surgery Unit, Second University of Naples, Naples, Italy, Piazza Miraglia 3, 83100 Naples, Italy. Tel: +39-081-5665228; fax: +39-081-5665230; e-mail: alfonso.fiorelli@unina2.it (A. Fiorelli).

Received 5 August 2015; received in revised form 28 October 2015; accepted 26 November 2015

ORIGINAL ARTICLE

Abstract

OBJECTIVES: The aim of the present study was to evaluate the diagnostic accuracy of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in differentiating benign from malignant exudative pleural effusions.

METHODS: This is a unicentre observational study including 97 consecutive patients with exudative pleural effusions. Metalloproteinase-9, tissue inhibitor of metalloproteinase-1, lactate dehydrogenase, ferritin, carcinoembryonic antigen and carbohydrate antigen 15-3 were measured in pleural effusion and serum by enzyme-linked immunosorbent assay. The activity of metalloproteinase-9 was also evaluated by substrate zymography. The data were correlated with final diagnosis of pleural effusions to evaluate the diagnostic accuracy.

RESULTS: Of the 97 eligible patients, 6 were excluded. Of the 91 patients included in the study, 70 had malignant pleural effusions and 21 had benign pleural effusions. Both in sera and pleural effusions, matrix metalloproteinase-9 ($P < 0.0001$), tissue inhibitor of metalloproteinase-1 ($P < 0.0001$) and carcinoembryonic antigen ($P < 0.0001$) levels were higher in neoplastic patients than in benign group. Zymography analysis showed a most prominent band at a molecular weight of 92 kDa (metalloproteinase-9) whereas a less intense band was observed at 72 kDa (metalloproteinase-2). A significant correlation was found between metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels in pleural effusion ($P < 0.0001$; $r = 0.8$) and serum ($P < 0.03$; $r = 0.2$). Pleural effusion metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels showed higher value of sensitivity (97 and 91%, respectively) and specificity (90 and 95%, respectively) compared with other standard markers. Serum metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels showed similar results. Among 70 neoplastic patients, 29 had negative pleural cytology. Of these, 25 presented elevated levels of metalloproteinase-9 and tissue inhibitor of metalloproteinase-1, whereas 4 patients had elevated levels of one of the two markers.

CONCLUSIONS: Our results showed that metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 might be valuable markers in differentiating benign from malignant pleural effusions. Their levels are neither influenced by the histology and tumour origin nor by the presence of tumour cells in pleural effusions. Thus, their use in clinical practice could help in the selection of patients needing more invasive procedures, such as thoracoscopic biopsy.

Keywords: Matrix metalloproteinase-9 • Tissue inhibitor of metalloproteinase-1 • Exudative pleural effusion

INTRODUCTION

Differential diagnosis of exudative pleural effusions (PEs) still remains a diagnostic problem. The diagnostic accuracy of cytological examination of PEs is about 60% and computed tomography-guided biopsy adds 7–13% of positive findings [1]. Video-assisted thoracic surgery (VATS) remains the gold standard procedure in the diagnosis of exudative PEs, which allows to perform biopsies in pathological pleural areas. However, VATS is a surgical procedure that may be contra-indicated in unfit patients.

In the last years, several new markers have been evaluated to increase the diagnostic accuracy of cytological examination of exudative PEs with controversial results [1, 2]. The exudative PEs, in fact, may have different pathogenesis related to the underlying

diseases. Thus, it is unlikely that a single mediator plays a key role in all types of exudative PEs.

The matrix metalloproteinases (MMPs) are a family of proteases capable of degrading all components of the extracellular matrix. Their activity is prevalently controlled by the tissue-specific inhibitors of metalloproteinases (TIMPs). The alteration of the balance between MMPs and their TIMPs has been suggested to play a role in the pathogenesis of benign and malignant PEs.

In humans, the MMP family is composed of at least 21 different members. Among these, metalloproteinase-9 (MMP-9) is one of the most powerful factors involved in angiogenesis and in degradation of the basement membrane. MMP-9 secretion is of particular interest in pleural diseases, since this gelatinase, by degrading Type IV collagen, contributes to loss of integrity of the basement membrane around blood vessels and under the mesothelial layer, leading to fluid accumulation in the pleural space [2–4].

The aim of the present study was to evaluate whether MMP-9 and its specific inhibitor TIMP-1 might have a role in the differential diagnosis of exudative PEs, in order to select which patients will benefit of invasive procedures such as VATS biopsy.

MATERIALS AND METHODS

Study design

This was a unicentre observational study. All consecutive patients with exudative PEs presented to our unit in the last two years (April 2013–May 2015) were eligible. Patients were excluded if (i) they refused invasive procedure; (ii) they did not have a definitive diagnosis at completion of diagnostic work-up; (iii) they received immune-stimulating agent, anti-cancer treatment, anti-tuberculosis treatment, corticosteroid or other non-steroid anti-inflammatory drug treatment; or (iv) serum and PE samples were spoiled for determination of markers.

MMP-9, TIMP-1, lactate dehydrogenase (LDH), ferritin, carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA 15-3) were measured in PE and in serum by enzyme-linked immunosorbent assay (ELISA). In addition, the activity of MMP-9 was also evaluated by substrate zymography.

PEs were split into two groups depending on diagnosis (PEs of benign origin versus PEs of malignant origin) and the intergroup difference of PE markers and serum markers was statistically analysed. The readers of the index tests were blind to the results of the other tests.

The protocol of this study was approved by the Hospital Ethics Committee of the Second University of Naples and written informed consent was obtained from all cases before being included in the study.

Patients

Ninety-seven consecutive patients with exudative PEs were eligible. All samples were designed as exudate according to the Light criteria [5]. The presence of malignant cells in the pleural fluid or in pleural biopsy indicated the malignant origin of PEs. Benign pleural effusion was defined of parapneumonic origin when associated with bacterial pneumonia, including empyema; of tubercular origin when mycobacterium tuberculosis was found in pleural fluid, sputum, bronchial lavage fluid or pleural biopsy specimen (positive smear or culture); or revealed granuloma and other granulomatous diseases.

Sample processing

PEs were collected via diagnostic thoracentesis, collected in sterile tube without anticoagulant and rapidly brought to our laboratory with a blood sample of the same patients. Pleural fluids and blood samples were immediately centrifuged at 1500 rpm for 7 min at 4°C and supernatants were aliquoted and stored at −70°C awaiting analysis of MMP-9 TIMP-1, LDH, ferritin and CA 15-3. Each aliquot was used only one in order to prevent enzyme activation due to freeze-thawing processes.

Markers measurements

The levels of MMP-9 (ng/ml), TIMP-1 (pg/ml), LDH (UI/l), ferritin (ng/ml), CEA (ng/ml) and CA 15-3 (kU/l) were determined within PE and serum using a 'sandwich' ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's guidelines.

Zymography

Gelatinases A and B were purchased from Hoffmann-La Roche Ltd (Basel, Switzerland). Triton X-100, Calcium chloride ($CaCl_2$), glycerol, gelatin, ethylene-diaminetetraacetic (EDTA) phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chemical Co. (St Louis, MO, USA). The protein assay reagent was from Bio-Rad Laboratories. All other reagents were available from commercial sources.

Gelatin Zymography: MMP activity was determined through zymographic analysis under denaturating but non-reducing conditions as previously described. Total serum proteins (25 µg) or pleural effusion proteins (15 µg) were mixed with sample buffer and applied directly without heating or reduction to 7.5% (w/v) acrylamide gels containing 0.1% (w/v) of gelatin. After the removal of SDS from gel by incubation in 2.5% (v/v) Triton X-100 for 1 h, the gels were incubated at 37°C for 18 h in 50 mM Tris-HCl pH 7.6 containing 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% (w/v) Brij 35. Gels were stained for 1 h in 30% methanol, 10% glacial acetic acid containing 0.5% (w/v) Coomassie Brilliant Blue G 250 and destained in the same solution without dye for several hours. The gelatinolytic activity of each collagenase was evident as a clear band against the blue background of stained gelatin. The molecular size of bands displaying enzymatic activity was identified in comparison with prestained standard protein, as well as with purified gelatinase A or gelatinase B. To normalize the possible difference between zymograms, an internal serum or pleural effusion sample from a patient was incorporated in every gel.

Control gels for MMPs. Control gels contained either of the MMP selective inhibitors, 20 mM EDTA or 10 mM 1,10 phenanthroline, in the MMP incubation buffer to confirm that lysis bands were the results of MMPs. Furthermore, the character of proteolytic bands was analysed by incubating the identical zymograms in 0.1 mg/ml of PMSF, a serine protease inhibitor; or 2 mM Pefabloc, an irreversible serine protease inhibitor (data not shown).

Analysis of the gels. Following zymography, the degree of gelatin digestion was quantified as previously described. Briefly, we used an image analysis software (ImageQuant TL, Amersham Bioscience, Chicago, IL, USA) according to the manufacturer's specifications. The image of the gel was inverted to reveal dark bands on a white background. The molecular weight, volume and background of each band were determined. The relative amounts of the different forms of both serum and pleural effusion gelatinases were expressed as the integrated density × 10^{-3} (volume) of all the pixels above the background of each band.

Statistical analysis

Data are presented as median and interquartile range (IRQ). Comparisons between different groups were performed using Mann–Whitney *U*-tests. In neoplastic patients, analysis of variance (ANOVA) test was used to compare the different origins of PEs. The correlation between MMP-9 and TIMP-1 levels was assessed with Spearman's rank correlation test. The accuracy of variables to distinguish malignant from benign PEs was calculated with receiver operating characteristics (ROC) curve. For the optimum cut-off point provided by ROC analysis, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. A value of $P < 0.05$ was considered statistically significant. MedCalc® statistical software was used for analysis.

RESULTS

Of the 97 eligible patients, 6 were excluded due to the lack of definitive diagnosis ($n = 1$) and spoiled samples ($n = 5$). Thus, our study population included 91 patients [65 male and 26 female, median age 64 (51–75)]; 21 patients with benign PEs (10 parapneumonic, 6 empyema, 5 tuberculosis) and 70 with PEs of malignant origin due to lung ($n = 27$; 38.5%), gastrointestinal ($n = 20$; 28.5%) and breast ($n = 5$; 7%) cancers and mesotheliomas ($n = 18$; 26%). The flow chart of study population is reported in Fig. 1.

All malignant patients underwent VATS which detected multiple pleural metastases. Of these, 41/70 (58%) patients also presented a positive fluid cytology for malignant cells. All patients with empyema and 9/10 (90%) patients with PEs of parapneumonic origin presented a macroscopic pus whereas only 1/10 (10%) patient presented clinical and radiological signs consistent with pneumonia and non-purulent pleural fluid. Tube thoracostomy and drainage were performed in 15 patients (9 with parapnuemonic PEs and 6 with empyema). Five patients with empyema required pleural decortication performed via VATS ($n = 4$) and thoracotomy ($n = 1$). In patients with no purulent fluid parapneumonic PEs, thoracentesis and medical therapy were performed.

Among patients with tuberculosis PEs, 2/5 (40%) had positive pleural fluid culture, 2/5 (40%) presented positive sputum culture and 1/5 (20%) had a granuloma on pleural biopsy which shows staining for the acid-fast bacilli. In all cases, PEs resolved with chest drainage and specific medical therapy.

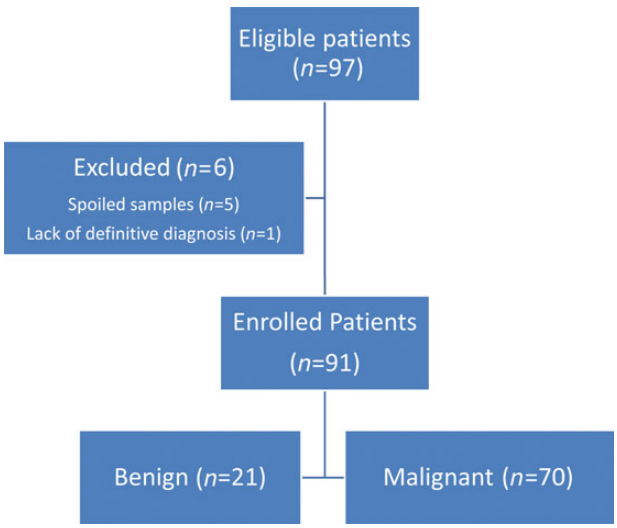

Figure 1: Flow chart of study population.

Marker levels in pleural effusion

The results are reported in Table 1 and Fig. 2. Neoplastic patients compared with control group had higher levels of MMP-9 ($P < 0.0001$, Fig. 2A), TIMP-1 ($P < 0.0001$, Fig. 2B) and CEA ($P < 0.0001$; Fig. 2C) while no significant difference was found regarding LDH ($P = 0.7$), ferritin ($P = 0.2$) and CA 15-3 ($P = 0.2$) levels.

Among neoplastic patients, lung cancer, extrathoracic cancer and mesothelioma showed similar value of MMP-9 levels (1927 ± 163 vs 2121 ± 266 vs 1949 ± 568; $P = 0.08$) and of TIMP-1 levels (1811 ± 529 vs 2100 ± 382 vs 2008 ± 458; $P = 0.1$).

Marker levels in serum

The results are reported in Table 1 and Fig. 2. Neoplastic patients compared with benign patients had higher levels of MMP-9 ($P < 0.0001$; Fig. 2D), TIMP-1 ($P < 0.0001$; Fig. 2E) and CEA ($P < 0.0001$; Fig. 2F) while no significant difference was found regarding LDH ($P = 0.1$), ferritin ($P = 0.6$) and CA 15-3 ($P = 0.7$) levels.

Table 1: Markers levels in pleural effusion and serum

Variable	Malignant	Benign	*P*-value
Pleural effusion			
MMP-9 (ng/ml)	2036 (1965–2177)	587 (533–647)	<0.0001
TIMP-1 (pg/ml)	2095 (1955–2204)	646 (582–706)	<0.0001
CEA (ng/ml)	1.7 (1.4–1.8)	1.1 (1.0–1.2)	<0.0001
LDH (UI/l)	220 (208–232)	224 (214–236)	0.7
Ferritin (ng/ml)	551 (497–602)	521 (491–565)	0.2
CA 15-3 (kU/l)	50 (45–55)	49 (41–52)	0.7
Serum			
MMP-9 (ng/ml)	185 (67–203)	71 (58–76)	<0.0001
TIMP-1 (pg/ml)	181 (174–184)	53 (47–83)	<0.0001
CEA (ng/ml)	1.2 (0.9–1.3)	0.6 (0.5–0.7)	<0.0001
LDH (UI/l)	147 (135–159)	140 (135–149)	0.1
Ferritin (ng/ml)	490 (436–541)	483 (440–517)	0.6
CA 15-3 (kU/l)	41 (36–46)	40 (38–44)	0.7

CEA: carcinoembryonic antigen; TIMP: tissue-specific inhibitors of metalloproteinases 1; CA 15-3: carbohydrate antigen 15-3; LDH: lactate dehydrogenase; MMP-9: metalloproteinase-9.

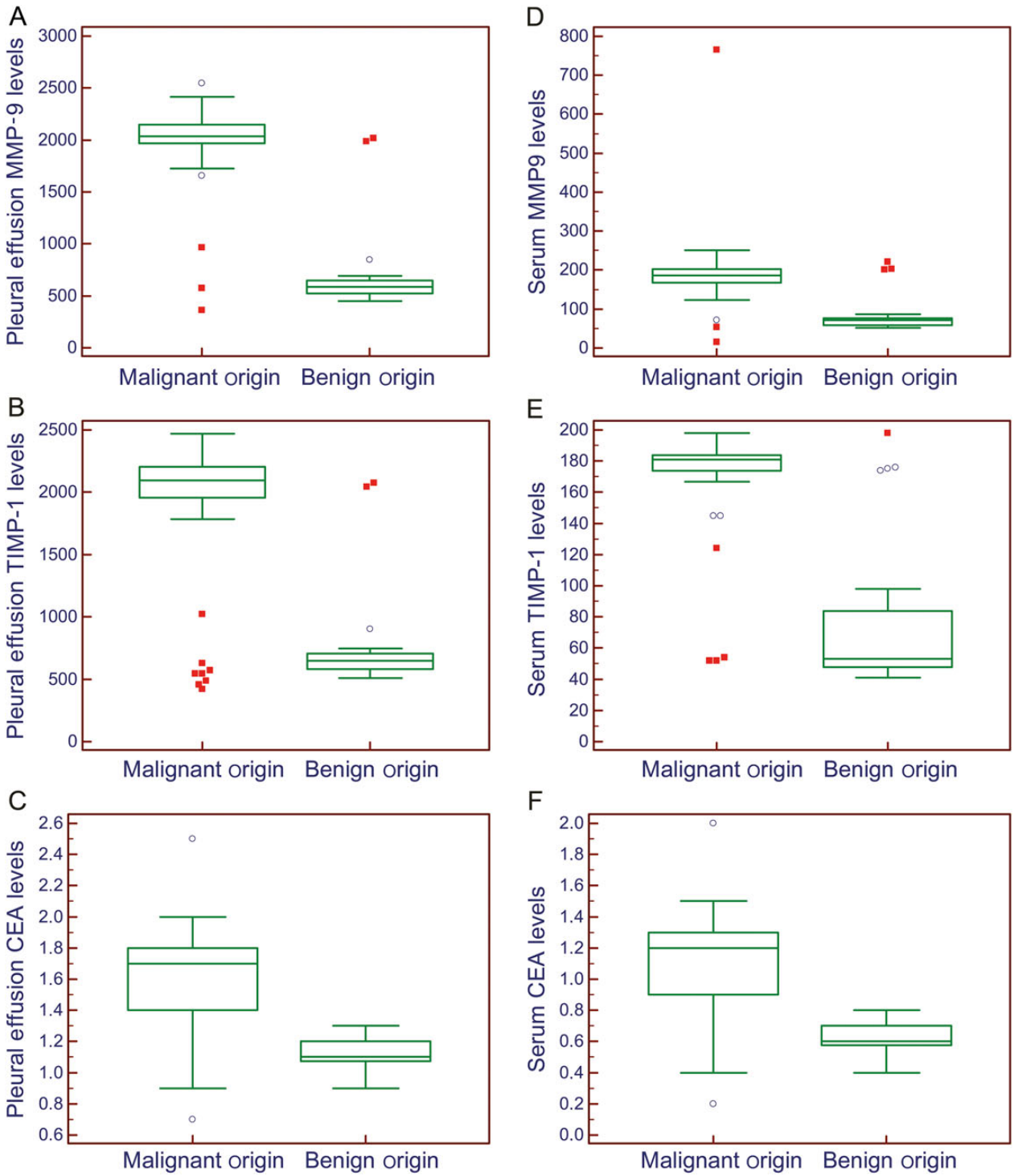

Figure 2: MMP-9 ($P < 0.0001$, **A**), TIMP-1 ($P < 0.0001$, **B**) and CEA ($P < 0.0001$; **C**) in pleural effusions and MMP-9 ($P < 0.0001$; **D**), TIMP-1 ($P < 0.0001$; **E**) and CEA ($P < 0.0001$; **F**). CEA: carcinoembryonic antigen; TIMP: tissue-specific inhibitors of metalloproteinases 1.

Among neoplastic patients, lung cancer, extrathoracic cancer and mesothelioma showed similar value of MMP-9 (208 ± 123 vs 185 ± 28 vs 180 ± 52; $P = 0.4$) and of TIMP-1 (173 ± 54 vs 183 ± 34 vs 175 ± 23; $P = 0.7$) levels.

In addition, PE MMP-9 ($P < 0.0001$) and PE TIMP-1 ($P < 0.0001$) levels were significantly higher than serum MMP-9 and TIMP-1 levels.

Zymography

In all neoplastic patients but one, multiple bands of gelatinolytic activity were evident on gelatin zymography of PE and serum. The most prominent band migrated at a molecular weight of 92 kDa (MMP-9). A less intense band was often, but not always, seen at 72 kDA [metalloproteinase-2 (MMP-2)].

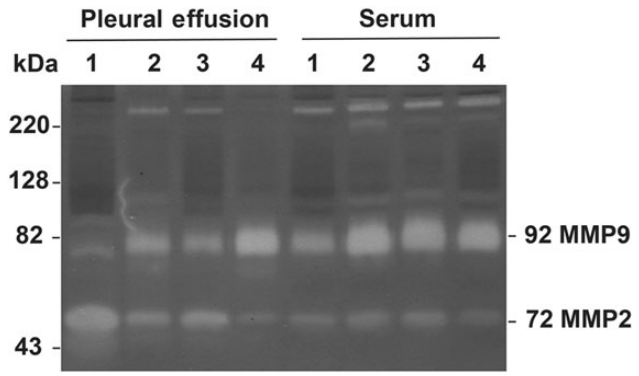

Figure 3: Typical gelatin zymographs in 4 PEs and serum samples. Lane 1 representative empyema; Lane 2 through 4 representative malignant disease. Lane 1 showed a predominant band at 72 kDa (MMP-2). The other lanes contained a more evident band at 92 kDa (MMP-9) and a less evident band at 72 kDa (MMP-2). PEs: pleural effusions.

Table 2: Characteristics of ROC curve

Variable	AUC	SE	95% CI
Pleural effusion			
MMP-9	0.946	0.0277	0.878–0.983
TIMP-1	0.912	0.0332	0.834–0.961
CEA	0.777	0.0513	0.678–0.858
LDH	0.523	0.0674	0.416–0.629
Ferritin	0.592	0.0664	0.484–0.694
CA 15-3	0.576	0.0675	0.468–0.679
Serum			
MMP-9	0.859	0.0628	0.770–0.923
TIMP-1	0.891	0.0523	0.808–0.947
CEA	0.807	0.0503	0.711–0.882
LDH	0.593	0.0630	0.484–0.694
Ferritin	0.537	0.0691	0.430–0.643
CA 15-3	0.521	0.0591	0.414–0.627

CA 15-3: carbohydrate antigen 15-3; CEA: carcinoembryonic antigen; TIMP: tissue-specific inhibitors of metalloproteinases 1; LDH: lactate dehydrogenase; ROC: receiver operating characteristics; SE: standard error of the mean; CI: confidence interval; AUC: area under curve; MMP-9: metalloproteinase-9.

Only one patient with empyema showed the presence of gelatinolytic band at a molecular weight of 72 kDa (MMP-2). A representative gel is reported in Fig. 3.

Diagnostic accuracy of pleural effusion markers

The ROC curves of PE markers are reported in Table 2 and Fig. 4A. PE MMP-9 levels reached a sensitivity of 97% (95% CI: 90.1–99.7), a specificity of 90% (95% CI: 69.6–99.8); a PPV of 97% (95% CI: 90.1–99.7); and a NPV of 90% (95% CI: 69.5–98.8) when a cut-off value >845 pg/ml was applied.

PE TIMP-1 levels reached a sensitivity of 91% (95% CI: 82.3–99.1), a specificity of 95% (95% CI: 76.2–99.9); a PPV of 98% (95% CI: 91.7–100); and a NPV of 76% (95% CI: 56.2–91) when a cut-off value >904 pg/ml was applied.

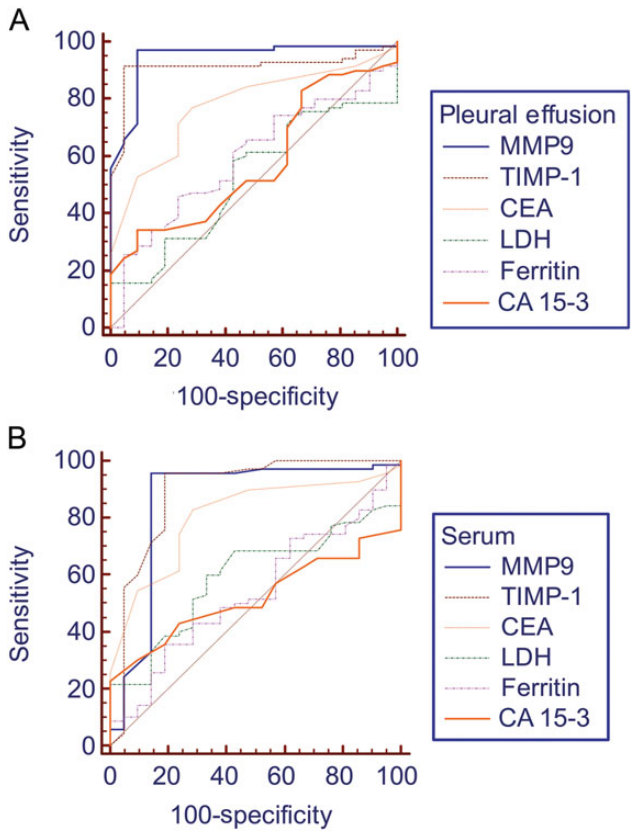

Figure 4: ROC curves of markers in pleural effusion (**A**) and serum (**B**). CEA: carcinoembryonic antigen; TIMP: tissue-specific inhibitors of metalloproteinases 1; ROC: receiver operating characteristics.

Area under curve (AUC) of PE MMP-9 levels was significantly higher compared with that of CEA ($P = 0.006$); of LDH ($P < 0.0001$), of ferritin ($P < 0.0001$); and of CA 15-3 ($P < 0.0001$).

AUC of PE TIMP-1 levels was significantly higher compared with that of CEA ($P = 0.02$); of LDH ($P = 0.003$), of ferritin ($P < 0.0001$); and of CA 15-3 ($P < 0.0001$).

No significant difference was found between AUC of PE MMP-9 and PE TIMP-1 levels ($P = 0.2$).

Diagnostic accuracy of serum markers

The ROC curves of serum markers are reported in Table 2 and Fig. 4B. Serum MMP-9 levels reached a sensitivity of 95% (95% CI: 88–99.1), a specificity of 85% (95%CI: 63.7–97); a PPV of 95% (95% CI: 88–99.1); and a NPV of 85% (95% CI: 63.6–96.9) when a cut-off value >86 pg/ml was applied.

Serum TIMP-1 levels reached a sensitivity of 95% (95% CI: 88–99.1), a specificity of 80% (95% CI: 58.1–94.6); a PPV of 94% (95% CI: 86.2–98.5); and a NPV of 84% (95% CI: 62–96.8) when a cut-off value >98 pg/ml was applied.

AUC of serum MMP-9 levels was significantly higher compared with that of LDH ($P = 0.008$), of ferritin ($P < 0.0001$); and of CA 15-3 ($P < 0.0001$).

AUC of serum TIMP-1 levels was significantly higher compared with that of LDH ($P < 0.0001$), of ferritin ($P = 0.0003$); and of CA 15-3 ($P < 0.0001$).

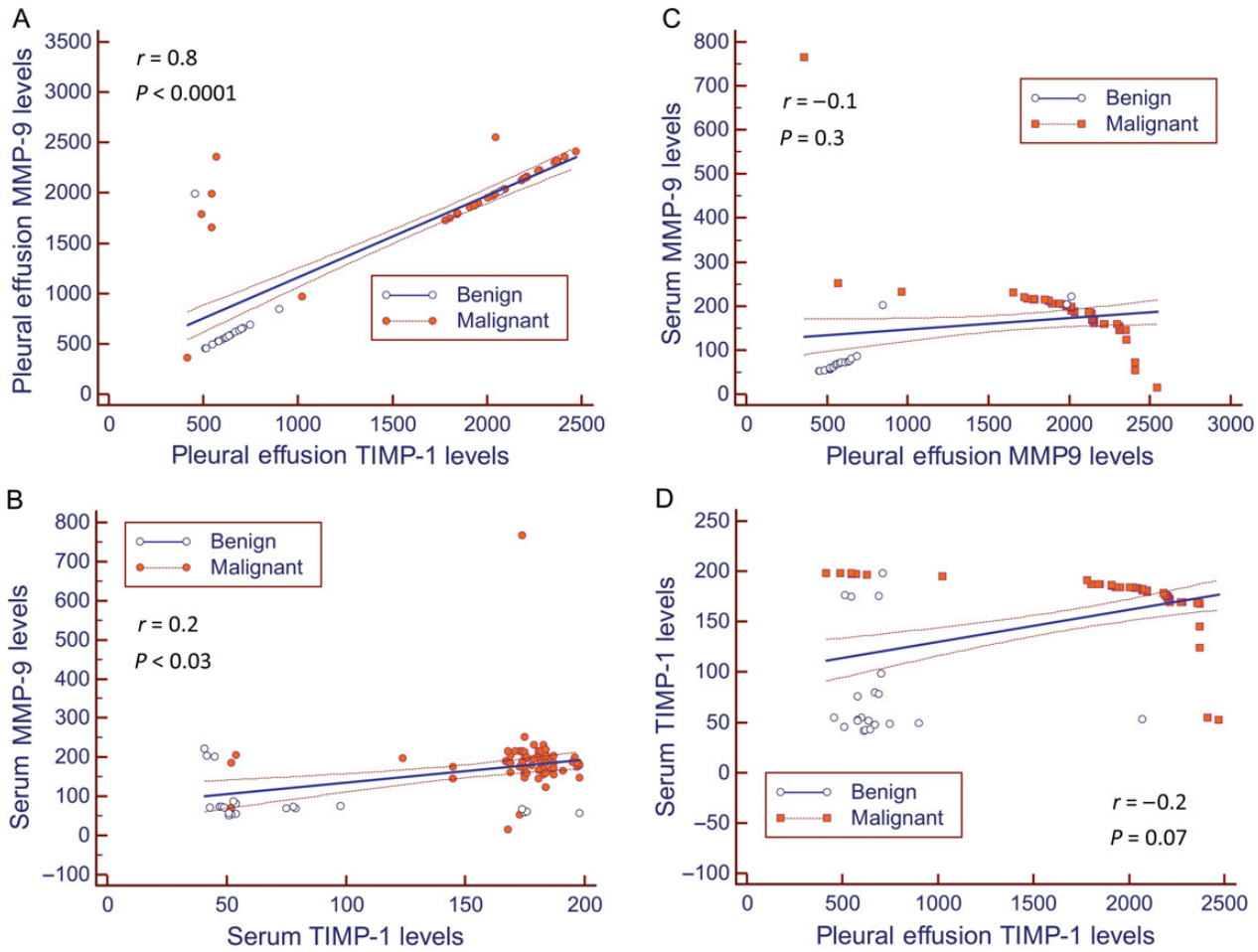

Figure 5: A significant correlation was found between PE MMP-9 and PE TIMP-1 levels (**A**) and serum MMP-9 and serum TIMP-1 levels (**B**). No significant correlation was found between PE MMP-9 and serum MMP-9 (**C**). Similar results were found for TIMP-1 (**D**). PE: pleural effusion; TIMP: tissue-specific inhibitors of metalloproteinases 1.

No significant difference was found between AUC of serum MMP-9 levels versus AUC of TIMP-1 levels ($P = 0.7$); of MMP-9 versus CEA levels (0.4); and of TIMP-1 versus CEA ($P = 0.7$).

Correlation between MMP-9 and tissue-specific inhibitors of metalloproteinases-1

A significant correlation was found between PE MMP-9 and PE TIMP-1 levels ($P < 0.0001$; $r = 0.8$; 95% CI: 0.84–0.93; Fig. 5A); and serum MMP-9 and serum TIMP-1 levels ($P < 0.03$; $r = 0.2$; 95% CI: 0.02–0.41; Fig. 5B).

No significant correlation was found between PE MMP-9 and serum MMP-9 ($P = 0.3$; $r = -0.1$; 95% CI: −0.30 to 0.10; Fig. 5C). Similar results were found for TIMP-1 ($P = 0.07$; $r = -0.2$; 95% CI: −0.39 to 0.112; Fig. 5D).

Pleural effusion tissue-specific inhibitors of metalloproteinases-1 and MMP-9 and cytological examination

Among malignant group, fluid cytology was positive for malignant cells in 41/70 (58%) whereas it was negative in the remaining 29/70 (42%) cases.

Malignant PEs and no-malignant PEs showed similar MMP-9 levels [2014 (1874–2145) vs 2143 (2014–2151); $P = 0.1$] and TIMP-1 [2073 (1933–2204) vs 2197 (2072–2205); $P = 0.1$].

Among 29 neoplastic patients with negative pleural cytology, 25 had PE MMP-9 and PE TIMP-1 levels above the cut-off levels, and 4 patients had at least one of the two PE marker levels (MMP-9 in 3 cases and TIMP-1 in 1 case) above the cut-off levels.

DISCUSSION

Infection and cancer are the main causes of exudative PEs. The differential diagnosis may be sometimes challenging because the mechanism of PE formation is multifactorial and not completely understood. The imbalance of MMP and TIMP enzymes favours the proteolitic processes that alter the integrity of underlying basement membrane facilitating fluid influx within pleural space in several diseases including cancer [6]. In the present paper, we hypothesized that MMP-9 and its specific inhibitor TIMP-1 could be useful markers in the differential diagnosis of exudative PEs.

We found higher levels of MMP-9 and TIMP-1 in PEs of malignant origin than in PEs of benign origin. These results are in agreement with other reports that found increased levels of MMP-9 in serum of patient with breast, gastric, colon, prostate and lung cancers [7–10]. The degradation of the extracellular matrix by tumour cells is a critical step in tumour metastasis, as the

basement membrane and interstitial stroma represent the first barrier to tumour spread. Although several proteolytic enzymes are involved in this process, MMP-9 plays a critical role, since it degrades Type IV collagen and contributes to loss of integrity of the basement membrane around blood vessels and under mesothelial layer. The alteration of vascular permeability leads to fluid accumulation in the pleural space [11].

Conversely, other studies have shown the presence of several MMPs, including MMP-9, in tuberculosis pleural fluid. Park *et al.* [12] found a significant increase in MMP-9 in tuberculosis PEs compared with transudates and malignant PEs. Similar results were also reported by Vatansever *et al.* [13] and by Sheen *et al.* [4].

The small number of patients with tuberculosis PEs and the different severity grades of disease may explain why our results are different from previous studies [4, 12, 13].

In all the above reported studies, the number of patients with tuberculosis PEs was similar to or higher compared with those with malignant PEs while in our study patients with tuberculosis PEs ($n = 5$) were lower than those with PEs of malignant origin ($n = 70$). The difference incidence towards neoplastic diseases compared with benign diseases probably is due to the fact that ours is a thoracic surgery unit where patients have been referred for receiving a surgical biopsy and/or treatment.

Mycobacterium tuberculosis is able to induce an extensive inflammatory response in the human host, which may reflect a particular involvement of MMPs in granuloma formation in this organism. Sheen *et al.* [4], in fact, observed that tuberculous granuloma formation was associated with increased MMP-9 levels in PEs. Among patients with pleural tuberculosis, MMP-9 concentrations were significantly higher in patients in whom granulomas were detected on biopsy compared with non-granulomatous pleural biopsies. In addition, cells adjacent to caseous necrosis in the granuloma presented high MMP-9 levels, which may be implicated in the process of caseation. Similarly, Park *et al.* [12] described that MMP-9 is produced predominantly by epithelioid cells in the granulomas of tuberculous pleural tissues, whereas lymphocytes are the major cellular source of MMP-9 in patients with malignant effusions. In our study population, only 1/5 patients with tuberculosis PEs presented with a granuloma on pleural biopsy.

Furthermore, in addition to data published by Iglesias *et al.* [6], we also observed a significant difference in MMP-9 and TIMP-1 levels between PEs of benign and of malignant origin. It is well known that the different extension of pleural involvement by neoplastic and infectious disease may be responsible for the different results. PEs of malignant origin, in fact, may be associated with several heterogeneous diseases and can occur as the initial presentation of cancer, as a delayed complication in patients with previously diagnosed malignancies, or as the first manifestation of cancer recurrence after therapy. In our study population, all neoplastic patients presented multiple pleural involvement during VATS. In addition, 18/70 (26%) patients were affected by mesothelioma with an extent pleural involvement. Similarly, among PEs of benign origin, all patients but one had parapneumonic effusions that required chest drainage and/or VATS debridement. Thus, the high levels of markers seen in our study may be related to the extension of pleural involvement whereas lower concentrations of MMP-9 and TIMP-1 would be expected when the pleura is only recently affected by tumour or infection.

An intriguing finding was the significant correlation between MMP-9 and TIMP-1 levels. Under normal physiological conditions, TIMP-1 reduces the activity of MMP-9. Thus, the imbalance between MMP-9 and TIMP-1 levels could provide a favourable biological environment for cancer cell growth and metastasis [6, 14]. Arnold *et al.* [15], in a study population of 39 samples, found that all metastatic brain tumours immunohistochemically expressed MMP-9, while 97% of samples were found to be negative for TIMP-1. Also Gonzales *et al.* [16] found a high MMP-9 activity without the increase of TIMP-1 levels in small-cell lung cancer, as a reflection of its clinical metastatic behaviour. Thus, since all our neoplastic patients had multiple pleural metastases, we would expect an alteration of balance between MMP-9/TIMP-1 towards MMP-9. In contrast, we found high TIMP-1 levels that were significantly correlated with MMP-9 which might suggest a strong response by organism for limiting MMP-9 gelatinolytic activity and, thus, tumour progression. Unfortunately, our data do not provide evidence for a causal relationship between the elevated levels of TIMP-1 and MMP-9. The mechanisms related to the observed correlations remain still unclear and need to be further investigated.

The evaluation of MMP-9 and TIMP-1 in PE revealed significantly higher levels than those observed in serum, but no significant correlations were found. These data may be explained by a possible compartmentalization of these enzymes in the pleural space, as previously observed by Hurewitz *et al.* [17]. The presence of MMP-9 and TIMP-1 in PEs is not simply due to the ultrafiltration from blood, but several cell types as mesothelial cells, monocyte/macrophages, lymphocytes, neutrophils and stromal cells have been reported as a source of MMP-9 and TIMP-1 in pleural cavity on the basis of underlying disease. Iisaza *et al.* [10], in fact, found that the frequency of tumour samples expressing MMP-9 was much lower than that of cases with elevated MMP-9 levels in plasma. Since macrophages stained positive for MMP-9 in tumour samples, despite the absence of inflammatory cell infiltrate, it is possible that macrophages release MMP-9 in the pleural space and are stimulated by the same tumour cells for producing regulatory factors, such as cytokines and chemokines. This hypothesis was also supported by the findings of Hrabec *et al.* [18]. Park *et al.* [13] who identified high rate of lymphocytes in malignant PEs compared with tuberculosis PEs, suggesting that, in neoplastic PEs, lymphocytes were the major cellular source of MMP-9.

These results also explained the similar MMP-9 and TIMP-1 levels observed in PEs with positive and negative cytology, since other cell types, as well as neoplastic cells, are able to release MMP-9 and TIMP-1 in pleural space.

The ELISA method measures the latent but not the active MMP-9 form, which is the only one capable of digesting collagen IV and is involved in metastatic processes. Ondo *et al.* [14] found no significant difference between MMP-9 levels, measured by ELISA, in NSCLC patients and healthy control. However, they showed that the active form of MMP-9 was an important marker for the evaluation of cancer progression. Thus, in order to quantify MMP-9 activity, we used gelatin zymography and we detected in all neoplastic patients two predominant MMPs isoforms, MMP-9 and MMP-2, which are both type IV collagenases, with different molecular weights, involved in the degradation of extracellular matrix. However, MMP-9 presented a higher enzymatic activity than MMP-2, probably because MMP-2 production seems to be related to physiological process of transudative PE formation, whereas MMP-9 is mostly active in exudative PE formation, as previously reported by Eickelberg *et al.* [19]. Also Hurewitz *et al.* [17] found the presence of MMP-2 and MMP-9 in 32 PEs, but could not correlate their pattern expression with the origin of pleural disease.

Compared with other tumour markers, MMP-9 and TIMP-1 present a high specificity with cut-off points that offer also a high

sensitivity. Probably, these differences could be a consequence of a slight difference in tumour origins in the sample of this study where mesothelioma represents the 26% of PEs and gastrointestinal tumour the 28.5%. Gastrointestinal tumours commonly present increased levels of CEA, whereas mesotheliomas have usually low levels of CEA and CA 15.3 [20]. Although the frequency of increased levels of each tumour marker depends on the tumour origin, in our analysis the increase of MMP-9 and TIMP-1 level seems not to be organ-specific, since different cancers showed similar levels.

In clinical practice, the dosage of MMP-9 and TIMP-1 could improve diagnostic value of pleural fluid analysis, considering that PEs of neoplastic origin with negative cytology are all diagnosed by measuring MMP-9 and TIMP-1 levels. In this way, VATS could be indicated in patients having exudative PEs with MMP-9 and TIMP-I above the cut-off points while a clinical follow-up could be preferred in other cases with suspicion of benignity according to clinical and radiological findings and low levels of MMP-9 and TIMP-1.

The evaluation of several tumour markers, including MMP-9 and TIMP-1, requires higher costs than the simple cytological analysis of PEs. The cost for measuring MMP-9 or TIMP-1 by ELISA method, in fact, is about 1.000 Euros for about 40 samples, which means that the cost for measuring each marker is 25 Euros per patient. However, the measurement of MMP-9 and TIMP-1 may avoid the necessity of performing VATS in selected cases economically, as VATS is a surgical procedure which requires an operative room with a dedicated staff.

Considering the different results from previous studies [4, 6, 12, 13], before proposing the standard measurements of these markers in diagnostic work-up of exudative PEs, our data should be corroborated with future larger studies.

CONCLUSIONS

In differential diagnosis of exudative PEs, the evaluation of MMP-9 and TIMP-1 may represent a rapid, inexpensive, practical and accurate tool for differentiating exudate PEs of benign origin from those of malignant origin. They presented a high specificity with cut-off points that offered also a high sensitivity. In addition, our data suggest that MMP-9 and TIMP-1 levels are not influenced by the histology and tumour origin neither by the presence of tumour cells in PEs. Thus, their use in clinical practice could help in the selection of patients needing more invasive procedures, such as thoracoscopic biopsy.

Conflict of interest: none declared.

REFERENCES

[1] Fiorelli A, Vicidomini G, Di Domenico M, Napolitano F, Messina G, Morgillo F *et al*. Vascular endothelial growth factor in pleural fluid for differential diagnosis of benign and malignant origin and its clinical applications. Interact CardioVasc Thorac Surg 2011;12:420–4.

[2] Fiorelli A, Rizzo A, Messina G, Izzo A, Vicidomini G, Pannone G *et al*. Correlation between matrix metalloproteinase 9 and 18F-2-fluoro-2-deoxyglucose-positron emission tomography as diagnostic markers of lung cancer. Eur J Cardiothorac Surg 2012;41:852–60.

[3] van Kempen LC, Coussens LM. MMP9 potentiates pulmonary metastasis formation. Cancer Cell 2002;2:251–2.

[4] Sheen P, O'Kane CM, Chaudhary K, Tovar M, Santillan C, Sosa J *et al*. High MMP-9 activity characterises pleural tuberculosis correlating with granuloma formation. Eur Respir J 2009;33:134–41.

[5] Light RW, Mac Gregor MI, Luchsinger PC, Ball WC. Pleural effusions: the diagnostic separation of transudates and exudates. Ann Intern Med 1972; 77:507–13.

[6] Iglesias D, Alegre J, Alemán C, Ruíz E, Soriano T, Armadans L *et al*. Metalloproteinases and tissue inhibitors of metalloproteinases in exudative pleural effusions. Eur Respir J 2005;25:104–9.

[7] Himelstein BP, Muschel RJ. Induction of matrix metalloproteinase 9 expression in breast carcinoma cells by a soluble factor from fibroblasts. Clin Exp Metastasis 1996;14:197–208.

[8] Jeziorska M, Haboubi NY, Schofield PF, Ogata Y, Nagase H, Woolley DE. Distribution of gelatinase B (MMP-9) and type IV collagen in colorectal carcinoma. Int J Colorectal Dis 1994;9:141–8.

[9] Sato H, Kida Y, Mai M, Endo Y, Sasaki T, Tanaka J *et al*. Expression of genes encoding type IV collagen-degrading metalloproteinases and tissue inhibitors of metalloproteinases in various human tumor cells. Oncogene 1992;7:77–83.

[10] Iizasa T, Fujisawa T, Suzuki M, Motohashi S, Yasufuku K, Yasukawa T *et al*. Elevated levels of circulating plasma matrix metalloproteinase 9 in non-small cell lung cancer patients. Clin Cancer Res 1999;5:149–53.

[11] Jin HY, Lee KS, Jin SM, Lee YC. Vascular endothelial growth factor correlates with matrix metalloproteinase-9 in the pleural effusion. Respir Med 2004;98:115–22.

[12] Park KJ, Hwang SC, Sheen SS, Oh YJ, Han JH, Lee KB. Expression of matrix metalloproteinase-9 in pleural effusions of tuberculosis and lung cancer. Respiration 2005;72:166–75.

[13] Vatansever S, Gelisgen R, Uzun H, Yurt S, Kosar F. Potential role of matrix metalloproteinase-2,-9 and tissue inhibitors of metalloproteinase-1,-2 in exudative pleural effusions. Clin Invest Med 2009;32:E293–300.

[14] Ondo K, Sugio K, Yamazaki K, Yamaguchi M, Yano T, Yoshino I *et al*. The significance of serum active matrix metalloproteinase-9 in patients with non-small cell lung cancer. Lung Cancer 2004;46:205–13.

[15] Arnold SM, Young AB, Munn RK, Patchell RA, Nanayakkara N, Markesbery WR. Expression of p53, bcl-2, E-cadherin, matrix metalloproteinase-9, and tissue inhibitor of metalloproteinases-1 in paired primary tumors and brain metastasis. Clin Cancer Res 1999;5:4028–33.

[16] González-Avila G, Iturria C, Vadillo F, Terán L, Selman M, Pérez-Tamayo R. 72-kD (MMP-2) and 92-kD (MMP-9) type IV collagenase production and activity in different histologic types of lung cancer cells. Pathobiology 1998;66:5–16.

[17] Hurewitz AN, Zucker S, Mancuso P, Wu CL, Dimassimo B, Lysik RM *et al*. Human pleural effusions are rich in matrix metalloproteinases. Chest 1992;102:1808–14.

[18] Hrabec E, Strek M, Nowak D, Hrabec Z. Elevated level of circulating matrix metalloproteinase-9 in patients with lung cancer. Respir Med 2001;95: 1–4.

[19] Eickelberg O, Sommerfeld CO, Wyser C, Tamm M, Reichenberger F, Bardin PG *et al*. MMP and TIMP expression pattern in pleural effusions of different origins. Am J Respir Crit Care Med 1997;156:1987–92.

[20] Villena V, López-Encuentra A, Echave-Sustaeta J, Martín-Escribano P, Ortuño-de-Solo B, Estenoz-Alfaro J. Diagnostic value of CA 549 in pleural fluid. Comparison with CEA, CA 15.3 and CA 72.4. Lung Cancer 2003;40: 289–94.