Soluble mesothelin in effusions – a useful tool for the diagnosis of malignant mesothelioma

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Abstract

Background Diagnosis of malignant mesothelioma is frequently difficult, the most common differential diagnosis being reactive pleural conditions and metastatic adenocarcinoma. Soluble mesothelin levels in serum have recently been shown to be highly specific and moderately sensitive for mesothelioma. So as most mesothelioma patients present with exudative effusions of either the pleura or peritoneum, we sought to determine if levels of mesothelin were elevated in these fluids and if any elevations could help distinguish mesothelioma from other causes of exudative effusion.

Patients and Methods Pleural fluid was collected from 192 patients who presented to respiratory clinics: 52 patients with malignant mesothelioma, 56 with non-mesotheliomatous malignancies and 84 with effusions of non-neoplastic origin. Peritoneal fluid was collected from 42 patients; 7 with mesothelioma, 14 with non-mesotheliomatous malignancies and 21 benign effusions. Mesothelin levels were determined on effusion and serum samples by ELISA.

Results Mesothelin was statistically significantly higher in effusions of patients with mesothelioma; with a specificity of 98% the assay had a sensitivity of 67% comparing mesothelioma patients to patients with effusions of non-neoplastic origin. In 7 out of 10 cases mesothelin was elevated in the effusion collected 3 weeks to 10 months before the diagnosis of mesothelioma was made, and in 4 out of 8 of these cases mesothelin was elevated in the effusion but not in the serum.

Conclusions Measurement of mesothelin concentrations in the pleural and/or peritoneal effusion of patients may aid in the differential diagnosis of mesothelioma in patients presenting with effusions.
INTRODUCTION
Malignant mesothelioma is an aggressive, asbestos-related tumour of the pleural and peritoneal cavities. Patients with pleural disease generally present with shortness of breath and those with peritoneal disease with abdominal swelling. Both are usually associated with the presence of exudative effusions. Cytological diagnosis at presentation can be difficult either because there are no malignant cells present in the fluid or because the cells present are difficult to distinguish from reactive mesothelial cells or other malignant cells (1).

Measurement of tumour markers in effusions has been reported to provide a complementary tool to aid in the diagnosis of the cause of an effusion. Differential levels of carcinoembryonic antigen (CEA), cancer antigen (CA) 15.3, CA72.4, CA19.9, CA549, neuron-specific enolase or cytokine fragment 19 (CYFRA 21-1) have been reported to differentiate malignant from benign effusions (2-5). However, there is less data available for the differential diagnosis of mesothelioma from other carcinomas. There are low levels of CEA in effusions of mesothelioma patients and elevated CEA levels provide a strong negative predictive value for this disease (4-7). However, there are few studies reporting markers with a high positive predictive value for mesothelioma. Elevated CA15-3 levels have been reported in mesothelioma (3-5) and in one study were able to differentiate between mesothelioma and bronchial cancer (5). Higher levels of hyaluronic acid have been reported in effusions from mesothelioma patients compared to those with other malignant diseases, however the difference was too small to be of diagnostic value (7). There is discrepancy in the literature on the ability of CYFRA 21-1 levels to differentiate between mesothelioma and other pleural malignancies (5, 7, 8). In summary, there are currently no reliable tumour markers used on pleural or peritoneal exudates in routine clinical practice for assisting in the diagnosing of mesothelioma.

We recently reported a new serum tumour marker with high specificity and moderate sensitivity for mesothelioma, soluble mesothelin related protein (SMRP), more simply described as mesothelin. Serum levels of these soluble mesothelin proteins were elevated in 84% of mesothelioma patients with established disease and only in 2% of patients with other cancers or other lung or pleural disease (9). Similar findings were recently reported in a French study (10). That the mesothelin protein itself is stable, with no significant difference in concentrations occurring following up to 10 freeze-thaw cycles (11), also suggests that mesothelin may be useful as a clinical marker. The current study was designed to assess whether measurement of soluble mesothelin levels in effusions can provide additional diagnostic value to the existing conventional diagnostic tools.

PATIENTS AND METHODS
Patients and Controls
From 1999 to 2005, we collected serum, pleural and peritoneal effusions samples from consecutive patients presenting at the respiratory clinics of either Sir Charles Gairdner Hospital or the Hollywood Specialist Centre in Perth, Western Australia. Effusions were obtained by routine pleurocentesis and were in excess to that required for diagnosis. The final diagnosis in all patients was confirmed by pathologists experienced in the diagnosis of effusions and included clinical follow-up of all cases until death or for an average of 6.8 months (range 1-42 months) following the effusion to confirm that the clinical pattern matched the diagnosis. Effusions were classified as being malignant or non-malignant on the basis of cytologic and immuno-histochemical features; and malignant effusions were further classified as mesothelioma, metastatic adenocarcinoma or other malignancy. Non-malignant effusions were classified as exudates or transudates on the basis of Light’s criteria (12). Effusions were classified as being associated with an infection if micro-organisms were detected in the fluid along with inflammatory cells or if the patient had pneumonia adjacent to
the effusion. We obtained written and oral informed consent from participants. This study was approved by the human ethics committees of Sir Charles Gairdner and Hollywood Hospitals.

All specimens were received as fresh effusions, with a volume range of 10-3,000mL. Specimens were centrifuged for 10 minutes at 2,000g. The resulting supernatant was stored at -80°C until assayed.

**Measurement of mesothelin**
Soluble mesothelin concentrations were determined in duplicate following the manufacturer’s instructions using a double determinant ELISA assay, the MESOMARK™ kit, supplied by Fujirebio Diagnostics, (Malvern PA). Mesothelin concentrations were determined from a standard curve performed on each plate and expressed as nM. Dilution of samples was carried out if necessary using the diluent supplied by the manufacturer. All assays were performed on coded samples by technical staff unaware of the patient’s diagnosis.

**Immunolocalisations**
Mesothelin staining was examined in a subset of cases. Formalin-fixed paraffin-embedded cell-block specimens of pleural effusions from patients with mesothelioma were retrieved from PathWest laboratories (Nedlands, Australia). Sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. Antigen-retrieval was performed using the pressure-cooking method (3 min, 1mM EDTA (pH 8.0)). Before staining, endogenous peroxidase was blocked. Sections were processed by standard methods and incubated with anti-mesothelin (clone 5B2, NovaCastra, Newcastle upon Tyne, UK) antibody (dilution 1:20) or anti epithelial membrane antigen (EMA) (clone E29, DakoCytomation, Glostrup, Denmark) for 60 min and washed in PBS. Immunodetection was performed using SuperPicTure Polymer detection reagent (Zymed, San Fransisco, CA). For negative controls, the primary antibody was omitted.

Staining was assessed by two independent observers (J.C. and A.S.). A positive result was defined as the presence of membranous stain in more than 20% of tumour cells. Staining intensity was graded semi-quantitatively as negative, weak (1+), moderate (2+) or strong (3+).

**Statistical Analysis**
Descriptive statistics and logistic regression analysis to predict cases from controls were performed using GraphPad Prism for Windows (GraphPad Software, San Diego CA). Differences between groups of patients were assessed by Student’s t-test after transforming mesothelin values to the log scale for which the distributions were closer to normality. For the same reason, median mesothelin values were estimated from the mean on the log scale and exponentiated to provide the estimate of the median on the original scale. All reported p-values are two sided. A level of p<0.05 was accepted as significant. Survival plots were generated by the Kaplan-Meier method and difference between patients groups determined by the log-rank test. Significance of the SMRP value nearest to mesothelioma diagnosis for survival was assessed using Cox proportional hazards regression (13). Receiver Operating Characteristic (ROC) curves display the trade-off between sensitivity and specificity for mesothelin differentiating between groups of patients. Cross-validated (14) estimates of sensitivity and specificity were obtained by the “leave-one-out” method to ameliorate over fitting bias using the Statistical Analysis System (SAS v 10, Cary NC), as the sample size was not sufficient to form independent training and validation data sets. Pearson’s correlation coefficient was used to assess correlations between mesothelin values (log scale) in serum and pleural effusions.
RESULTS

Characteristics of patients

Pleural effusions were collected from 192 patients, of these 52 were diagnosed with mesothelioma, 84 with effusions of non-neoplastic origin and 56 with non-mesotheliomatous malignancy (Table 1). Of the 52 patients with mesothelioma, 15 cases were of epithelial histology, 5 biphasic, and 9 sarcomatoid. In 23 cases diagnosis was made on the basis of cytological findings (15) in which case the histological subtype could not be confirmed.

Peritoneal fluid and effusions were collected from 42 patients, of these 7 were diagnosed with mesothelioma, 14 with non-mesotheliomatous malignancy, 6 with effusions of non-neoplastic origin and 15 with end stage renal failure (Table 1).

Soluble mesothelin levels in pleural effusions

Pleural effusions from patients with mesothelioma had significantly higher concentrations of mesothelin than those from patients with non-malignant effusions; the median mesothelin values (± SE) being 27.7 ± 1.34 versus 4.1 ± 0.8, respectively ($p<0.0001$) and from patients with non-mesothelioma malignancies (6.3 ± 1.2) ($p<0.0001$) (Figure 1A, Table 1).

Mesothelioma patients with predominantly sarcomatoid histology had a significantly lower concentration of mesothelin in their effusions than mesothelioma patients with predominantly epithelial histology, medians 46.9 ± 1.1 versus 4.5 ± 1.4 respectively, ($p<0.0001$) (Figure 1B, Table 1). There was no significant difference in the levels of mesothelin in effusions from patients with sarcomatoid mesothelioma and non-malignant effusions.

As an indicator of the precision of the assay, control samples assayed in duplicate over twenty assays had for the high control (mean (± SD) mesothelin concentration 12.7 ± 0.67) a coefficient of variation of 5.3% and for the low control (mesothelin concentration 4.4 ± 0.25) a CV of 5.7%.

ROC curves for mesothelin in effusions comparing different cohorts of patients showed (Figure 1C) that when compared with patients with non-malignant conditions patients with mesothelioma (including those with the sarcomatoid variant) had an area under the curve of 0.898. At a cut-off value of 20nM the diagnostic specificity was 98% and the corresponding cross-validated sensitivity in the combined histological groups of mesothelioma was 67%. When data were analysed excluding mesothelioma patients with sarcomatoid histology, the area under the ROC curve was 0.964. At the same threshold value of 20nM the diagnostic specificity remained 98% and cross-validated sensitivity increased to 77%. In this group when specificity was reduced to 90%, the cross-validated sensitivity was 86%. Using a 20nM cut-off, 8 out of the 56 patients with non-mesothelioma malignancies had elevated SMRP in their effusions, giving a specificity of 86% for mesothelioma.

Of the two patients with lung cancer and effusion mesothelin concentrations greater than 50 nM, one was diagnosed with bronchioalveolar carcinoma and the other with lung adenocarcinoma. Of the two patients with other malignancies and mesothelin concentrations above 50 nM, one was diagnosed with likely low grade Non-Hodgkins lymphoma and the other with pancreatic carcinoma.

Correlation of mesothelin levels in pleural effusions and serum

In order to determine whether mesothelin concentrations in pleural effusion added diagnostic value to those from serum, we compared mesothelin concentrations in matching fluid and serum samples from 41 patients with mesothelioma (Figure 2A), 22 out of 41 patients had elevated mesothelin in both the effusion and in the serum, and 2 patients had elevated mesothelin in the serum only. Six patients had elevated mesothelin in the effusion but not in the serum. Of the eleven patients who were negative for mesothelin in the serum and effusion,
five were of predominantly the sarcomatoid variant. There was a significant \( p < 0.0001 \) correlation between serum and pleural effusion mesothelin concentrations (Pearson’s correlation coefficient 0.6656, CI 95% 0.45 – 0.81).

There were matching effusion and serum samples available for 31 non-mesothelioma individuals, 16 with benign effusions and 15 with patients with malignant effusions (Figure 2B). Four out of 15 patients with non-mesothelioma malignant effusions had elevated mesothelin in the pleural effusion, of which two were also elevated in the serum. Two of these three patients had lung adenocarinaoma and the other had non-Hodgkin’s lymphoma. The patient with elevated mesothelin levels in the effusion but not in the serum had a diagnosis of pancreatic carcinoma. One patient with melanoma, metastatic to the lungs had mesothelin elevated in the serum but not in the effusion. There was no correlation between serum and effusion mesothelin concentrations in patients with non-mesothelioma malignancy.

**Mesothelin levels in effusions at and prior to mesothelioma diagnosis**

Effusion samples were available from 13 individuals who were subsequently diagnosed with mesothelioma obtained 1 - 39 weeks prior to eventual diagnosis. No definite malignant cells were visible in these samples by cytological examination. Mesothelin was elevated in 10 of these 13 pre-diagnosis samples (Figure 2C). To determine if serum mesothelin alone would have made the diagnosis without the need to examine fluid mesothelin levels, serum mesothelin concentrations in those patients with elevated pre-diagnosis mesothelin levels in their fluid was determined. Four of these patients had elevated serum and effusion levels, four had elevated levels in the effusion only and two had no matching serum sample available.

**Relation of mesothelin levels in effusions with mesothelioma patient survival**

Survival data for mesothelioma patients with mesothelin concentrations above the median level of the entire cohort (from Table 1, 26 nM) were plotted against that of patients with mesothelin concentrations below the median and tested for statistically significant difference using the Kaplan-Meier method (Figure 3). The median survival of patients was 14 months for those with high effusion mesothelin levels (\( n = 27 \)) and 8 months for those with low levels (\( n = 25 \)). As patients with mesothelioma with sarcomatoid histology have poorer prognosis than those with other histologies, we also examined the survival of non-sarcomatoid mesothelioma patients with mesothelin concentrations below 26 nM. The median survival of this group of patients was 12 months (\( n = 16 \)). There was no significant difference in survival of mesothelioma patients in relation to the earliest available mesothelin effusion concentration using the Kaplan-Meier method or Cox’s proportional hazards regression (data not shown).

**Mesothelin levels in peritoneal effusions and fluid**

Mesothelin concentrations elevated above 20nM were found in 5 out of 7 patients with mesothelioma and all four with ovarian cancer, with a median estimate of 48 ± 0.86 nM and 73.7 ± 0.77 nM, respectively. All 10 patients with malignant effusions of other histologies were mesothelin negative, all non-malignant exudates were negative, with median estimates being 3.58 ± 0.71 and 2.96 ± 0.6, respectively. Mesothelin levels in continuous ambulatory peritoneal dialysis (CAPD) fluid from patients with chronic renal failure, fluid in which large numbers of reactive mesothelial cells are usually found, had no detectable mesothelin (Figure 4, Table 1).

**Mesothelin staining**

Sarcomatous mesothelioma tissue does not stain with mesothelin (data not shown). To determine whether high or low mesothelin levels in the effusions of patients with non-sarcomatoid mesothelioma reflected the pattern of mesothelin expression in the tumour cells
present in the effusion, immunohistochemical staining was done on a subset of samples from these patients (Table 3; Figure 5). Of the 6 samples from patients with pleural effusion mesothelin concentrations greater than 26 nM, five stained positive for mesothelin. In the sixth sample staining was equivocal. Mesothelin staining was predominantly membranous and may be associated with the microvilli. Of the seven patients with mesothelin effusion concentrations less than 26 nM, only two had positive staining for mesothelin, and this staining was weak.

DISCUSSION
Given that an elevated mesothelin level in serum is relatively specific and sensitive for mesothelioma (9, 10) and given that the majority of such patients present with exudative effusions (1), we investigated whether mesothelin levels in effusions added value to the analysis of serum mesothelin levels. In this study we show that mesothelin levels greater than 20nM in effusions are highly suggestive of malignancy, particularly of mesothelioma, and that measurement of effusion mesothelin levels could facilitate earlier diagnosis. There are several previous reports describing the use of tumour markers for the differential diagnosis of pleural effusions but none have entered routine clinical use (2, 16, 17). In the current study effusion levels of mesothelin have a sensitivity of 67% at the high specificity of 98% for distinguishing mesothelioma from non-malignant effusions, and these levels of sensitivity and specificity indicate that this marker is useful in the investigation of patients with undiagnosed pleural or peritoneal effusions. The ROC curve generated for this data had an AUC of 0.898 (95% CI 0.839-0.958), a result very similar to that reported by Scherpereel and colleagues who generated a ROC curve from patients entered on a multicenter study in France with an AUC of 0.831 (95% CI 0.734-0.927) (10). The concordance of the results from these independent studies is promising for the future use of mesothelin in effusion diagnosis. Also 15% of non-mesothelioma tumours had elevated mesothelin effusion levels suggesting that an mesothelin level above 20nM should be considered suspicious of malignancy, particularly, but not exclusively, malignant mesothelioma. Elevated fluid mesothelin levels in the absence of cytologically visible malignant cells, suggests that mesothelin measurement should be performed when there is an index of suspicion of cancer regardless of the absence of malignant cells.

That some patients with non-mesothelioma malignant effusions have elevated effusion mesothelin levels is consistent with the knowledge that mesothelin staining has been reported on approximately 40% of lung and 85% of pancreatic adenocarcinomas (18, 19). To our knowledge mesothelin expression has not been examined in Non-Hodgkin’s lymphoma and it is unclear why one of the six patients with this malignancy in the present study had elevated mesothelin levels. Of note, a negative mesothelin result does not rule out malignancy. The biological reason why some epithelial mesothelioma cases do not produce mesothelin is currently under investigation. It is likely that the reason the sarcomatous mesothelioma patients have low levels of mesothelin in the serum and effusions is the lack of that protein in the tissue based on histological staining (20).

As mesothelin is expressed on the surface of normal mesothelial cells lining the serosal cavities and can be enzymatically cleaved from the cell surface by trypsin or proteinase K (21), it was conceivable that inflammatory conditions would produce elevated mesothelin levels in effusions. The fact that mesothelin levels in exudative effusions, with or without infection, were not elevated increases the likelihood that measurement of effusion mesothelin levels is a useful diagnostic test. This is in contrast to other markers such as CA125 and CA19-9 which are also released by normal mesothelial cells but are elevated in inflammatory states reducing their diagnostic specificity (2).
It has not been possible to recover any pleural fluid from normal pleural cavities for analysis so it is impossible to define a normal pleural mesothelin level. At a cut-off value of 20nM the specificity of mesothelin in non-malignant effusions was high (98%) suggesting that mesothelin levels were not elevated as a non-specific effect of inflammatory or reactive mesothelial processes.

The finding that mesothelin levels in effusions generally correlate with levels in the serum is to be expected given the size of the molecule and the location of the tumour. However, mesothelin levels were elevated before diagnosis in the effusions of 4 out of 8 patients when serum mesothelin levels were normal. The measurement of mesothelin in the fluid of patients with suspected mesothelioma may prove, therefore, to be of use even when serum mesothelin levels remain normal. Not all patients with mesothelioma present with an effusion therefore the measurement of a serum biomarker such as mesothelin will be the only systemic aid for the diagnosis of this disease in such patients.

Elevated effusion levels of mesothelin before a definitive cytological and/or histological diagnosis may provide an early suggestion of the presence of malignancy and indicate the need for active invasive investigation such as thoracoscopy to establish a diagnosis. Early diagnosis could reduce the costs of subsequent hospitalizations and investigations, reduce the ‘anxiety of not knowing’ in those patients who know they have a potentially malignant effusion (eg those with substantial asbestos exposure whose colleagues may have died of mesothelioma) and provide the opportunity for early treatment using new treatments regimens which have proven to be of some value (22). Whether early intervention achieves these goals is yet to be proven.

The current data indicates that mesothelin levels provide no prognostic information in patients with mesothelioma. This is predictable given that a low concentration of mesothelin in an effusion may either reflect a small tumour burden (which may have a better prognosis) or a less differentiated tumour such as sarcomatoid mesothelioma, which has a worse prognosis (23).

Patients with benign peritoneal effusions did not show elevated mesothelin levels, although benign peritoneal effusions are less frequent so are harder to obtain than benign pleural effusions. Mesothelin levels were not elevated even in patients undergoing CAPD in which a marked reactive mesothelial cell proliferation is characteristically seen in the peritoneal fluid (24). Although this confirms that reactive mesothelial cells in this clinical situation do not release large amounts of mesothelin into the fluid, it is possible that mesothelin protein would not have had time to accumulate into the dialysate or indeed if the dialysate dilutes mesothelin concentrations beyond the detection levels of this assay. Patients with ovarian cancer, which has a number of biological similarities to mesothelioma and who also exhibit elevated serum mesothelin levels (25), also exhibit elevated peritoneal fluid mesothelin levels. None of the patients studied showed elevated mesothelin levels in peritoneal fluid though not all possible cancer types that can involve peritoneum have been studied so far. It appears that the value of measuring mesothelin in peritoneal fluid is similar to that in pleural fluid for patients with suspected mesothelioma or ovarian carcinoma.

Our results showing a correlation between tissue mesothelin expression and pleural fluid mesothelin levels suggest that the tumour or the tumour microenvironment is responsible for producing the mesothelin protein. This could be by cleavage of surface-bound mesothelin (29, 30) by tumour proteases or by generation of a soluble form of mesothelin by alternative splicing of the mesothelin mRNA (25). As the antibodies in the mesothelin assay have the potential to react with both surface bound mesothelin and soluble mesothelin, this study does not directly resolve this issue. Given that mesothelin staining was observed on the surface of mesothelioma cells present in two of seven pleural effusions with low mesothelin concentrations examined both scenarios are possible. The absence of elevated fluid levels of
mesothelin in inflammatory states suggests that it is not easily cleaved from the surface and that there is something particular about the malignant state that generates high mesothelin levels in effusions.

Other markers for mesothelioma are currently under study eg, osteopontin (31) and RCAS1, a type II membrane protein (32). Mesotheliomas of sarcomatoid histology stain positively for RCAS, however, in the small sample set published levels in effusions were not elevated compared to those in lung cancer (32). Given that a panel of tumour markers would improve specificity and specificity (3, 16) further work is required to determine which of these and other markers could improve the sensitivity and specificity of mesothelin in diagnosing mesothelioma.

Mesothelin measurements in serum are now undertaken in a number of centres in the world as an aid to the diagnosis of mesothelioma using a standardized, commercially available ELISA assay. Until now the measurement of tumour markers in pleural effusions has not become routine clinical practice (17). The data presented here argue that measurement of mesothelin in effusions, pleural or peritoneal, might be a useful adjunct to serum analysis in patients with suspected malignancy, particularly if the index of suspicion for mesothelioma is high. As effusion fluid is routinely sent for pathological, biochemical and often microbial analysis it is a simple matter to undertake mesothelin analysis at the same time. Clearly, in cases where fluid cytology shows mesothelioma cells with a high degree of certainty mesothelin measurement would add little, however, cases with a high level of mesothelin in the fluid especially those with no malignant cells present suggests a diagnosis of malignancy, mesothelioma in particular, and the need for early biopsy. The availability of this simple test may be particularly useful in those centers not experienced in the cytopathological diagnosis of this disease and where diagnosis is often complicated and time consuming.
### TABLE 1: PATIENT CHARACTERISTICS AND MESOTHELIN VALUES

<table>
<thead>
<tr>
<th>Type of Effusion</th>
<th>Number of cases</th>
<th>Median age, Years (range)</th>
<th>mesothelin (nM) a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLEURAL EFFUSIONS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mesothelioma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial</td>
<td>12M:3F</td>
<td>66.5 (44-94)</td>
<td>46.9 ± 1.1</td>
</tr>
<tr>
<td>Biphasic</td>
<td>4M:1F</td>
<td>68 (48-88)</td>
<td>30.1 ± 0.8</td>
</tr>
<tr>
<td>Sarcomatoid</td>
<td>9M:0F</td>
<td>77 (57-82)</td>
<td>4.5 ± 1.38</td>
</tr>
<tr>
<td>Cytology only</td>
<td>21M:2F</td>
<td>68.5 (44-89)</td>
<td>39.2 ± 0.96</td>
</tr>
<tr>
<td><strong>All</strong></td>
<td>52</td>
<td>68.5 (44-94)</td>
<td>27.7 ± 1.34</td>
</tr>
<tr>
<td><strong>Non-malignant effusions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transudate</td>
<td>22M:13F</td>
<td>75 (48-93)</td>
<td>4 ± 0.73</td>
</tr>
<tr>
<td>Exudate – noninfection</td>
<td>24M:6F</td>
<td>75 (34-94)</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>Exudate - infection</td>
<td>13M:6F</td>
<td>72 (34-91)</td>
<td>4.3 ± 0.85</td>
</tr>
<tr>
<td><strong>All</strong></td>
<td>84</td>
<td>75 (12-94)</td>
<td>4.1 ± 0.82</td>
</tr>
<tr>
<td><strong>Non-mesothelioma malignant effusions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung cancer</td>
<td>22</td>
<td>71 (51-79)</td>
<td>9.4 ± 1.1</td>
</tr>
<tr>
<td><strong>Non SMALL CELL</strong></td>
<td>19 (15M:4F)</td>
<td>70.5 (51-79)</td>
<td>10.4 ± 1.1</td>
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<tr>
<td>Adenocarcinoma</td>
<td>14M:3F</td>
<td>65 (51-79)</td>
<td>11.8 ± 1.1</td>
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<tr>
<td>Squamous Cell</td>
<td>1M:0F</td>
<td>76</td>
<td>5.5</td>
</tr>
<tr>
<td>Large Cell</td>
<td>0M:1F</td>
<td>71</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>SMALL CELL.</strong></td>
<td>3 (3M:0F)</td>
<td>76.5 (74-79)</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td><strong>Other Cancer</strong></td>
<td>34</td>
<td>66 (33-93)</td>
<td>4.8 ± 1.1</td>
</tr>
<tr>
<td>Colorectal</td>
<td>2M:3F</td>
<td>75 (50-88)</td>
<td>5 ± 0.6</td>
</tr>
<tr>
<td>Hodgkin’s lymphoma</td>
<td>0M:1F</td>
<td>33</td>
<td>9.7</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma</td>
<td>2M:4F</td>
<td>67.5 (52-93)</td>
<td>5.4 ± 1.4</td>
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<tr>
<td>Breast</td>
<td>0M:10F</td>
<td>63 (47-74)</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>Gastric</td>
<td>1M:0F</td>
<td>67</td>
<td>4.1</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1M:1F</td>
<td>69 (49-81)</td>
<td>1.1 ± 1.1</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>2M:0F</td>
<td>74.5 (74-75)</td>
<td>24.3 ± 2.7</td>
</tr>
<tr>
<td>Hepatocellular</td>
<td>1M:0F</td>
<td>46</td>
<td>3.8</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>1M:0F</td>
<td>82</td>
<td>4.3</td>
</tr>
<tr>
<td>Adenoid cystic</td>
<td>1M:0F</td>
<td>48</td>
<td>4.9</td>
</tr>
<tr>
<td>Waldenstrom’s</td>
<td>0M:1F</td>
<td>70</td>
<td>7.4</td>
</tr>
<tr>
<td>Unknown primary</td>
<td>2M:1F</td>
<td>72 (70-74)</td>
<td>7.4 ± 1.0</td>
</tr>
<tr>
<td><strong>All</strong></td>
<td>56</td>
<td>66 (33-93)</td>
<td>6.3 ± 1.2</td>
</tr>
<tr>
<td><strong>PERITONEAL EFFUSIONS AND FLUID</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mesothelioma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial</td>
<td>1M:1F</td>
<td>67 (64 – 70)</td>
<td>27.9 ± 0.92</td>
</tr>
<tr>
<td>Cytology only</td>
<td>5M:0F</td>
<td>68 (45 – 75)</td>
<td>59.7 ± 0.83</td>
</tr>
<tr>
<td><strong>All</strong></td>
<td>1F:6M</td>
<td>68 (45 – 75)</td>
<td>48 ± 0.86</td>
</tr>
<tr>
<td><strong>Non-mesothelioma malignant effusions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>0M:4F</td>
<td>65.5 (54 – 70)</td>
<td>73.7 ± 0.77</td>
</tr>
<tr>
<td><strong>Other cancer</strong></td>
<td>7M:3F</td>
<td>62.5 (48 – 76)</td>
<td>3.58 ± 0.71</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>0M:3F</td>
<td>66 (48 – 76)</td>
<td>5.03 ± 0.55</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>5M:0F</td>
<td>57 (56 – 74)</td>
<td>4.35 ± 0.22</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>1M:0F</td>
<td>69</td>
<td>2.9</td>
</tr>
<tr>
<td>Non- hodgkin’s lymphoma</td>
<td>1M:0F</td>
<td>59</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Non-malignant effusions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver disease</td>
<td>2F:4M</td>
<td>57 (32 – 73)</td>
<td>2.96 ± 0.6</td>
</tr>
<tr>
<td><strong>Dialysis fluid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>End Stage Renal Failure</td>
<td>9F:6M</td>
<td>64.5 (36 – 85)</td>
<td>0.2 ± 1.08</td>
</tr>
</tbody>
</table>

a  Exponentiated mean of log transformed data plus/minus standard error
### TABLE 2: CORRELATION BETWEEN MESOTHELIN STAINING AND MESOTHELIN LEVEL IN PATIENTS WITH MESOTHELIOMA

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient number</th>
<th>Histology</th>
<th>Effusion mesothelin concentration (nM)</th>
<th>Mesothelin staining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Staining intensity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 25 nM</td>
<td>1</td>
<td>Mixed</td>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Cytology only</td>
<td>12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Cytology only</td>
<td>17</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Cytology only</td>
<td>21</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Mixed</td>
<td>22</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Cytology only</td>
<td>23</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Cytology only</td>
<td>25</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>&gt; 25 nM</td>
<td>1</td>
<td>Cytology only</td>
<td>42</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Cytology only</td>
<td>52</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Epithelial</td>
<td>83</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Epithelial</td>
<td>110</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Cytology only</td>
<td>127</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Cytology only</td>
<td>198</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mesothelin staining - A positive result was defined as the presence of membranous stain in more than 20% of tumour cells.

<sup>b</sup> Staining intensity was graded semi-quantitatively as negative, weak (1+), moderate (2+) or strong (3+).
SPONSERS AND ACKNOWLEDGEMENTS: Supported in part by research grants from the National Health and Medical Research Council of Australia and the Insurance Commission of Western Australia, the Raine Foundation, and in part by Fujirebio Diagnostic Malvern, PA, USA. Biospecimens were provided by members of the ABN-Oncology Group which is funded by the National Health and Medical Research Council of Australia.

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DISCLOSURE STATEMENT: JC and BWR have received consultancy fees from FDI. The remaining authors have no conflicts to disclose.
FIGURE LEGENDS

Figure 1(A). Mesothelin concentrations in pleural effusions of patients. Mesothelin values were determined at least in duplicate by ELISA and individual patient values are plotted on the graph. Effusions were defined as being, transudate or exudate in nature, and as being benign or resulting as a consequence of malignancy. (B). Mesothelin concentrations in pleural effusions of patients with malignant mesothelioma further characterised by the histology of the tumour (sarcomatoid, biphasic or epithelial) or those cases where diagnosis was made without histology being reported (cytology only). (C). Receiver operating characteristic (ROC) curve showing accuracy of mesothelin effusion concentration in differentiating between (a.) all patients with mesothelioma, (b.) patients with epithelial, biphasic and cytologically defined mesothelioma and (c.) all patients with malignancy from individuals with effusions of a non-malignant nature. Furthermore curve (d.) shows the accuracy of mesothelin differentiating between patients with mesothelioma and non-mesothelioma malignancies. The area under the ROC curve was for (a) 0.898 (CI 95% 0.839-0.958); (b) 0.964 (CI 95% 0.934-0.994); (c) 0.748 (CI 95% 0.681- 0.816) and (d) 0.896 (CI 95% 0.731 - 0.896).

Figure 2. (A&B) Correlation of mesothelin concentration determined in serum and pleural effusion from individual patients. (A) In patients with mesothelioma and (B) In patients with non-mesothelioma malignant conditions (▲) and with benign conditions (△). (C) Mesothelin concentrations in pleural effusions of mesothelioma patients before pathological diagnosis of mesothelioma. A threshold value of 20nM for mesothelin concentrations in effusions and of 2.5nM in serum are indicated on the graph by dashed lines.

Figure 3. Median survival of mesothelioma patients according to the mesothelin concentration in the first available pleural effusion sample for each individual.

Figure 4. Mesothelin concentrations in peritoneal effusions of patients with malignant and benign disease, and in dialysis fluid of patients with renal failure. Mesothelin values were determined at least in duplicate by ELISA and individual patient values are plotted on the graph.

Figure 5. Immunostaining of sections of formalin fixed paraffin-embedded cell pellets from pleural fluid specimens of patients diagnosed with mesothelioma of unknown histology. Panels A & B are cells from a sample with a mesothelin concentration of 52 nM. Panels C & D are cells from a sample with a mesothelin concentration of 12 nM. Panels E & F show cells from a sample with a mesothelin level of 23 nM. Panels A, C and E were stained with an antibody against mesothelin; Panels B, D and F were stained with an antibody against EMA to demonstrate the presence of malignant mesothelioma cells.
REFERENCES

A.

B.

C.

mesothelin (nM)

Benign

Malignant

Exudate

Transudate

Non-infection

Infection

Lung Cancer

Other Cancer

MM

mesothelin (nM)

Epithelial

Biphasic

Cytology only

Sarcomatoid

a.

b.

c.

d.

mesothelioma vs all non-malignant

non-sarcomatoid mesothelioma vs all non-malignant

all malignant vs all non-malignant

mesothelioma vs non-mesothelioma malignant

Sensitivity

1 - Specificity

1 - Specificity
A. Serum mesothelin (nM) vs. Effusion mesothelin (nM)
   - Malignant mesothelioma

B. Serum mesothelin (nM) vs. Effusion mesothelin (nM)
   - Non-mesothelioma
   - Malignant effusion
   - Benign effusion

C. Time (weeks) vs. mesothelin (nM)
   - Malignant mesothelioma
Soluble mesothelin in effusions - a useful tool for the diagnosis of malignant mesothelioma

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