Combined blood and pleural levels of mesothelin and osteopontin for the diagnosis of malignant pleural mesothelioma

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Abstract  Background: Malignant pleural mesothelioma (MPM) is a highly aggressive tumor with poor survival rate. It is difficult to diagnose MPM at an early stage. Soluble mesothelin remains the best available biomarker for MPM, however the lack of sensitivity for early stage disease provides a motivation for the search of an additional marker that could be combined with mesothelin for early malignancy detection.

Aim of work: The aim was to evaluate the diagnostic value of soluble mesothelin and osteopontin both in blood and pleural fluid of MPM patients and to assess whether combination of these markers could improve the diagnostic accuracy of mesothelin.

Methodology: In this study mesothelin and osteopontin were measured by ELISA method in 197 samples (123 blood and 74 pleural) obtained from 123 participants, divided into 4 groups: 38 MPM patients, 24 patients with metastatic pleural effusion (Mets) of various carcinomas, 29 patients with hydrothorax and 32 healthy asbestos exposed subjects. Receiver operating characteristic (ROC) curves were generated to compare the diagnostic capability of these biomarkers. Combination of markers was done through logistic regression analysis.

Results: The median blood and pleural levels of the two markers were significantly higher in MPM patients than in hydrothorax or asbestos exposure groups (P < 0.0001), however the difference between MPM and Mets group was not significant. Combining the data from blood mesothelin and
Introduction

Malignant pleural mesothelioma (MPM) is a highly aggressive tumor with poor survival rate. The latency period between first exposure to asbestos and the development of mesothelioma has a wide range with an average of 15–40 years. The median survival time after diagnosis is less than 18 months [1]. Worldwide, the incidence of MPM has increased and is expected to increase for at least the next 10 years as a result of widespread exposure to asbestos in past decades [2].

Management of patients with MPM remains difficult as they are often referred in a late stage. Moreover MPM exhibits high resistance to radiotherapy and chemotherapy [3]. Therefore the discovery of a serum marker that permits earlier diagnosis or can accurately predict response to treatment will be a great advance in the management of this malignancy [4].

Several reports have raised interest about soluble mesothelin [2,3,5] and osteopontin [4] as possible markers for diagnosing MPM. However questions were raised about the clinical utility of these markers because the early reports did not include other pleural malignancies and non malignant pleural disease as controls [4,6]. Mesothelin is a physiologically expressed membrane-bound peptide on the surface of normal mesothelial cells and is found expressed in various cancers, including MPM, pancreatic, ovarian cancers, sarcomas, and in some gastrointestinal and pulmonary Carcinoma [7]. A soluble form released from the membrane-bound mesothelin can be detected in blood, and have been found highly increased in the blood of mesothelioma patients [2,5].

Osteopontin is an extra cellular adhesion protein involved in non-mineral bone matrix formation, and is a key cytokine in mediating type 1 immune response [8]. Osteopontin is also a regulator of inflammation, regulator of macrophage differentiation and recruitment, and is implicated in potentiating metastatic spread of tumor cells [9]. In fact osteopontin was first described as being secreted by transformed malignant epithelioid cells [10].

Aim of work

The aim is to evaluate the diagnostic value of soluble mesothelin and osteopontin levels in blood and pleural fluid of mesothelioma patients so as to assess whether combination of these markers can provide additional diagnostic value to the existing conventional diagnostic tools.

Subjects and methods

Starting from January 2008 till March 2010, 62 patients were recruited whose diagnosis was consistent with mesothelioma. They were admitted in Chest Diseases Department in Kasr Al-Aini hospital, Cairo University. All these patients had chest pain, dyspnea associated with pleural thickening and or pleural effusion on thoracic computed tomography scan. Pleural biopsies were taken by thoracoscopy, thoracotomy or US guided biopsy for final histo-pathological diagnosis. Exclusion criteria were any concomitant infectious or suppurative lung disease. Based on the histo-pathological diagnosis the 62 patients were divided into 2 groups: Group 1: 38 patients with confirmed MPM; Group 2: 24 patients with pleural metastases (Mets) of various carcinomas.

We also included 29 patients with hydrothorax as Group 3. The pleural fluid was confirmed to be non-malignant by cytology.

Group 4: By the aid of the occupational disease physicians, we recruited 32 healthy subjects living, for at least 10 years, in a residential area (AlMaasara) that surrounds one of the well known asbestos factories in Helwan, Cairo. Subjects were recruited from the houses facing the factory. Those subjects were supposed to be environmentally exposed to asbestos. They had no clinical complaint, no chest or any systematic disease and none of them developed MPM or another malignancy. Only serum samples were available from this group.

Informed consent was obtained from all participants.

Sampling

Blood samples (EDTA- anticoagulated plasma for osteopontin and serum for mesothelin assay) were collected from all participants. Also pleural samples (if available) were collected for both markers assay. From 123 participants, 197 samples were available (123 blood and 74 pleural samples)

Osteopontin is cleaved by thrombin after blood coagulation and therefore serum levels of osteopontin are much lower than the corresponding plasma levels [11,12]. Therefore we preferred to measure plasma and not serum osteopontin. Blood and pleural fluid samples were centrifuged for 10 min at 4000 rpm and the supernatant was stored in aliquots at −80 °C until the time of analysis.

Soluble mesothelin assay

Serum and pleural levels of soluble mesothelin were determined using sandwich-type ELISA kit (Mesomark, Fujirebio...
Diagnostics Inc. Malvern, USA) according to manufacturer’s instructions; results were expressed in nanomoles/L.

**Osteopontin assay**

Plasma and pleural levels of osteopontin were determined using human osteopontin kit (R&D systems, Inc., Minneapolis, USA) which employs the quantitative sandwich ELIZA technique; results were expressed in ng/ml.

**Statistics**

Quantitative non-normal data were expressed as median and interquartile range (25–75th percentiles). Comparison between groups was carried out using Kruskal–Wallis or Mann–Whitney tests. Chi-square test was used to assess the differences between different variables. Linear regression analysis was done to determine the correlation between different variables. To assess the clinical potential of each marker at a time, receiver operating characteristic (ROC) curves were plotted, and the areas under curves (AUC) were calculated with their 95% confidence intervals (95% CI) using standard techniques to evaluate sensitivity and specificity. Logistic regression analysis was run to calculate the probability of MPM diagnosis using different combinations of blood and pleural levels of mesothelin and osteopontin. ROC curves for the combinations of markers were plotted with calculation of the AUC and the best cut-off values. Statistical analysis was run on SPSS for Windows, release 17.0 (SPSS Inc., Chicago, Ill, USA). *P* values ≤0.05 were considered statistically significant.

**Results**

Demographic and laboratory data of the studied groups are summarized in Table 1.

### Diagnostic value of serum mesothelin

MPM group showed the highest median level of serum mesothelin (1.1 nmol/L). Serum mesothelin had a good capability to distinguish MPM from hydrothorax and asbestos exposure groups with AUC of 0.785 and 0.752, respectively, although it showed low capability in differentiating MPM from Mets group (AUC 0.577) (Table 2). Mesothelin level at 0.55 nM was determined to be the optimal cutoff value with a sensitivity of 79% and specificity of 60% for the diagnosis of mesothelioma.

### Diagnostic value of pleural mesothelin

In the three groups of patients having pleural samples pleural mesothelin levels were higher than respective serum values (Table 1). Pleural mesothelin median value was highest in MPM group (5.8 nmol/L). Pleural mesothelin showed its best discriminating power when ROC curve was drawn for differentiating MPM from hydrothorax group as AUC was 0.871. The optimal cutoff value was 3.0 nmol/L with 73% Sensitivity and 82% specificity. Pleural mesothelin had better diagnostic value than serum mesothelin as all its AUCs were better than corresponding serum mesothelin AUCs among different comparisons (Table 2).

### Diagnostic value of plasma osteopontin

The highest median level was found in MPM group (126 ng/ml). Serum osteopontin had an excellent ability to distinguish between MPM and asbestos exposure groups with AUC of 0.943 (Table 2).

Plasma osteopontin showed significant positive correlation with the duration of asbestos exposure (r = 0.48, *P* = 0.005) among asbestos exposure group.

| Table 1 Demographic data and levels of mesothelin and osteopontin among studied groups. |
|----------------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| MPM group *n* = 38 (for serum markers) | Mets group *n* = 24 (for pleural markers) | Hydrothorax group *n* = 29 | Asbestos exposure group *n* = 32 | *P* value |
| Age (years) Median (25–75th) | 56 (44–64) | 58 (50–70) | 56 (51–62) | 45 (35–54) |
| Gender | | | | |
| Male n (%) | 27 (71%) | 9 (37%) | 15 (52%) | 22 (69%) |
| Female n (%) | 11 (29%) | 15 (63%) | 14 (48%) | 10 (31%) |
| Confirmed Asbestos exposure n (%) | 8 (21%) | – | – | 32 (100%) |
| Duration of exposure in years Median (25–75th) | 41 (26–58) | – | – | 20 (15–25) |
| Primary disease n (%) | Epithelioid 17 (45%) | Bronchogenic 14 (58%) | Liver cirrhosis 21 (72%) | – |
| | Mixed 11 (29%) | Breast 8 (34%) | Collagen disease 6 (21%) | – |
| | Sarcomatoid 10 (26%) | Unknown 2 (8%) | Heart failure 2 (7%) | – |
| Serum mesothelin (nmol/L) | 1.1 (0.6–2.7) | 0.7 (0.3–1.7) | 0.2 (0.1–0.8) | 0.5 (0.4–0.9) | <0.0001 |
| Pleural mesothelin (nmol/L) | 5.8 (2.5–18.6) | 4.7 (0.6–11.8) | 0.7 (0.4–2.7) | – | <0.0001 |
| Plasma osteopontin (ng/ml) | 126 (81–200) | 94 (47–170) | 82 (44–144) | 34 (29–57) | <0.0001 |
| Pleural osteopontin (ng/ml) | 2135 (218–2802) | 490 (150–1504) | 81 (35–417) | – | <0.0001 |

Blood and pleural levels are presented as Median and percentiles (25–75th).

* a,b,c Groups comparing bearing same initials do not differ statistically at *P* < 0.05.
nient cutoff value was 73 ng/ml with 84% sensitivity and 72% specificity in confirming mesothelioma.

**Diagnostic value of pleural osteopontin**

The highest median level of pleural osteopontin was recorded in the MPM group (2135 ng/ml). Pleural osteopontin showed its best differentiating capability between MPM and hydrothorax groups with AUC of 0.847 (Table 2). At a cutoff value of 720 ng/ml, pleural osteopontin had a sensitivity of 73% and specificity of 88% for the diagnosis of mesothelioma.

**Combining mesothelin and osteopontin**

A) **Combining serum mesothelin and plasma osteopontin.** A bivariate scatter plot of serum mesothelin vs plasma osteopontin levels in MPM, hydrothorax and asbestos exposure subjects revealed that 35% of patients were positive for both markers, 20% were positive for only mesothelin, 14% were positive for only osteopontin, and 31% were negative for both markers (Fig. 1-A). So both markers were concordant in 66% of those 3 groups of patients.

The probability of the risk of developing MPM was calculated using logistic regression formula

\[
\text{Logit}(P) = (0.628X1) + (0.015X2) - 2.645
\]

where \(X1 = \text{serum mesothelin}\) and \(X2 = \text{plasma osteopontin}\). Logit (\(P\)) was converted into probability of the risk by using the following equation: \(P = \frac{1}{1+e^{-\text{logit}(P)}}\). According to this formula the AUC of the ROC curve of combined markers was increased from 0.774 for serum mesothelin and from 0.828 for plasma osteopontin to 0.867. Also the combination of these two blood markers had a better performance in terms of sensitivity (which was raised from 79% for serum mesothelin to 84%)

![Figure 1-A](image-url)
and in terms of specificity (which was raised to 79%) in differentiating MPM from hydrothorax and asbestos exposed subjects (Fig. 2-A, Table 3).

B) Combining pleural mesothelin and pleural osteopontin. A bivariate scatter plot of pleural mesothelin vs pleural osteopontin levels in MPM (only patients with pleural effusion) and hydrothorax patients revealed that 29% of patients were positive for both markers, 18% were positive for only pleural mesothelin, 14% were positive for only pleural osteopontin, and 39% were negative for both markers (Fig. 1-B). So both pleural markers were concordant in 68% of those two groups of patients.

The probability of the risk of developing MPM was calculated using logistic regression formula:

\[
\text{Logit}(P) = (0.416X1) + (0.007X2) - 2.324
\]

where \(X1\) = pleural mesothelin and \(X2\) = pleural osteopontin. Logit \((P)\) was converted into probability of the risk by using the equation: \(P = \frac{1}{1 + e^{-\text{Logit}(P)}}\). The AUC of the ROC curve of combined pleural markers was raised from 0.871 for pleural mesothelin and from 0.847 for pleural osteopontin to 0.905 in differentiate MPM from hydrothorax group. Combined sensitivity yielded a higher percentage (77%) than either marker alone, the specificity was raised from 82% for pleural mesothelin to 88% for combined pleural markers (Fig. 2-B, Table 3)

**Discussion**

Soluble mesothelin remains the best available biomarker for MPM [13]. However the lack of sensitivity for early stage disease provides a motivation for the search of an additional marker that could be combined with mesothelin for early malignancy detection.

In agreement with other reports [2,5,7], we confirmed here that serum mesothelin level is significantly higher in MPM patients than in subjects exposed to asbestos \((P < 0.0001)\) or those with hydrothorax \((P < 0.0001)\), but was insignificantly higher than in Mets group \((P = 0.0001)\).

When we tried to optimize both sensitivity and specificity of serum mesothelin, the best cutoff value was 0.55 nM with 79% sensitivity and 60% specificity. In concordance with our results portal et al. [14] chose 0.55 nM/L to be the best cutoff level of serum mesothelin with 72% sensitivity and 72% specificity.

**Table 3**  Sensitivity, Specificity and AUC of each marker and after combination.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum mesothelin (cutoff point 0.55 nmol/L)</td>
<td>79</td>
<td>60</td>
<td>0.774 (0.678–0.852)</td>
</tr>
<tr>
<td>plasma osteopontin (cutoff point 73 ng/ml)</td>
<td>84</td>
<td>72</td>
<td>0.828 (0.739–0.897)</td>
</tr>
<tr>
<td>Combined blood markers (when cutoff combined probability of risk &gt;0.313)</td>
<td>84</td>
<td>79</td>
<td>0.867 (0.784–0.927)</td>
</tr>
<tr>
<td>Pleural mesothelin (cutoff point 3.0 nmol/L)</td>
<td>73</td>
<td>82</td>
<td>0.871 (0.778–0.964)</td>
</tr>
<tr>
<td>pleural osteopontin (cutoff point 720 ng/ml)</td>
<td>73</td>
<td>88</td>
<td>0.847 (0.738–0.956)</td>
</tr>
<tr>
<td>Combined pleural markers (when cutoff combined probability of risk &gt;0.559)</td>
<td>77</td>
<td>88</td>
<td>0.903 (0.789–0.969)</td>
</tr>
</tbody>
</table>

**Figure 1-B**  Plotted concentrations of Pleural mesothelin and pleural osteopontin levels in 26 patients with MPM (closed diamonds) and 29 hydrothorax patients (open diamonds). Cut-off values for each assay are designated by lines.
However Scherpered et al. [2] reported higher sensitivity and specificity (80% and 82%, respectively) at different cutoff value which was 0.93 nmol/L. To achieve similar specificity, we had to set the cutoff at 0.95 nmol/L but this resulted in only 50% sensitivity. On the basis of our study the use of serum mesothelin alone as MPM screening marker may not reach sufficient sensitivity with adequate specificity. To reach a sensitivity of > 90–95%, the cut off value would be 0.25 nmol/L (sensitivity 96%), again the specificity would fall down to 56%. However, established tumor markers such as prostate specific antigen (PSA) exhibit similar or even lower test performances; PSA has sensitivity for detecting prostate cancer of 75% but a low specificity of only 40%, even if the identification of other molecular forms of PSA has led to a new era in PSA markers [15].

Given that the majority of MPM patients present with exudative effusions [3], we investigated whether mesothelin levels in effusions would add to the diagnostic value of serum mesothelin levels. In the current study pleural mesothelin level above 3.0 nmol/L is highly suggestive of MPM (73% sensitivity and 82% specificity) with a resulting AUC of 0.871 when differentiating MPM from hydrothorax group. This result is very similar to that reported by Scherpered et al. [2] who generated a ROC curve for pleural mesothelin in the setting of MPM diagnosis in a multicentre study in France with an AUC of 0.831.

Creaney et al. [13] showed a better AUC (0.890) for pleural mesothelin to diagnose MPM. This concordance of the results from these independent studies is promising for the future use of pleural mesothelin in diagnosing pleural effusions. Notably we have also shown that pleural mesothelin had AUCs higher than that of serum mesothelin among our different comparisons. This further underlines the fact that measurement of effusion mesothelin is a useful diagnostic test. However negative results should be interpreted cautiously as it does not rule out malignancy.

In fact, not all patients with mesothelioma present with an effusion, so the measurement of a serum biomarker such as...
mesothelin will be the only systemic aid for the diagnosis in such patients.

When measuring plasma osteopontin our results were consistent with Pass et al. [4] and Grigoriu et al. [7] studies who reported that significant higher levels of plasma osteopontin were recorded in MPM patients than asbestos exposure group. In our study, ROC analysis comparing MPM group with asbestos exposure subjects showed an excellent ability of plasma osteopontin (AUC = 0.943) in differentiating between the two groups.

However the utility of plasma osteopontin alone is hampered by the insufficient specificity to MPM as osteopontin was found to be elevated in other types of cancers including gastrointestinal, laryngeal, and urinary neoplasms which would result in a very high number of false positive results [4]. Osteopontin is also a cytokine that has been involved in a broad range of biological processes as cellular immune response and inflammation, cancer progression and metastases [16]. Moreover in our results significant positive correlation was found between the duration of exposure and plasma osteopontin among the asbestos exposed subjects ($r = 0.488$, $P = 0.005$). This means that the level of osteopontin is affected by the ongoing inflammatory process along the years of exposure, however serum mesothelin did not show this positive correlation with the duration of exposure in asbestos exposed subjects ($r = 0.194$; $P = 0.50$). This could be an advantage of mesothelin over osteopontin as it is not easily cleaved from the surface of mesothelial cells by the inflammatory process and there is something particular about the malignant state to increase mesothelin level. This is in contrast to other markers such as CA125 and CA19-9 which are also released by normal mesothelial cells and are increased in inflammatory states, reducing their diagnostic specificity for MPM [17].

There is considerable interest in screening asbestos exposed individuals for the early detection of MPM. Combining markers often improves the performance of diagnosis and screening strategies, and the use of independent biomarkers can significantly enhance sensitivity [18]. However, several reports failed to present suitable combination of markers that is able to increase diagnostic accuracy of mesothelin [19–21].

Cristaudo et al. [22] in their recent report mentioned that their study was the first to show that combined serum mesothelin and plasma osteopontin through logistic regression analysis can increase both the sensitivity and specificity. And here we also succeeded to show higher sensitivity, specificity and higher AUC by calculating the probability of risk through logistic regression analysis. We also add here that combination of pleural levels of mesothelin and osteopontin increased the sensitivity and the AUC for diagnosing MPM.

Usually, the application of the two traditional ways of combining multi-tests (parallel assessment or serial assessment) improves either sensitivity or specificity. However estimation of the sensitivity and specificity after calculation of the probability of risk through logistic regression analysis avoided this limitation.

**Conclusion**

Combined blood mesothelin and osteopontin measurement improved the diagnostic ability of serum mesothelin in terms of sensitivity, specificity and AUC. Pleural mesothelin is a biological marker as interesting as serum mesothelin and is potentially better. Until now the measurement of tumor markers in pleural effusions has not been part of routine clinical practice. The data presented here argue that measurement of pleural mesothelin and osteopontin 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 and biochemical analysis, it is simple to undertake mesothelin and or osteopontin analysis at the same time and not to be satisfied with blood level only.

Additional investigations in a larger panel or maybe multicentre study will be necessary to demonstrate the usefulness of blood and pleural mesothelin and osteopontin combination in the management of MPM.

**References**