

# **Title: 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 (PF) (n=206) mesothelin (by ELISA) during the initial consultation.

28/230 (12%) 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 PF, 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 PF 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 PF levels were unaffected. 19/35 (54%) patients with a benign pleural effusion and  $EGFR \leq 59$  ml/min 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 unhelpful.

Malignant pleural mesothelioma 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 malignant pleural mesothelioma (MPM) can be elusive, particularly in frail patients who are unfit for the most invasive of available diagnostic procedures such as video assisted thoracoscopy (VATS) 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]. CT scan appearances, while of reasonable specificity for identifying malignant thickening of the pleura, distinguish poorly between MPM and adenocarcinoma[5].

An asbestos exposure history 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 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 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 demonstrated promising diagnostic characteristics for pleural fluid mesothelin in the clarification of non-diagnostic pleural fluid cytology[9].

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 more than 50% has been shown in control populations with stage 3 chronic kidney disease or greater [10, 11]. A further 2011 study by Hollevoet et al demonstrated an independent association between age, glomerular filtration rate and body mass index and serum mesothelin[12]. 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 (08/H0102/11).

Consecutive patients presenting to the pleural disease service of a large teaching UK hospital between July 2008 and 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 online depository 1 and 2.

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

**Mesothelin quantification:** Paired serum and PF samples were collected in serum gel separator tubes and centrifuged at 1000g for 20 minutes. The supernatant was stored at -70<sup>oc</sup> for later assay. Mesothelin concentrations were measured with a commercially available ELISA (Mesomark<sup>®</sup>, FUJIREBIO diagnostics inc, Malvern, USA). Clinical team members were blind to the mesothelin results.

Method of estimated glomerular filtration rate (eGFR) calculation is given in online depository 3.

**Final diagnosis and follow-up:** Patients were followed-up to a histological or microbiological diagnosis, complete resolution of the pleural effusion, to death or to 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 online depository 4.

**Statistical analysis:** Analyses were performed using STATA version 12.0 and Graphpad Prism 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 and 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 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 used the test manufacturer's cut-off level of 1.5 nM, and pleural fluid 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 subgroup analysis was performed to determine whether significant chronic kidney disease (CKD) affected test results. Patients were classified into groups based on EGFR results ( $\leq 59$  ml/min (stage 3 CKD or greater) vs  $> 59$  ml/min), 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 kappa concordance score of 0.96 (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 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 PF 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 26/69 (38%) and positive PF results in 19/71 (26%) patients with non-MPM malignant pleural effusions including those with adenocarcinoma of unknown primary, breast carcinoma, cholangiocarcinoma, chronic lymphocytic leukaemia, B cell lymphoma, non-small cell lung cancer (NSCLC), ovarian carcinoma, primary peritoneal carcinoma, renal cell carcinoma and small cell lung cancer (figure 2). A full breakdown of malignant histological sub-types is given in online depository 5.

False negative serum mesothelin was seen in 3/3 patients with sarcomatoid MPM, 1/2 biphasic and 7/22 patients with epithelioid MPM while a false negative PF mesothelin was seen in 3/3 sarcomatoid and 4/20 epithelioid MPM cases for whom samples were available.

**Table 1: Final diagnosis and serum and pleural fluid mesothelin levels**

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

***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 1.5nM, yielding sensitivity 59.3% (38.8 -77.6), specificity 64.7 % (57.2-72.0), PPV 20.5% (12.2 – 31.1), NPV 91.2% (84.8 – 95.5) (table 2a).

PF mesothelin was examined at a cut-off of 20nM, giving sensitivity 72.0% (50.6 – 87.9), specificity 87.5% (81.5 -92.8), PPV 46.2% (30.1-62.8), NPV 95.5% (90.8-98.2) (table 2b).

**Table 2a. Diagnostic characteristics of serum mesothelin at cut off 1.5nM. \*False negative results were seen in 3 patients with sarcomatoid, 1 biphasic and 7 epithelioid MPM.\*\* False positive results occurred in 36 patients with benign pleural effusions and 26 with non-MPM malignant pleural effusions.**

Serum mesothelin	MPM	Not MPM	Total
≥ 1.5 nM	16	62**	78
< 1.5 nM	11*	114	125
Total	27	176	203

**Table 2b. Diagnostic characteristics of pleural fluid mesothelin at cut off 20 nM. \*False negative results were seen in 3 patients with sarcomatoid and 4 epithelioid MPM. \*\*False positive results occurred in 2 patients with benign pleural effusions and 19 with non-MPM malignant pleural effusions.**

Pleural fluid mesothelin	MPM	Not MPM	Total
≥ 20.0 nM	18	21**	39
< 20.0 nM	7*	147	154
Total	25	168	193

***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 benign asbestos related pleural effusion are given in online depositories 6-9.

### ***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 PF mesothelin results (24 with MPM), the area under the receiver operating characteristic curve (AUC) for serum mesothelin in the diagnosis of mesothelioma, was 0.61 (95% CI: 0.46 to 0.77) which is significantly inferior to the AUC for PF of 0.85 (95% CI: 0.77 to 0.94),  $P=0.0001$ . See figure 3.

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

### ***The impact of significant chronic kidney disease (CKD)***

Contemporaneous creatinine and estimated glomerular filtration rate (EGFR) was available for 229/230 patients at the time of sample collection.

74/229 (32%) patients had an  $EGFR \leq 59$  ml/min (stage 3 CKD or greater).

Specificity of serum mesothelin was 72.3% (63.3-80.1) in patients with an  $EGFR > 59$  ml/min but 49.1% (35.6 – 62.7) in those with  $EGFR \leq 59$  ml/min. (tables 3a /3b). 19/35(54%) patients with a benign cause for pleural effusion but  $EGFR \leq 59$  ml/min had a false positive serum mesothelin result.

Diagnostic characteristics of PF mesothelin were unaffected by renal function. Specificity with  $EGFR > 59$  ml/min was 86.0% (78.2-91.8) and with  $EGFR \leq 59$  ml/min, 87.0 % (75.1-94.6).

In patients with a confirmed non MPM diagnosis, median serum mesothelin was 1.54 nM (1.00-2.40) in patients with an EGFR  $\leq$  59 ml/min, significantly higher than with an EGFR > 59ml/min at 1.1nM (0.80 – 1.59). Median PF mesothelin was not significantly different with (6.22 nM (3.0-11.07)) and without (5.41 nM (2.66-10.99)) renal impairment (figure 4).

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.

**Table 3a. Diagnostic accuracy of serum mesothelin in patients with and EGFR > 59 ,  
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
<b>Total</b>	19	141	138
<b>Sensitivity:</b> <b>47.4%</b> (24.5-71.2)	<b>Specificity:</b> <b>72.3%</b> (63.3 – 80.1)	<b>PPV:</b> <b>21.0%</b> (10.3-36.8)	<b>NPV:</b> <b>89.6%</b> (81.6 – 94.9)

**Table 3b. Diagnostic accuracy of serum mesothelin in patients with and EGFR ≤ 59 (CKD3  
or greater), 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
<b>Total</b>	8	57	65
<b>Sensitivity:</b> <b>88.9%</b> (51.8-99.7)	<b>Specificity:</b> <b>49.1%</b> (35.6 – 62.7)	<b>PPV:</b> <b>21.6%</b> (9.8-38.2)	<b>NPV:</b> <b>96.6%</b> (82.2-99.9)

## 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 PF 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 over expression 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; 36/107(34%) patients with a benign diagnosis had a positive serum mesothelin at a cut-off level of 1.5nM. PF mesothelin was positive in only 2 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 (5/13 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 'false' negative test results shown in this and previous

studies are expected[20]. Serum and PF 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 PF 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 examined serum mesothelin levels in 144 patients with stable chronic kidney disease (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[10].

Hollevoet et al measured serum mesothelin and 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[11].

In our series, specificity of serum mesothelin was 72.3% in patients with an EGFR > 59 ml/min, compared to 49.1% in patients with CKD stage 3 or greater. 20/36 of 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 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 to most previous studies that have selected samples from established tissue banks by diagnosis. This proportion, however, 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 malignant pleural mesothelioma 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 positive predictive value of mesothelin is 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  $\text{eGFR} \leq 59 \text{ ml/min}$ . A negative result in a patient with a low pretest probability of MPM is reassuring for an absence of epithelioid mesothelioma and negative predictive value appears 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 benign asbestos related pleural effusions. 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.

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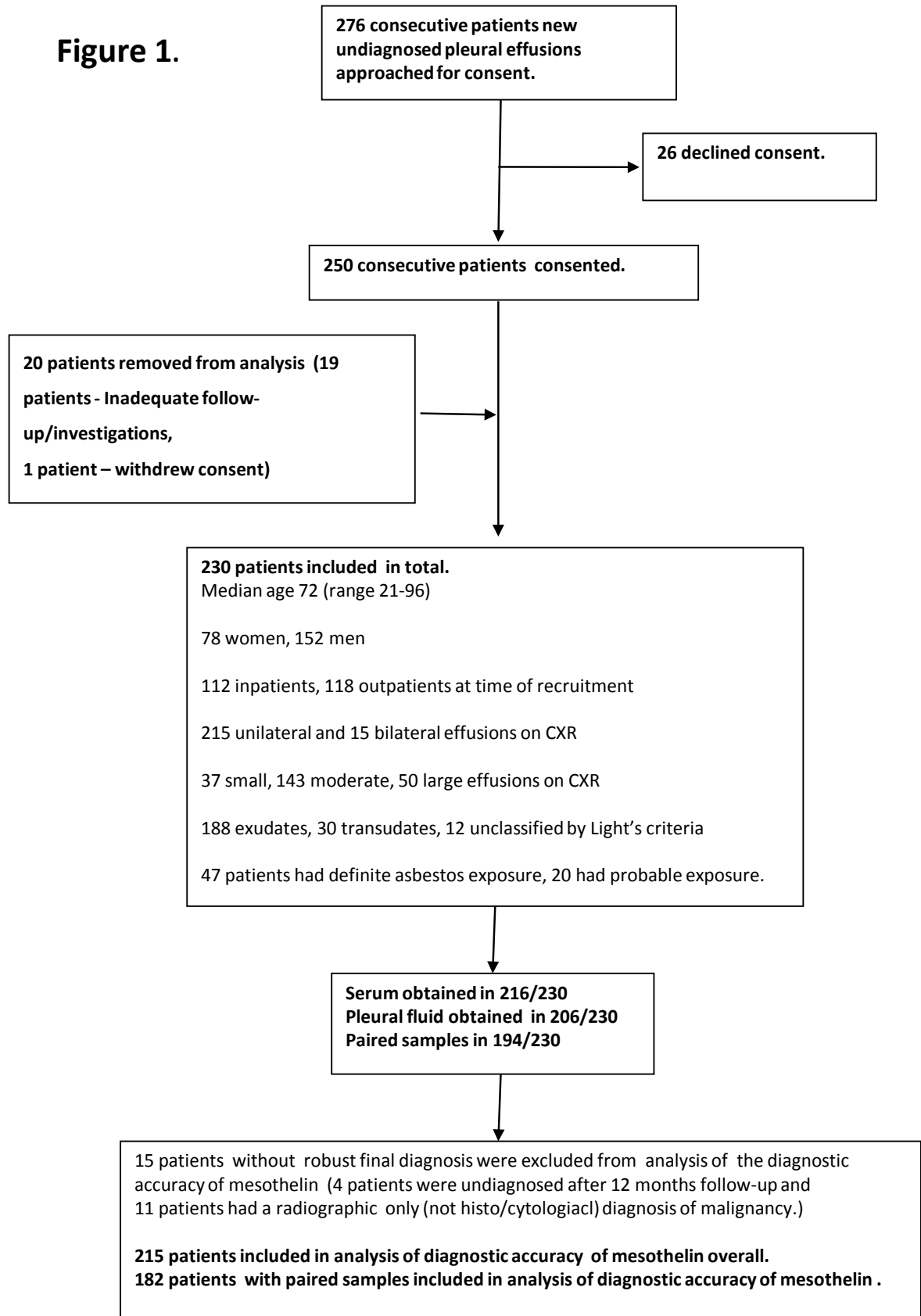
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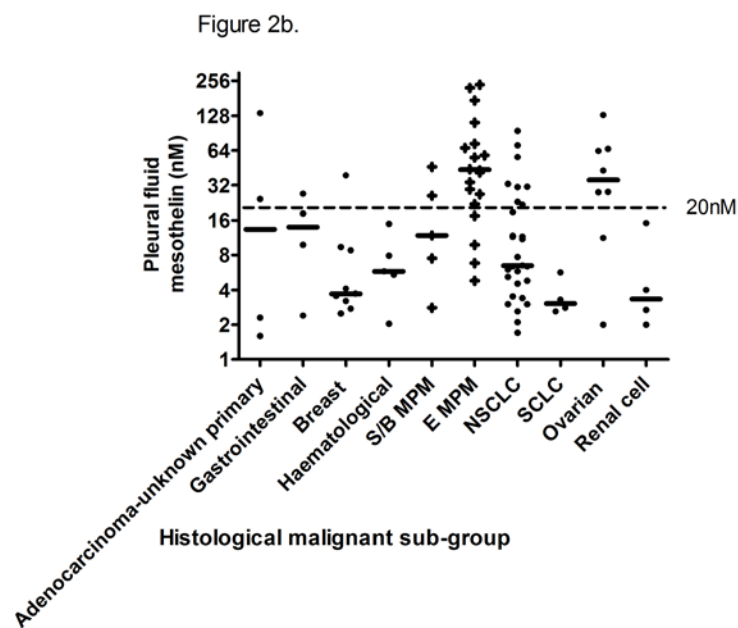
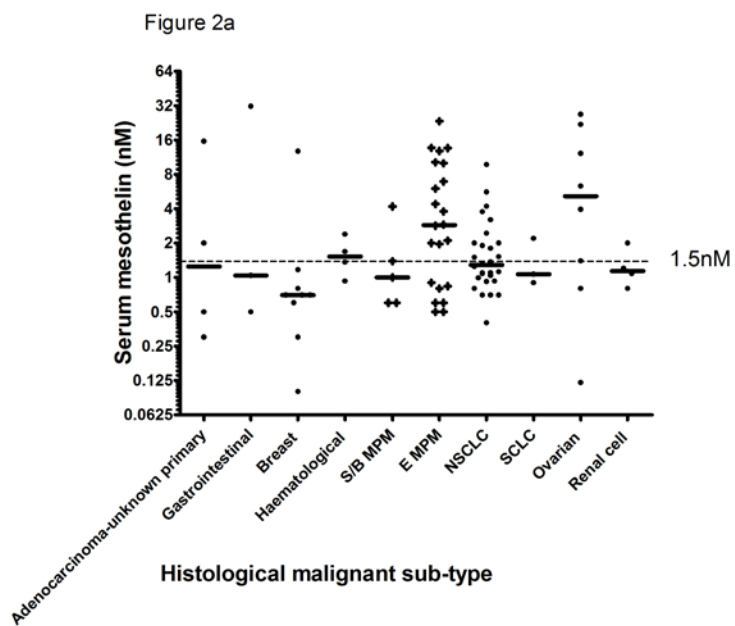
24. Hollevoet K, Nackaerts K, Gosselin R, De Wever W, Bosquée L, De Vuyst P, Germonpré P, Kellen E, Legrand C, Kishi Y, Delanghe JR, van Meerbeeck JP. Soluble mesothelin, megakaryocyte potentiating factor, and osteopontin as markers of patient response and outcome in mesothelioma. *Journal of clinical oncology*. 2011. 6(11): p. 1930-7.

**Figure 1. Patient recruitment, inclusions, exclusions and demographics.**

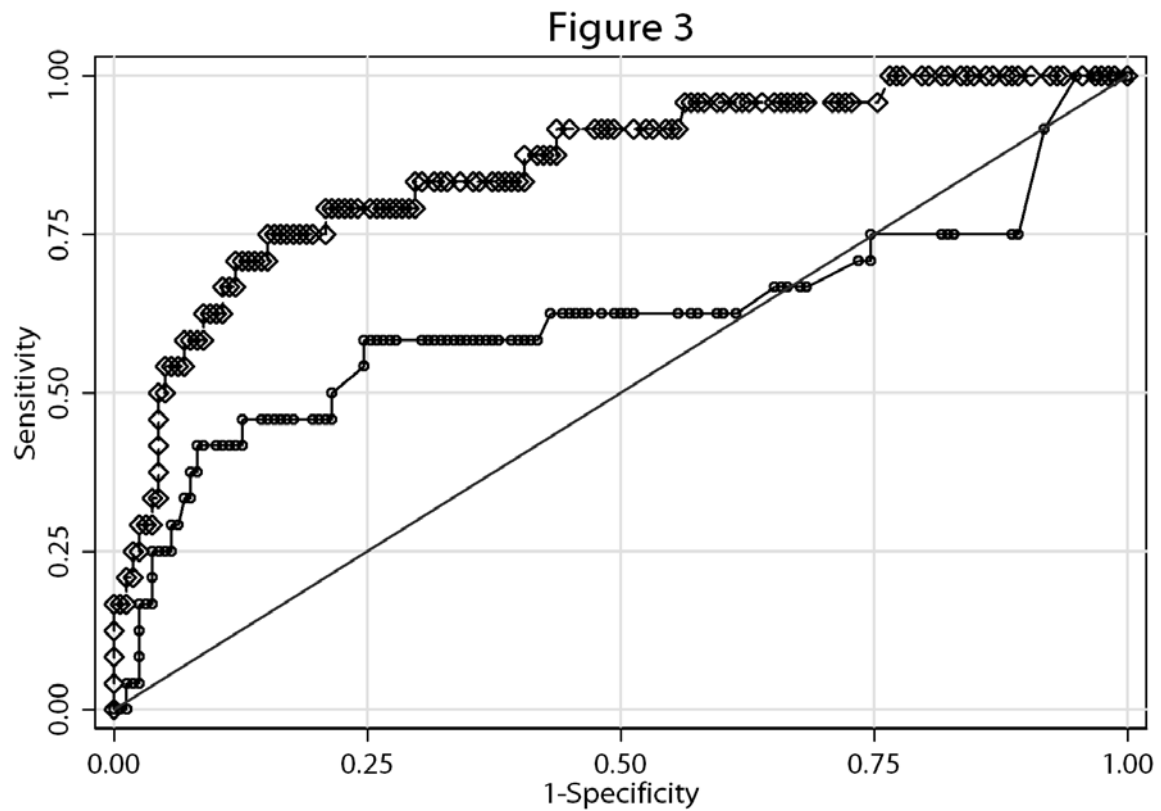
**Figure 1.**



**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.**



**Figure 3. Receiver operating characteristics curves comparing the diagnostic accuracy of serum (  $\diamond$  ) and pleural fluid (  $\circ$  ) mesothelin testing for the diagnosis of MPM. Patients with both sample types and a definitive final diagnosis included (n= 182)**



**Figure 4 a: Serum (SMes) and b: pleural fluid (Pmes) mesothelin in patients with a non-MPM diagnosis with ( $EGFR \leq 59$ ) and without ( $EGFR > 59$ ) significant renal failure.**

Figure 4a.

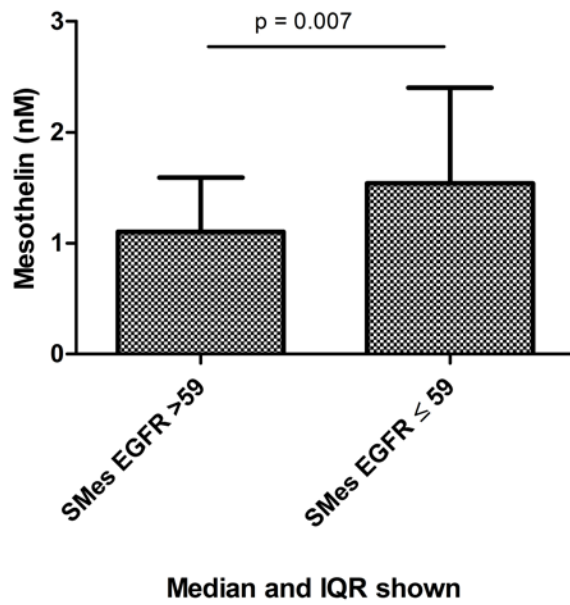


Figure 4b.

