A prospective trial evaluating the role of mesothelin in undiagnosed pleural effusions

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ABSTRACT: Mesothelin has been proposed as a useful tool in the diagnosis of malignant pleural mesothelioma (MPM). We aimed to examine its diagnostic utility and the impact of renal impairment on results.

We prospectively recruited 230 patients with new undiagnosed pleural effusions, testing serum (n=216) and pleural fluid (n=206) mesothelin (by ELISA) during the initial consultation.

28 (12%) out of 230 patients had MPM. Serum mesothelin gave sensitivity 59.3%, specificity 64.7%, negative predictive value (NPV) 91.2%, positive predictive value (PPV) 20.5%, and pleural fluid sensitivity 72.0%, specificity 87.5%, NPV 95.5%, PPV 46.2% for distinguishing effusions due to MPM. In a matched comparison, diagnostic characteristics of pleural fluid mesothelin were superior to serum (p=0.0001). Serum mesothelin levels in patients without MPM were higher in patients with renal impairment (p=0.007) while pleural fluid levels were unaffected. 19 (54%) out of 35 patients with a benign pleural effusion and an estimated glomerular filtration rate $\leqslant$59 mL·min^{-1} had a false-positive serum mesothelin result.

The diagnostic accuracy of pleural fluid mesothelin is superior to that of serum and is unaffected by renal function. In patients with a low pre-test probability of mesothelioma, a negative mesothelin test could be reassuring, because of its high NPV. Routine use of mesothelin testing in undiagnosed pleural effusions at presentation appears to be unhelpful.

KEYWORDS: Benign asbestos-related effusion, biomarker, diagnosis, malignancy, mesothelioma, predictive value

Malignant pleural mesothelioma (MPM) is an increasingly common malignancy worldwide with a predicted peak in incidence between 2015 and 2025 in many countries [1]. Most patients present with a pleural effusion and associated breathlessness, a clinical picture with a wide range of possible malignant and benign aetiologies [2]. The diagnosis of MPM can be elusive, particularly in frail patients who are unfit for the most invasive of available diagnostic procedures such as video-assisted thoracoscopy and biopsy [3]. Pleural fluid cytology is of low sensitivity and discrimination between mesothelial cellular atypia due to inflammation and MPM can be challenging [4]. Computed tomography (CT) scan appearances, while of reasonable specificity for identifying malignant thickening of the pleura, distinguish poorly between MPM and adenocarcinoma [5].

A history of asbestos exposure is common amongst patients presenting with a new pleural effusion, many of whom are ultimately diagnosed with benign disease but must be investigated extensively to exclude a malignant diagnosis [3]. In particular, the diagnosis of benign asbestos-related pleural effusion (BAPE) can currently only be made with confidence following benign pleural biopsy and prolonged radiographic follow-up [6].

Serum or pleural fluid biomarkers that could rule out MPM in high-risk groups, accurately raise diagnostic suspicion in lower risk patients and/or clarify non-diagnostic test results would be valuable.

There have been several studies examining the use of serum mesothelin, a 40-kDa glycoprotein product of mesothelial cells, as a diagnostic and/or screening tool for mesothelioma, and a recent meta-analysis concluded summary estimates of

This article has supplementary material available from www.erj.ersjournals.com

64% sensitivity and 89% specificity [7] for the test, which can be performed as a commercially available ELISA. Most studies have employed tissue bank samples, selecting defined diagnostic sub-groups rather than true consecutive series of patients presenting in clinical practice.

The two largest studies to date, examining the use of pleural fluid for mesothelin testing, gave sensitivities of 67% and 71% and specificities of 98% and 89% for the diagnosis of mesothelioma [8, 9]. DAVIES *et al.* [9] demonstrated promising diagnostic characteristics for pleural fluid mesothelin in the clarification of non-diagnostic pleural fluid cytology.

Renal impairment has recently been shown to be an independent predictor of elevated serum mesothelin in patients without pleural disease and false-positive rates of >50% have been shown in control populations with stage 3 chronic kidney disease (CKD) or greater [10, 11]. A further study by HOLLEVOET *et al.* [12] demonstrated an independent association between age, glomerular filtration rate and body mass index and serum mesothelin. The effect of clinical variables such as renal function on mesothelin levels in pleural fluid has not been fully explored.

The aim of the current study was to apply serum and pleural fluid mesothelin testing to a ''real-world'' prospective consecutive series of patients presenting with an undiagnosed pleural effusion and examine diagnostic utility throughout the standard investigation pathway, compare accuracy between serum and pleural fluid and establish the impact of renal impairment on results.

MATERIALS AND METHODS
The study was approved by North Bristol NHS Trust Research Ethics Committee, Bristol, UK (08/H0102/11).

Consecutive patients presenting to the pleural disease service of a large UK teaching hospital between July 2008 and July 2010 with an undiagnosed pleural effusion requiring investigation were approached for consent. All recruited patients gave informed written consent.

All patients underwent a comprehensive clinical assessment adhering to British Thoracic Society Guidelines [6].

Cytology/histology techniques and CT scan reporting protocol are described in the online supplementary material.

Clinical data collection
Clinical data were collected prospectively throughout the patients' involvement in the study. Case notes were reviewed a further 12 months after trial entry to establish the final diagnosis.

Mesothelin quantification
Paired serum and pleural fluid samples were collected in serum gel separator tubes and centrifuged at $1,000 \times g$ for 20 min. The supernatant was stored at -70°C for later assay. Mesothelin concentrations were measured with a commercially available ELISA (Mesomark®; Fujirebio Diagnostics Inc., Malvern, PA, USA). Clinical team members were blind to the mesothelin results.

Method of estimated glomerular filtration rate (eGFR) calculation is given in the online supplementary material.

Final diagnosis and follow-up
Patients were followed-up to a histological or microbiological diagnosis, to complete resolution of the pleural effusion, to death or for a minimum of 12 months. The final diagnosis of each effusion was established independently by two consultant chest physicians using predefined diagnostic criteria by comprehensive review of investigation results and case notes, blind to the mesothelin result. Patients who did not fulfil any diagnostic category following exhaustive investigation and 12 months follow-up were considered ''undiagnosed''. Full diagnostic criteria are described in the online supplementary material.

Statistical analysis
Analyses were performed using STATA (StataCorp, College Station, TX, USA) version 12.0 and Prism (GraphPad Software, La Jolla, CA, USA) version 5.0. Differences in the distribution of serum mesothelin and pleural fluid mesothelin levels were compared between patients with and without MPM. Due to the non-normality of the data (based on the D'Agostino–Pearson omnibus normality test), the Mann–Whitney U-test was used to compare medians.

The diagnostic accuracy of serum and pleural fluid was then compared first using a fixed specificity of 95% (obtained from a receiver operating characteristic (ROC) analysis), including patients for whom both sample types was available, and secondly, including all available results, using pre-defined cut-off levels that have been previously recommended for use in practice. Serum testing used the test manufacturer's cut-off level of 1.5 nM and pleural fluid testing used the best cut-off established in previous studies of 20 nM [8, 9, 13]. 95% confidence intervals are given for measures of diagnostic accuracy. Non-parametric ROC curves were obtained, and the area under the curve (AUC) was compared between serum and pleural fluid for the paired data. Patients who were undiagnosed or had ''radiographic only'' diagnoses of malignancy were excluded from all analysis of diagnostic accuracy.

A sub-group analysis was performed to determine whether significant CKD affected test results. Patients were classified into groups based on eGFR results ($\leqslant 59$ mL·min^{-1} (stage 3 CKD or greater) *versus* >59 mL·min^{-1}), and the two groups were compared in terms of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

RESULTS
Patient recruitment, inclusions, exclusions and demographics are detailed in figure 1.

Agreement between reviewing consultants with regards to the final diagnosis for cause of effusion was good with an un-weighted κ concordance score of 0.96 (95% CI 0.93–0.99).

Table 1 summarises patient diagnoses and mesothelin levels.

Serum and pleural fluid mesothelin levels in diagnostic patient sub-groups
The median (interquartile range) serum mesothelin level amongst patients with MPM (n=27) was 2.10 (0.80–6.91) nM, significantly greater than in those with confirmed non-MPM diagnosis (n=176) (1.17 (0.90–1.91) nM; p=0.04). Median
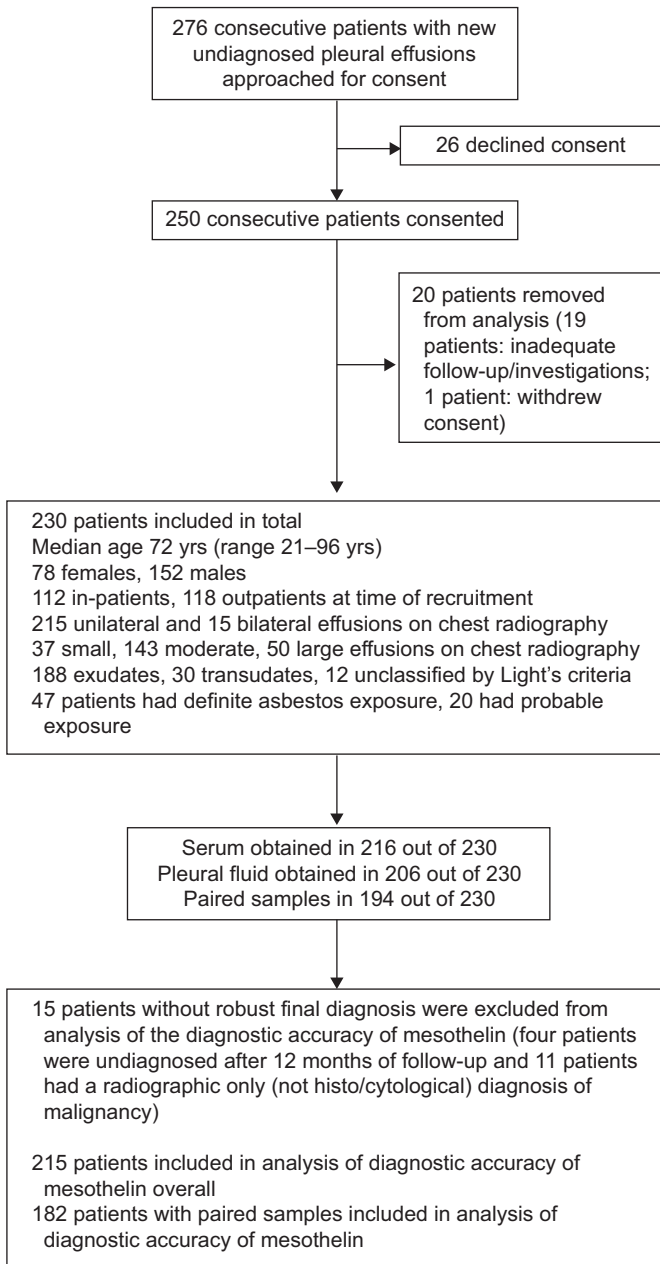
276 consecutive patients with new undiagnosed pleural effusions approached for consent

→ 26 declined consent

250 consecutive patients consented

→ 20 patients removed from analysis (19 patients: inadequate follow-up/investigations; 1 patient: withdrew consent)

230 patients included in total
Median age 72 yrs (range 21–96 yrs)
78 females, 152 males
112 in-patients, 118 outpatients at time of recruitment
215 unilateral and 15 bilateral effusions on chest radiography
37 small, 143 moderate, 50 large effusions on chest radiography
188 exudates, 30 transudates, 12 unclassified by Light's criteria
47 patients had definite asbestos exposure, 20 had probable exposure

Serum obtained in 216 out of 230
Pleural fluid obtained in 206 out of 230
Paired samples in 194 out of 230

15 patients without robust final diagnosis were excluded from analysis of the diagnostic accuracy of mesothelin (four patients were undiagnosed after 12 months of follow-up and 11 patients had a radiographic only (not histo/cytological) diagnosis of malignancy)

215 patients included in analysis of diagnostic accuracy of mesothelin overall
182 patients with paired samples included in analysis of diagnostic accuracy of mesothelin

FIGURE 1. Patient recruitment, inclusions, exclusions and demographics.

pleural fluid mesothelin level in patients with MPM (n=25) was 41.2 (14.7–70.0) nM, also significantly greater than non-MPM patients (n=168) (5.90 (2.85–11.0) nM; p<0.0001).

Positive serum mesothelin results were observed in 26 (38%) out of 69 patients and positive pleural fluid results in 19 (26%) out of 71 patients with non-MPM malignant pleural effusions, including those with adenocarcinoma of unknown primary, breast carcinoma, cholangiocarcinoma, chronic lymphocytic leukaemia, B-cell lymphoma, nonsmall cell lung cancer, ovarian carcinoma, primary peritoneal carcinoma, renal cell carcinoma and small cell lung cancer (fig. 2). A full breakdown of malignant histological sub-types is given in the online supplementary material.

TABLE 1 Final diagnosis and serum and pleural fluid mesothelin levels

Diagnosis	Patients	Serum mesothelin		Pleural fluid mesothelin	
		Patients	nM	Patients	nM
MPM (all biopsy proven)	28 (23 epithelioid, 3 sarcomatoid, 2 biphasic)	27	2.10 (0.80–6.91)	25	41.2 (14.7–70.2)
Malignant effusion (not MPM) (all histology or cytology proven)	74	69	1.12 (0.80–2.10)	71	6.50 (3.29–23.0)
Radiographic diagnosis of pleural malignancy (unconfirmed malignant effusion)	11	9	1.09 (0.80–2.15)	9	10.9 (4.24–20.0)
Benign asbestos related effusion	13	13	1.45 (0.90–2.10)	10	9.50 (7.4–18.9)
Cardiac cause	26	24	1.03 (0.91–2.06)	24	5.70 (3.41–9.07)
Pleural infection	33	31	1.2 (0.80–1.56)	30	5.70 (3.41–9.07)
Simple parapneumonic	11	11	1.59 (1.10–2.00)	8	4.55 (3.25–10.0)
Idiopathic pleuritis	8	8	0.80 (0.55–1.70)	5	8.70 (3.70–13.1)
Other benign causes	22	20	1.11 (0.83–1.98)	20	8.85 (4.87–10.9)
Undiagnosed	4	4	1.20 (0.52–10.5)	4	4.30 (3.20–17.7)
Total	230	216		206	

Data are presented as n or median (interquartile range). MPM: malignant pleural mesothelioma.
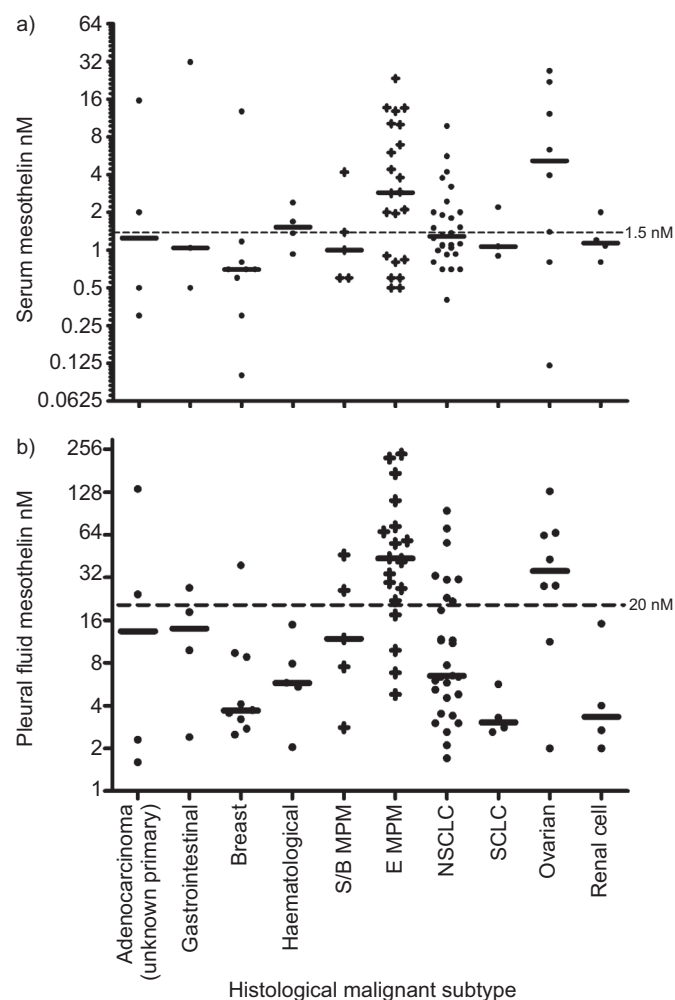
FIGURE 2. a) Serum and b) pleural fluid mesothelin levels in histological sub-types of patients with malignant pleural effusions. Median levels in each group and positive cut-off level for mesothelin are shown. S/B MPM: sarcomatoid/biphasic malignant pleural mesothelioma; E MPM: epithelioid MPM; NSCLC: nonsmall cell lung cancer; SCLC: small cell lung cancer.

False-negative serum mesothelin was seen in three out of three patients with sarcomatoid MPM, one out of two biphasic MPM patients and seven out of 22 patients with epithelioid MPM, while a false-negative pleural fluid mesothelin was seen in three out of three sarcomatoid MPM and four out of 20 epithelioid MPM cases for whom samples were available.

Diagnostic characteristics of mesothelin for distinguishing MPM from other causes of pleural effusion

Excluding unconfirmed malignancy (radiographic diagnosis) and undiagnosed patients, serum mesothelin was examined in the group as a whole at a cut-off of 1.5 nM, yielding sensitivity 59.3% (95% CI 38.8–77.6%), specificity 64.7% (95% CI 57.2–72.0%), PPV 20.5% (95% CI 12.2–31.1%) and NPV 91.2% (95% CI 84.8–95.5%) (table 2).

Pleural fluid mesothelin was examined at a cut-off of 20 nM, giving sensitivity 72.0% (95% CI 50.6–87.9%), specificity 87.5% (95% CI 81.5–92.8%), PPV 46.2% (95% CI 30.1–62.8%), NPV 95.5% (95% CI 90.8–98.2%) (table 3).

TABLE 2	Diagnostic characteristics of serum mesothelin at a cut-off of 1.5 nM		
Serum mesothelin	**MPM**	**Not MPM**	**Total**
⩾1.5 nM	16	62[#]	78
<1.5 nM	11[¶]	114	125
Total	27	176	203

Data are presented as n. MPM: malignant pleural mesothelioma. [#]: false-positive results occurred in 36 patients with benign pleural effusions and 26 with non-MPM malignant pleural effusions; [¶]: false-negative results were seen in three patients with sarcomatoid, one biphasic and seven epithelioid MPM.

Mesothelin testing within the investigation pathway for an undiagnosed pleural effusion

Data regarding the diagnostic accuracy of mesothelin testing in the context of cytology, CT scan and pleural biopsy results, and in patients with a final diagnosis of BAPE are given in the online supplementary material.

Comparative diagnostic accuracy of mesothelin measured in serum and pleural fluid

Considering only the 182 patients with a confirmed final diagnosis and both serum and pleural fluid mesothelin results (24 with MPM), the AUC for serum mesothelin in the diagnosis of mesothelioma was 0.61 (95% CI 0.46–0.77), which is significantly inferior to the AUC for pleural fluid at 0.85 (95% CI 0.77–0.94; p=0.0001) (fig. 3).

Diagnostic cut-off levels derived from both ROC curves at 95% specificity gave sensitivity 25.0% (95% CI 12.0–45.0%), PPV 46.0% (95% CI 23.0–71.0%) and NPV 89.0% (95% CI 84.0–93.0%) for serum (cut-off 0.975 nM), and sensitivity 50.0% (95% CI 31.0–69.0%), PPV 63.0% (95% CI 41.0–81.0%) and NPV 93.0% (95% CI 87.0–96.0%) for pleural fluid (cut-off 43.0 nM).

The impact of significant CKD

Contemporaneous creatinine and eGFR measurements were available for 229 out of 230 patients at the time of sample collection.

74 (32%) out of 229 patients had an eGFR ⩽59 mL·min^{-1} (stage 3 CKD or greater).

TABLE 3	Diagnostic characteristics of pleural fluid mesothelin at a cut-off of 20 nM		
Pleural fluid mesothelin	**MPM**	**Not MPM**	**Total**
⩾20.0 nM	18	21[#]	39
<20.0 nM	7[¶]	147	154
Total	25	168	193

Data are presented as n. MPM: malignant pleural mesothelioma. [#]: False-positive results occurred in two patients with benign pleural effusions and 19 with non-MPM malignant pleural effusions; [¶]: false-negative results were seen in three patients with sarcomatoid MPM and four patients with epithelioid MPM.
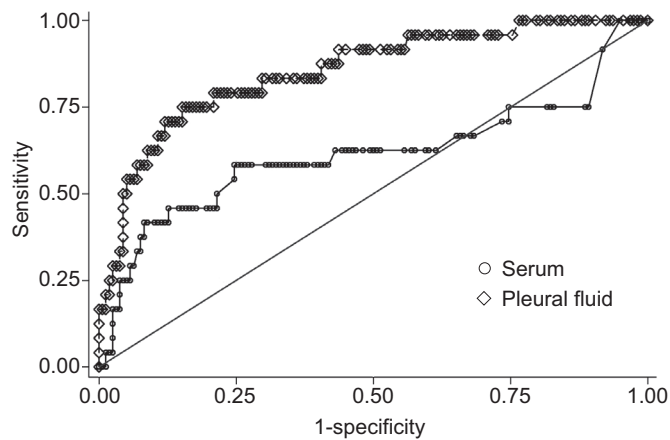
FIGURE 3. Receiver operating characteristics curves comparing the diagnostic accuracy of serum and pleural fluid mesothelin testing for the diagnosis of malignant pleural mesothelioma. Patients with both sample types and a definitive final diagnosis were included (n=182).

Specificity of serum mesothelin was 72.3% (95% CI 63.3–80.1%) in patients with an eGFR >59 mL·min^{-1} but 49.1% (95% CI 35.6–62.7%) in those with eGFR ≤59 mL·min^{-1} (tables 4 and 5). 19 (54%) out of 35 patients with a benign cause for pleural effusion but eGFR ≤59 mL·min^{-1} had a false-positive serum mesothelin result.

Diagnostic characteristics of pleural fluid mesothelin were unaffected by renal function. Specificity with eGFR >59 mL·min^{-1} was 86.0% (95% CI 78.2–91.8%) and with eGFR ≤59 mL·min^{-1}, 87.0% (95% CI 75.1–94.6%).

In patients with a confirmed non-MPM diagnosis, median serum mesothelin was 1.54 (1.00–2.40) nM in patients with eGFR ≤59 mL·min^{-1}, significantly higher than with an eGFR >59 mL·min^{-1} at 1.1 (0.80–1.59) nM. Median pleural fluid mesothelin was not significantly different with (6.22 (3.0–11.07) nM) and without (5.41 (2.66–10.99) nM) renal impairment (fig. 4).

TABLE 4	Diagnostic accuracy of serum mesothelin in patients with estimated glomerular filtration rate >59 mL·min^{-1}, excluding those undiagnosed or with unconfirmed malignancy		
Serum mesothelin	**MPM**	**Not MPM**	**Total**
≥1.5 nM	9	33	42
<1.5 nM	10	86	96
Total	19	141	138
Sensitivity %	47.4 (24.5–71.2)		
Specificity %	72.3 (63.3–80.1)		
PPV %	21.0 (10.3–36.8)		
NPV %	89.6 (81.6–94.9)		

Data are presented as n or % (95% CI). MPM: malignant pleural mesothelioma; PPV: positive predictive value; NPV: negative predictive value.

TABLE 5	Diagnostic accuracy of serum mesothelin in patients with estimated glomerular filtration rate ≤59 mL·min^{-1} (chronic kidney disease stage ≥3), excluding those undiagnosed or with unconfirmed malignancy		
Serum mesothelin	**MPM**	**Not MPM**	**Total**
≥1.5 nM	7	29	36
<1.5 nM	1	28	29
Total	8	57	65
Sensitivity %	88.9 (51.8–99.7)		
Specificity %	49.1 (35.6–62.7)		
PPV %	21.6 (9.8–38.2)		
NPV %	96.6 (82.2–99.9)		

Data are presented as n or % (95% CI). MPM: malignant pleural mesothelioma; PPV: positive predictive value; NPV: negative predictive value.

There was no statistically significant association between age and serum or pleural fluid mesothelin when adjustment was made for the association between falling eGFR and increasing age.

DISCUSSION

This study has examined the practical application of mesothelin testing in serum and pleural fluid, in consecutive patients presenting with an undiagnosed pleural effusion.

Consistent with previous investigators, we have demonstrated significantly higher levels of mesothelin in serum and pleural fluid in patients with histologically confirmed mesothelioma than with pleural effusions of another cause, but a clinically important rate of false-positive results in patients with a wide range of non-MPM malignant sub-types as well as benign disease [8, 9, 13–17].

Mesothelin is expressed on normal mesothelium [18] and gene expression studies confirm overexpression on malignant cells of many histological sub-types including MPM, ovarian, pancreatic, endometrial, lung, oesophageal, colonic, vulval and cervical malignancies [19]. Lack of specificity amongst non-MPM malignant pleural effusions is therefore explicable and well documented, but does represent a substantial limitation to the clinical utility of the test.

False-positive serum results in patients with benign diagnoses were also very common in our series; 36 (34%) out of 107 patients with a benign diagnosis had a positive serum mesothelin at a cut-off level of 1.5 nM. Pleural fluid mesothelin was positive in only two patients with benign disease; both had a final diagnosis of robustly ascertained BAPE.

Positive results in patients with BAPE were also seen commonly with the serum test (five out of 13 patients). All patients had a lack of radiographic progression on serial CT scan for at least 12 months and all but one had irrefutable histological confirmation of benign disease.

Immuno-histochemical studies have shown that most sarcomatoid sub-type MPM tissue does not express mesothelin, such that the "false" negative test results shown in this and
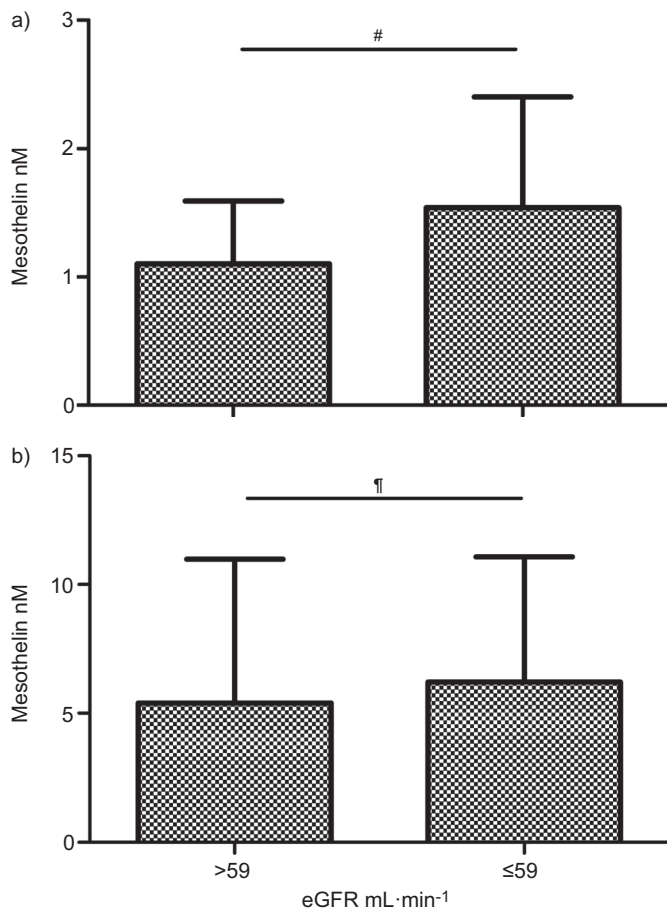
a)

b)

FIGURE 4. a) Serum and b) pleural fluid mesothelin in patients with a non-malignant pleural mesothelioma diagnosis with (estimated glomerular filtration rate (eGFR) $\leqslant$59 mL·min^{-1}) and without (eGFR >59 mL·min^{-1}) significant renal failure. Data are presented as median and interquartile range. #: p=0.007; ¶: p=0.605.

previous studies are to be expected [20]. Serum and pleural fluid mesothelin were also negative in 32% and 20% of patients with epithelioid sub-type disease, presumably reflecting variable gene expression.

We have demonstrated superior diagnostic characteristics for pleural fluid mesothelin above serum within paired results alone, at a fixed specificity of 95%, and using established cut-off levels, at each stage in the diagnostic pathway. This contrasts with the conclusion of several earlier series examining smaller numbers of paired results that have not demonstrated a significant difference between pleural fluid and serum analysis [8, 13].

Overall the NPV of mesothelin when measured in serum (91.2%) and pleural fluid (95.5%) is good, but the PPV (20.5% and 46.2%, respectively) is not within a clinically useful range.

A recent study by BOUDVILLE et al. [10] examined serum mesothelin levels in 144 patients with stable CKD but no clinically apparent pleural disease and demonstrated significantly higher levels in patients with CKD stage 3 or greater compared to those with CKD stage 2, suggesting that mesothelin, released from normal mesothelial cells, undergoes a degree of renal clearance, consistent with its small molecular

mass. HOLLEVOET et al. [11] measured serum mesothelin and glomerular filtration rate (GFR) in 66 control patients, demonstrating a positive correlation between GFR and the reciprocal of serum mesothelin and a false-positive rate of 52% in patients with CKD stage 3 or greater.

In our series, specificity of serum mesothelin was 72.3% in patients with an eGFR >59 mL·min^{-1}, compared with 49.1% in patients with CKD stage 3 or greater. 20 out of 36 positive serum tests in patients with benign pleural effusions may be explained by significant renal failure. Levels of mesothelin measurable in pleural fluid in patients without mesothelioma were not affected by renal function and the diagnostic accuracy of the test when applied to pleural fluid was not significantly different between the two renal function groups. While accumulation of mesothelin in pleural fluid as a result of diminishing renal clearance would not necessarily be expected (as it would in serum), this is the first study to demonstrate this potential advantage of pleural fluid testing.

There are several notable limitations to this study. While it is one of the largest reported series of patients presenting with pleural effusions to undergo mesothelin testing, the proportion of patients with mesothelioma was low when compared with most previous studies that have selected samples from established tissue banks by diagnosis. However, this proportion accurately reflects our consecutive patient population. We studied the diagnostic accuracy of mesothelin at previously established cut-off levels, which, while achieving the study aim (to examine the test in a clinically relevant manner), may produce inferior diagnostic characteristics than derivation of cut-off levels specific to our patient population or according to a desired sensitivity or specificity. Finally, conclusions regarding the utility of the test when applied to pleural fluid are tempered by the current lack of validation of commercially available ELISAs for this sample type.

The strength of this study is the recruitment of an unselected consecutive series of patients with pleural effusions requiring investigation and the robust application of diagnostic criteria, diagnosis of MPM exclusively on histological grounds and patient follow-up to 12 months in the presence of benign pleural disease. Within this complex "real world" sample, the specificity and PPV of mesothelin are challenged.

In conclusion, these results suggest that the routine testing of mesothelin in all undiagnosed pleural effusions is unhelpful. If the test is performed for diagnostic purposes, pleural fluid should be used instead of serum, particularly in patients with an eGFR $\leqslant$59 mL·min^{-1}. A negative result in a patient with a low pre-test probability of MPM is reassuring for an absence of epithelioid mesothelioma and NPV appears to be relatively consistent between serum and pleural fluid. Positive test results, however, should be interpreted with caution, as false positives are common, including in patients with BAPE. The role of the test in MPM diagnosis therefore appears limited. Serum mesothelin has shown promise in monitoring treatment response and disease progression [21–24] and this should be the focus of future studies.

CLINICAL TRIAL

This study is registered at UK Clinical Research Network with adoption number 8960.

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