

Research Article

Comparison of the Diagnostic Accuracy of the *MSLN* Gene Products, Mesothelin and Megakaryocyte Potentiating Factor, as Biomarkers for Mesothelioma in Pleural Effusions and Serum

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The *MSLN* gene products, soluble mesothelin and megakaryocyte potentiating factor (MPF), are being investigated as biomarkers for the asbestos-related cancer malignant mesothelioma (MM). Pleural fluid biomarkers of MM can be elevated when serum levels remain normal. The aim of this study was to determine if this was true for MPF and to compare levels of mesothelin. Biomarker concentrations were compared in 66 MM patients, 39 patients with other malignancies, 37 with benign disease, 18 asbestos-exposed healthy individuals, and 53 patients with chronic kidney disease. In pleural effusions, MPF and soluble mesothelin concentrations were both significantly elevated in MM patients relative to controls. No significant difference between the area under the receiver operator curve (AUC) for MPF (0.945 ± 0.02) and mesothelin (0.928 ± 0.03) when distinguishing MM from all other causes of effusion was observed. MPF and mesothelin serum concentrations were highly correlated and of equivalent diagnostic accuracy with AUCs of 0.813 ± 0.04 and 0.829 ± 0.03 , respectively. Serum levels of both markers increased with decreasing kidney function. In conclusion, MPF is elevated in the pleural effusions of MM patients similar to that of mesothelin. Mesothelin and MPF convey equivalent diagnostic information for distinguishing MM from other diseases in pleural effusions as well as serum.

1. Introduction

Malignant mesothelioma (MM) is an aggressive asbestos-induced cancer affecting the mesothelial cells lining the serosal cavities of the body including the pleura and peritoneum. The median survival is generally less than one year [1, 2]. The study of MM-specific biomarkers is being undertaken in order to improve diagnosis, assessment of disease progression, and response to treatment. Mesothelin

family proteins in particular have been identified as likely biomarkers for this disease, particularly in a diagnostic setting.

The *MSLN* gene is located on chromosome 16p13.3 and encodes at least four protein products: megakaryocyte potentiating factor (MPF) [3, 4] and three isoforms of mesothelin: variant 1 (mesothelin) [5], the as yet uncharacterised, variant 2 [5, 6], and variant 3 (soluble-mesothelin-related protein (SMRP)) [7].

TABLE 1: Comparison of antibody-based assay results for megakaryocyte potentiating factor.

Report	Manufacturer of assay	Antibodies used	Sensitivity for MM	cf benign and controls	Specificity cf malignancy	Overall
Onda et al., 2006 [12]	Authors	MPF25 and MPF49	51/56 (91%)	0/70	—	100%
Shiomi et al., 2006* [13]	Authors	7E7 and PoAb-282	7/7	0/13	0/3	100%
Shiomi et al., 2008* [14]	Authors	7E7 and PoAb-282	29/39 (74%)	—	—	87% (46/356)
Shiomi et al., 2008* [14]	Authors	7E7 and 16K16	28/39 (72%)	—	—	96% (14/356)
Creaney et al., 2008 [11]	IBL, Japan	7E7 and PoAb-282	21/66 (32%)	3/70	2/30	95%
Iwahori et al., 2008 [15]	Authors	20-10 and 41-28	20/27 (74%)	1/47	14/82	88%
Hollevoet et al., 2010* [16]	MBL, Japan	20-10 and 41-28	58/85 (68%)	10/359	3/63	97%

* Values extrapolated from the presented data.

IBL: Immuno-Biologic Laboratories (Gunma, Japan).

MBL: Medical & Biological Laboratories (Nagano, Japan).

MPF is a 31 kDa secreted cytokine-like protein which has been shown to stimulate colony formation of mouse bone marrow cells in the presence of interleukin 3 [3, 4]. The three mesothelin isoforms share a common N-terminal region. Mesothelin is a glycoposphatidylinositol-(GPI-) linked cell-surface glycoprotein of approximately 40 kDa and considered a differentiation molecule of the mesothelium [5]. Mesothelin can be cleaved or shed from the cell surface [8, 9]. SMRP lacks the GPI-anchor region responsible for cell surface attachment and has a unique C-terminal hydrophilic tail that may make the protein soluble [7].

There is in general good correlation between laboratories for the sensitivity and specificity of mesothelin to differentiate MM from benign conditions and other malignancies (reviewed in [10]); however there is disparity on the sensitivity of MPF in this setting with, at high specificities, sensitivities ranging from 32% [11] to over 90% [12, 13] (Table 1), perhaps due to the use of different antibody clones in the four different reported MPF ELISA-based assays.

Pleural effusion MPF levels should logically provide more sensitive information than serum, given the close relationship between the fluid and the tumour. Mesothelin levels were elevated in the effusions of 15% of MM patients with normal serum levels [17]. Effusion MPF levels are likely to be clinically important but have not been reported to date; therefore the *primary* aim of this study was to address this question.

Recent studies using the commercialized assay of Iwahori and colleagues [15] have found that serum mesothelin and MPF are highly correlated and have an equivalent diagnostic accuracy for MM [16, 18]. Both biomarkers were similarly affected by covariates such as age, kidney function, and body mass index as assessed in controls and by tumour stage and kidney function in MM patients [19, 20]. Although the statistical significance was unclear, MPF was twice as sensitive as serum mesothelin for stage I MM [20] when compared at the specificity of 95% in control subjects. The authors thus recommended that further research in a large cohort be undertaken, and so the *secondary* aim of the current study was to evaluate MPF in a setting for MM diagnosis and compare the results obtained with two commercially produced assays and with soluble mesothelin in a large cohort of patients.

2. Materials and Methods

2.1. Patients. Serum and pleural effusion samples were collected from patients who attended Sir Charles Gairdner Hospital, Royal Perth Hospital, or the Hollywood Specialist Centre in Perth, Western Australia. All patients provided written informed consent. The study was approved by the human research ethics committees of the three hospitals. The diagnosis was confirmed by pathologists experienced in the diagnosis of MM, lung cancer, and effusion cytology. The diagnosis of pleural plaques and asbestosis was based on accepted radiological and clinical criteria [21–23]. Nonmalignant effusions were classified as exudates or transudates on the basis of Light's criteria [24]. Effusions were classified as being infection associated if microorganisms were detected in the fluid along with inflammatory cells or if the patient had pneumonia adjacent to the effusion. Clinical followup was until death or for a median of 20 (range 1–167) months to confirm that the clinical pattern of the illness matched the diagnosis. To assess biomarkers in pleural fluid, samples from 43 MM patients were examined: 28 with epithelioid histology, 2 with biphasic, and 13 with histological data unavailable because diagnosis was made based upon clinical, radiological, and cytological evidence. For comparison, effusion samples from a group of 79 patients with non-MM malignant effusions and 143 with Nonmalignant effusions were examined (Table 2).

MPF concentrations determined using the Human N-ERC/mesothelin assay from Immuno-Biological Laboratories (IBL) were reported in a series of MM and control patients previously [11]. To assess the diagnostic accuracy of the Medical & Biological Laboratories (MBL) Molecular MPF assay, the results of the two assays were compared in a subset of the samples previously reported on. The sample set consisted of 66 MM patients: 32 were epithelioid, 3 biphasic, and 9 sarcomatoid; no histological data was available for further 22 patients who were diagnosed on clinical, radiological, and cytological evidence. The other asbestos-related disease (ARD) patients consisted of a mixture of asbestosis ($n = 3$), pleural plaques ($n = 2$), and a mixture of asbestosis and pleural plaques ($n = 9$). The group of patients designated Cancer Other consisted of breast ($n = 3$), melanoma ($n = 2$),

TABLE 2: Patient demographics and effusion mesothelin and MPF concentrations in MM patients and controls.

Group	Number of cases (number of females)	Mean age, years (range)	Effusion mesothelin (nM) ^{a,c}	Effusion MPF (ng/mL) ^{a,b,c}
MM	43 (6)	71 (42–88)	35.1 ± 7.2	1464 ± 219
Non-MM malignant effusions	79 (47)	70 (37–96)	7.1 ± 0.7***	210 ± 23***
Lung cancer	42 (17)	72 (48–92)	6.4 ± 0.9***	198 ± 25***
Breast cancer	19 (19)	65 (37–96)	5.8 ± 0.8***	201 ± 32***
Ovarian cancer	11 (11)	66 (42–86)	12.2 ± 4.1 ^{ns}	188 ± 93***
Pancreatic cancer	7 (0)	64 (56–75)	9.9 ± 2.6 ^{ns}	420 ± 147**
Benign effusions	143 (39)	66 (19–96)	2.7 ± 0.8***	96 ± 10***
ARD	16 (1)	74 (50–91)	5.5 ± 0.8***	170 ± 20***
Exudate	51 (12)	65 (19–94)	2.9 ± 0.4***	107 ± 14***
Infection	42 (10)	64 (30–96)	2.0 ± 0.3***	48 ± 13***
Transudate	34 (16)	71 (12–93)	4.0 ± 0.6***	148 ± 14***

^aexponentiated mean of log transformed data plus/minus standard error of log transformed data *100.

^bMPF concentrations determined from Human MPF Assay (MBL).

^csignificant difference between indicated cohorts and mesothelioma group as a whole ($n = 43$) as determined by Student's t -test (ns is not significant; ** is $P < 0.001$; and *** is $P < 0.0001$).

TABLE 3: Patient demographics and serum mesothelin and MPF concentrations in MM patients and controls.

Group	Number of cases (females)	Mean age, years (range)	Serum mesothelin (nM) ^{a,d}	Serum N-ERC (MPF) (ng/mL) ^{a,b,d}	Serum MPF (ng/mL) ^{a,c,d}
MM	66 (5)	66 (48–84)	4.29 ± 0.7	0.16 ± 0.05	40.37 ± 6.1
Cancer lung	19 (7)	72 (58–82)	1.77 ± 0.4**	0.23 ± 0.1**	15.13 ± 3.4**
Cancer other	10 (5)	73 (50–82)	0.87 ± 0.3***	0.58 ± 0.19***	13.07 ± 2.3**
Asbestos exposed	18 (8)	61 (37–86)	0.41 ± 0.1***	0.04 ± 0.02***	9.66 ± 1.6***
ARD	14 (1)	73 (60–82)	1.07 ± 0.1***	0.10 ± 0.06***	12.19 ± 2.3***
PE-benign	23 (7)	68 (32–86)	0.70 ± 0.2***	0.07 ± 0.3***	9.53 ± 1.2***

^aexponentiated mean of log transformed data plus/minus standard error of log transformed data *100.

^bMPF concentrations determined from Human N-ERC/Mesothelin Assay (IBL).

^cMPF concentrations determined from Human MPF Assay (MBL).

^dsignificant difference between indicated cohorts and the mesothelioma cohort as a whole ($n = 66$) as determined by Student's t -test (** is $P < 0.001$; *** is $P < 0.0001$).

pancreatic ($n = 2$) and one each of lymphoma, melanoma, and thyroid cancers. The group of patients with benign pleural effusions consisted of exudates ($n = 18$), including 8 related to infection, and transudates ($n = 5$) (Table 3).

Kidney function effects on MPF levels were determined on a subset of randomly selected samples previously assessed for serum mesothelin [25], with approximately 30 patients in each of the Stages II to V chronic kidney disease (CKD) [26]; none of the participants had a previous history of malignancy (Table 4).

2.2. Biomarker Assays. Soluble mesothelin concentrations were determined using the MESOMARK assay (Fujirebio Diagnostics, Malvern, PA, USA). MPF concentrations were determined using either the Human N-ERC/Mesothelin Assay kit (Immuno-Biological Laboratories (IBL), Gunma, Japan) or the Ab-Match ASSEMBLY Human MPF kit and Ab-Match UNIVERSAL kit (Medical & Biological Laboratories (MBL), Nagano, Japan). Biomarker concentrations were performed in duplicate according to the manufacturers' protocols. Concentrations were determined from a standard

curve performed on each plate. Dilution of samples was carried out if necessary using the diluent supplied by the manufacturer. The limit of detection (LOD) for the assays was reported to be 0.3 nM for the MESOMARK, 0.024 ng/mL for N-ERC/Mesothelin, and 0.03 ng/mL for the MPF assay. For the purposes of data analysis, concentrations of samples below LOD were assigned the value of the respective LOD.

2.3. Statistical Analysis. Selection of random subsets of patients was performed using the random sampling function in Microsoft Excel (Redmond, WA, USA). Differences between groups of patients were assessed by Student's t -test on log scale values to normalize the distribution. A two-sided P value < 0.05 was accepted as significant. The area under the curve (AUC) of Receiver Operating Characteristic (ROC) curves was compared using the method of DeLong et al. [28] and results reported as AUC ± 95% confidence interval (CI). Spearman's correlation coefficient was used to assess correlations between different biomarkers. Statistical analysis was performed using GraphPad Prism (California, USA) and Medcalc software (Mariakerke Belgium).

TABLE 4: Patient demographics and serum creatinine, mesothelin, and MPF concentrations in patients with chronic kidney disease (CKD).

CKD stage	Number of cases (number of females)	Mean age, years (range)	Median eGFR ^a (mL/min/1.73 m ²) (range)	Serum mesothelin (nM) ^{b,d}	Serum MPF (ng/mL) ^{b,c,d}
II	30 (12)	68 (25–95)	74 (62–89)	1.05 ± 0.09	16.5 ± 1.8
III	32 (16)	68 (42–91)	40 (30–59)	1.4 ± 0.15*	32.1 ± 2.86***
IV	30 (12)	64 (32–96)	22 (16–28)	2.4 ± 0.19***	46.2 ± 5.4***
V	29 (13)	66 (46–96)	12 (3–14)	2.8 ± 0.29***	53.6 ± 7.6***

^aestimated glomerular filtration rate was calculated using the Modified Diet in Renal Disease (MDRD) Formula [27].

^bexponentiated mean of log transformed data plus/minus standard error of log transformed data *100.

^cMPF concentrations determined from Human MPF Assay (MBL).

^dsignificant difference between indicated cohorts and the Stage II CKD patient group ($n = 30$) as determined by Student's t -test (* is $P < 0.05$; *** is $P < 0.0001$).

3. Results

3.1. Pleural Effusion Biomarker Concentrations. As MPF has been reported to have cytokine-like functions and also suggested to be associated with thrombocytosis, MPF concentrations were examined in effusions from patients with inflammatory and infective conditions in addition to patients with non-MM malignancies.

The estimated median concentration of MPF in MM patients was 1464 ± 219 ng/mL and was significantly higher than that in patients with benign effusions grouped together (96 ± 10 ng/mL; $P < 0.0001$) and also higher than that in all patients with other malignancies grouped together (210 ± 23 ng/mL; $P < 0.0001$) (Table 2; Figure 1). There was no significant difference between the effusion MPF concentration in patients with benign effusions related to infection and those of other benign causes.

The estimated median concentration of effusion mesothelin in MM patients was 33 ± 7.2 nM and was significantly higher than that in patients with benign effusions grouped together (3 ± 0.8 nM; $P < 0.0001$) and also higher than that in all patients with other malignancies grouped together (7.1 ± 0.7 nM; $P < 0.0001$) (Table 2; Figure 1). There was a significant correlation between effusion MPF and mesothelin results ($r = 0.809$; $P < 0.0001$).

ROC curves were generated to assess the ability of the effusion markers to distinguish between patients with MM from all the other patients ($n = 265$). The AUC (95% CI) of MPF was 0.945 (0.923–0.977), not significantly different from that of mesothelin (0.928; (0.890–0.956)) (Figure 1). At a specificity of 95% relative to healthy or patients with benign disease ($n = 55$), sensitivity of effusion MPF was 81% (threshold = 600 ng/mL) and that of mesothelin was 70% (threshold = 20 nM).

3.2. Serum MPF. Serum MPF was measured using two different commercial assays. Using the N-ERC assay (IBL) N-ERC/MPF concentrations in the serum of patients with MM ranged from undetectable to 5.9 ng/mL. The estimated median concentration of N-ERC/MPF in MM patients was 0.16 ± 0.05 ng/mL and was not significantly different from that in the healthy individuals and patients with benign conditions grouped together (0.06 ± 0.02 ng/mL) nor was it

different from that in all patients with other malignancies grouped together (0.34 ± 0.1 ng/mL) (Figure 2).

In the same sample set, using the MPF assay (MBL), MPF concentrations in the serum of MM patients ranged from 2.6 to 622 ng/mL and were several orders of magnitude higher than those observed with the IBL assay. The mean concentration of MPF in MM patients was 40.4 ± 6.1 ng/mL and was significantly higher than that in the healthy individuals and patients with benign conditions grouped together (Figure 2). There was a significant but weak correlation between the results of the MPF assays ($r = 0.263$; $P < 0.001$) (Figure 2).

3.3. Serum Mesothelin and MPF. Serum mesothelin was measured using the MESOMARK assay. Concentrations in the serum of patients with MM ranged from undetectable to 85 nM. The mean concentration of mesothelin in MM patients was 4.3 ± 0.7 nM and was significantly higher than that in patients with other malignancies grouped together (1.39 ± 0.3 nM; $P < 0.0001$) and also higher than that in the patients with benign conditions and healthy individuals grouped together (0.66 ± 0.1 nM; $P < 0.0001$) (Figure 2). There was a significant correlation between MPF (MBL assay) and mesothelin results in these patients ($r = 0.706$; $P < 0.0001$) (Figure 2).

ROC curves were generated to assess the ability of the markers to distinguish between patients with MM from all the other cases in this study set ($n = 150$). The AUC for the N-ERC/MPF was 0.549 (0.465–0.630), for the MPF was 0.813 (0.741–0.872), and for mesothelin was 0.829 (0.759–0.886) (Figure 2). The AUC for N-ERC/MPF was significantly less than that for MPF and for mesothelin ($P < 0.0001$). There was no difference in the AUC for serum MPF and for mesothelin in this patient set ($P = 0.623$).

At a specificity of 95% relative to healthy or patients with benign disease ($n = 55$), sensitivity of serum N-ERC was 29% (threshold = 1.6 ng/mL), of serum MPF was 52% (threshold = 33.2 ng/mL), and of serum mesothelin was 61% (threshold = 2.4 nM).

3.4. Kidney Function and Serum Biomarker Values. Serum mesothelin levels increased with decreasing kidney function, as previously reported [25] (Figure 3). In these patients, serum MPF concentrations increased with diminishing renal

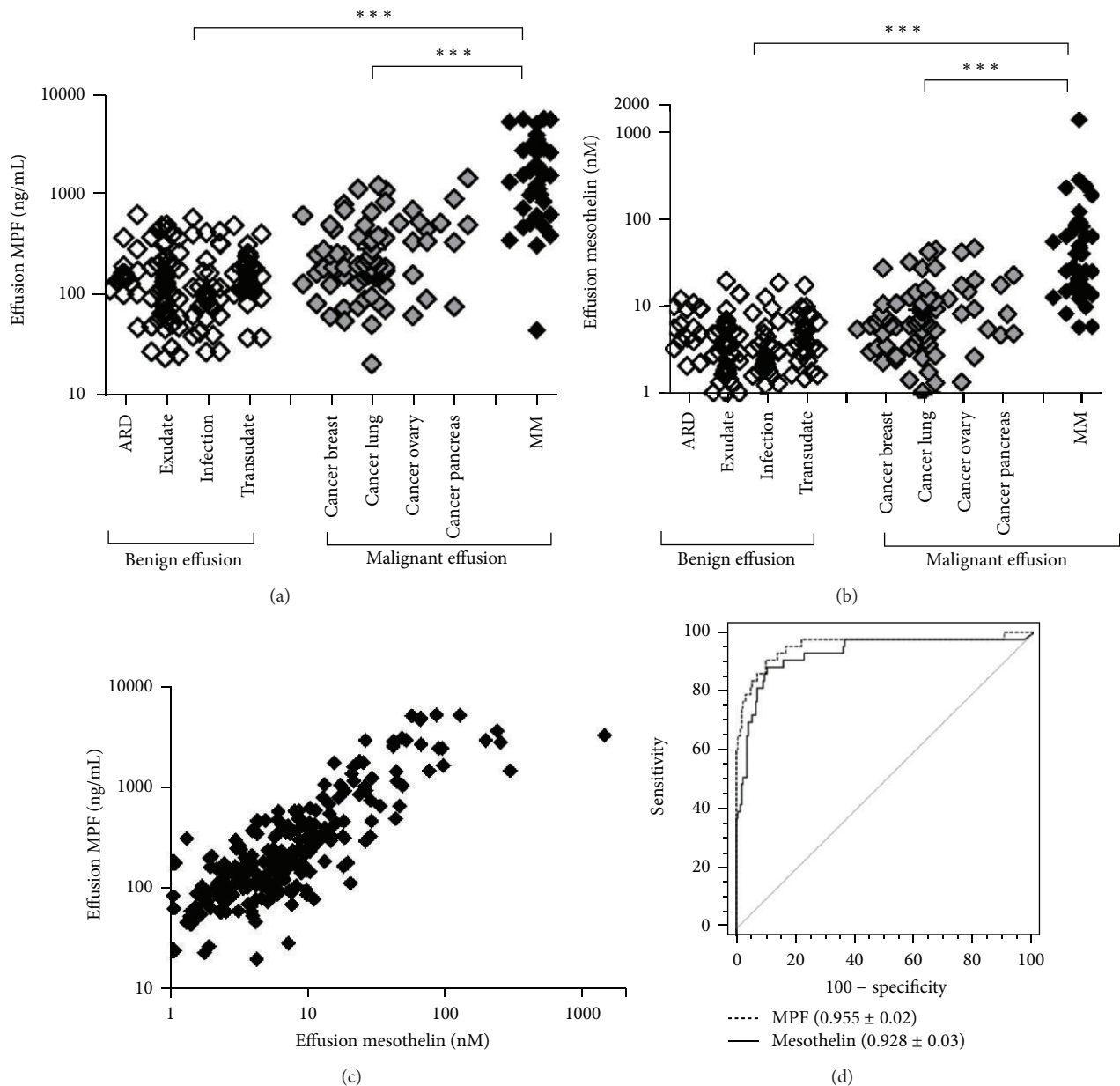


FIGURE 1: Biomarker concentrations in pleural effusions: (a) MPF as determined using assay from MBL; (b) soluble mesothelin concentrations; (c) correlation between MPF concentrations determined by MBL assays and mesothelin; and (d) Receiver Operating Characteristic (ROC) curves showing accuracy of effusion biomarkers in differentiating all patients with MM ($n = 43$) from all other cases ($n = 222$). (Significant difference between indicated cohorts: *** $P < 0.0001$.)

function (range 4.5 to 173 ng/mL) (Figure 3). The group mean MPF level for patients with Stage II kidney disease (16.5 ± 1.8 ng/mL) was significantly lower than that for patients with Stages III, IV, and V ($P < 0.001$). There was a strong correlation between mesothelin and MPF concentrations in these samples ($r = 0.731$; $P < 0.0001$). Patient age, sex, smoking, asbestos exposure, or diabetes were not associated with MPF concentration. Of the 121 samples from patients with CKD Stages II–V in this study, 70 (58%) were found to have MPF concentration greater than 30 ng/mL.

4. Discussion

The main products of the *MSLN* gene on chromosome 16p13.3, mesothelin and MPF, are elevated in the serum of MM patients. This study demonstrated that MPF is also elevated in the pleural effusions of MM patients and that there is strong correlation between the two markers. There was no difference in the diagnostic accuracy for MM in this study between soluble mesothelin and MPF.

The choice of antibody pair in ELISA can influence the accuracy of a test. Previous studies using assays based

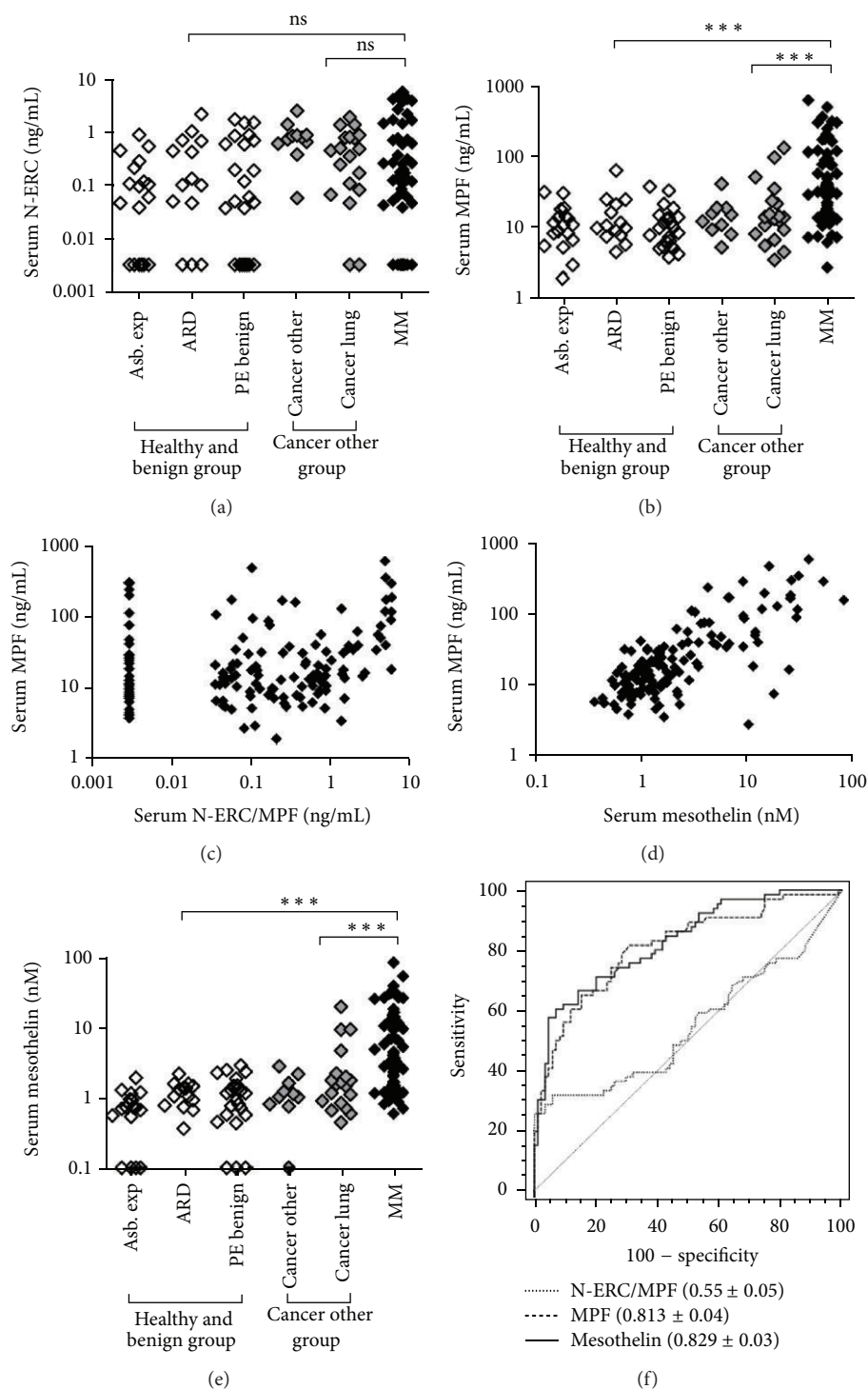


FIGURE 2: Biomarker concentrations in serum: biomarker concentrations were determined at least in duplicate by ELISA and individual patient values plotted. (a) MPF as determined using N-ERC assay from IBL; (b) MPF as determined using assay from MBL; (c) correlation between MPF concentrations determined by the two different independent assays; (d) correlation between MPF concentrations determined by MBL assays and mesothelin; (e) soluble mesothelin concentrations; and (f) Receiver Operating Characteristic (ROC) curves showing accuracy of serum biomarkers in differentiating all patients with MM ($n = 66$) from all other cases ($n = 84$). (Significant difference between indicated cohorts: ns is not significant; *** $P < 0.0001$.)

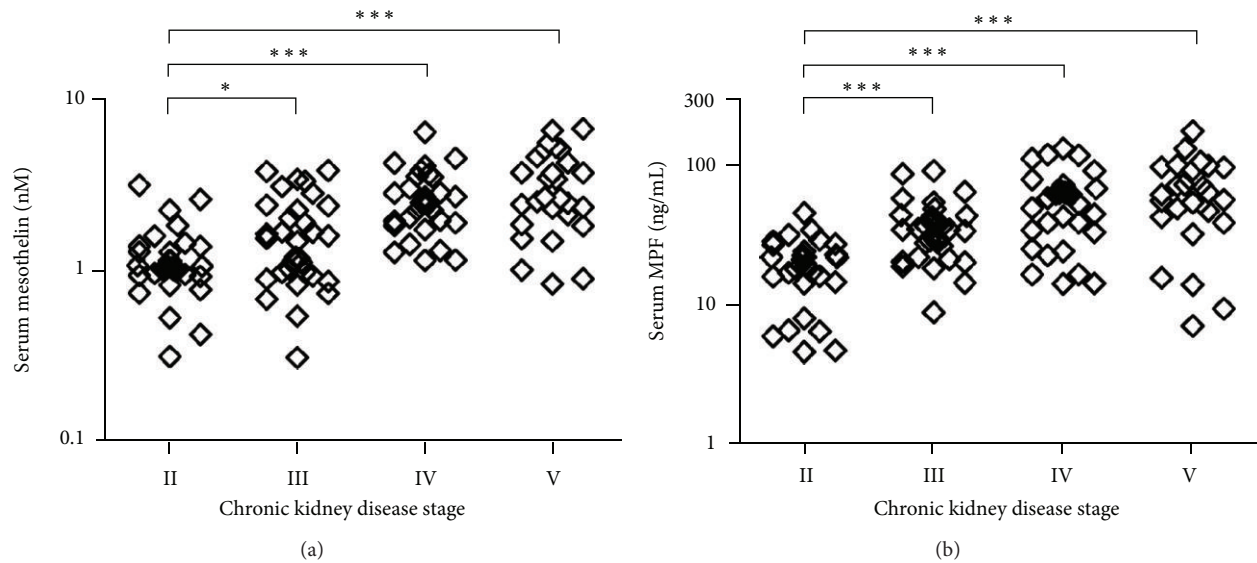


FIGURE 3: Biomarker concentrations in serum of patients with chronic kidney disease: (a) soluble mesothelin concentrations and (b) MPF concentrations as determined using assay from MBL. (Significant difference between indicated cohorts: * $P < 0.05$; *** $P < 0.0001$.)

on four different anti-MPF antibody pairs have generally reported favourable results for MPF as an MM biomarker (Table 1) [12, 14–16]. The current study directly compared two of these assays and found that the assay using the antibodies 20–10 and 41–28 (as developed by [15] and manufactured by MBL) was superior to the other assay examined with comparable diagnostic accuracy and clinical performance to that of mesothelin, the most well characterised biomarker for MM.

The previous inconsistent results regarding the diagnostic accuracy of serum MPF as a marker for MM, where several studies found greater than 90% sensitivity for MM at high specificity [12, 13], compared to one study reporting a sensitivity of 32% [11] are likely attributable to the use of different assays. To our knowledge the MPF assay utilising the antibody pair 7E7 and PoAb-282, as used in the latter study, is no longer commercially available. The current findings support a previous work that found serum MPF to be a viable biomarker for MM [16].

Over 70% of MM patients present with an effusion [29, 30], and such effusions provide specimens that can be used for diagnosis and clinical management decisions. This study has shown for the first time that MPF is elevated in the pleural fluid of MM patients. Based upon the fact that effusions in MM are in contact with the tumour and that mesothelin has previously been shown to be elevated in effusions [17, 31, 32], this was an expected finding. However, some other MM-associated biomarkers, for example CA125 and osteopontin, are, in addition to being elevated in MM effusions, also elevated in various benign conditions thus limiting their clinical utility [33, 34]. Given that MPF was originally identified because of its cytokine-like activity *in vitro* [3, 4], a function not previously associated with the related gene product mesothelin, it was considered important

to examine the association between MPF concentrations in effusions and inflammatory conditions. In the current study MPF concentrations were of a similar magnitude in benign effusions related to infective or other causes. Any cytokine-associated function of MPF occurring in infective conditions did not affect the diagnostic utility of the marker for MM.

A strong correlation between MPF and mesothelin in serum was found in this study, consistent with previous studies [18]. There was no significant difference in the diagnostic accuracy of the two markers for MM, and both markers behaved in a very similar fashion in relation to changes in kidney function. Whilst little is known about the transcriptional and/or translational regulation of these two proteins, as both MPF and mesothelin are products of the *MSLN* gene [35], conceivably they could be produced in equimolar amounts by a cell, as reflected by the equivalence in serum levels.

Similarly, there was a strong correlation between effusion MPF and mesothelin concentrations, with no significant difference in their diagnostic accuracy for MM. This raises the possibility that the assays are measuring the same protein; however no cross-reactivity was observed between the antibodies and the standards of the MPF and mesothelin assay kits (data not shown). Why there is such concordance between the two proteins is difficult to envision given that cell-bound mesothelin must be cleaved or shed from the cell surface to be detected in solution. This cleavage must be tightly regulated to account for the strong correlation between mesothelin and MPF expressions. There is therefore the technical possibility that the MPF-mesothelin preprotein is not being cleaved (at the putative furin-cleavage site [35]) and that the approximately 70 kDa molecule is being detected by both assays. This is currently under investigation.

In conclusion MPF is elevated in the pleural effusions of MM patients, and there is a strong correlation between

mesothelin and MPF. Mesothelin and MPF convey equivalent diagnostic data in pleural effusions as well as serum and thus, in a clinical setting, could be used interchangeably.

Conflict of Interests

All of the authors declare no conflicting interests.

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