YKL-40 and Mesothelin in the Blood of Patients with Malignant Mesothelioma, Lung Cancer and Asbestosis

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Abstract. *Background/Aim: In the diagnosis of malignant mesothelioma (MM) there still is a lack of specific and sensitive screening biomarkers: this study examined the discriminatory power of a panel of serum/plasma biomarkers. Patients and Methods: The study involved four groups: (a) individuals previously exposed to asbestos with asbestosis; (b) patients with MM; (c) patients with non-small cell lung cancer; and (d) controls without any evidence of malignancy. The concentrations of mesothelin, chitinase-3-like-1 (YKL-40), vascular endothelial growth factor (VEGF), endothelin-1, interleukin-8 (IL-8) and fibulin-3 in the serum of patients were determined. Results: Patients with MM had significantly higher serum levels of mesothelin (p<0.001), YKL-40 (p<0.001), IL-8 (p<0.001) and VEGF (p<0.01) than controls. The cut-off point for MM was 1.26 nM for mesothelin alone, and 167 pg/ml for YKL-40 alone; the presence of both markers above these cut-off levels improved diagnostic specificity. Conclusion: The addition of YKL-40 may improve the specificity of mesothelin measurements alone for detecting patients with MM.*

Malignant mesotheliomas (MMs) are highly aggressive tumours that lead to a median patient survival of 6-18 months (1). The vast majority of cases are related to asbestos exposure, although other aetiopathogenetic factors cannot be ruled out.

The usefulness of MM screening in individuals previously exposed to asbestos is controversial because only a few studies have demonstrated that early therapeutic intervention is effective. However, people experiencing occupational or non-occupational asbestos exposure are concerned by their greater risk of developing MM, and it has been reported that patients with stage IA disease can survive for five years or more if the tumour is promptly removed (2). Effective preventive protocols may include frequent imaging diagnostic tests over a long period of time (decades), but this would be neither economic nor ethical, particularly since available tests have failed to detect malignancy early and there are still frequent difficulties in distinguishing benign from malignant disease (3).

It would, therefore, be very useful to identify sensitive and specific biomarkers. The limited invasiveness and acceptability of blood tests makes the use of serum-based biomarkers an attractive strategy that would be relatively cheap for national health services. However, although many such candidates have been reported, their individual value has not yet been confirmed and therefore more substantial scientific evaluation is required before promoting a screening programme (4).

There are promising published data indicating that mesothelin could be used as a biomarker of MM but, when used alone, it has a high rate of false-positives in healthy individuals and, as mesothelin levels are also high in patients with non-malignant pleural effusion or other malignancies, it is unlikely to be diagnostically useful (5, 6). This has led to various attempts to find other markers that could improve the diagnostic specificity of mesothelin. However, Creneay *et al.* found that combining serum mesothelin and plasma osteopontin levels did not significantly increase the area under the receiver operating characteristic (ROC) curve using a logistic regression model (7), and the same is true of megakaryocyte-potentiating factor, hyaluronic acid, carcinoembryonic antigen, CYFRA 21.1 and Cancer antigen 125 (8, 9).

YKL-40 (a chitinase-like protein) is an inflammatory biomarker that is associated with the pathogenesis of lung lesions. It is produced at the site of disease by various cells, including cancer cells and cancer-associated macrophages

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(10,11). It has recently been shown that pleural YKL-40 levels are higher in patients with exudative pleural effusion than in those with cardiogenic transudative pleural effusions (12), and that serum YKL-40 levels predict a poor prognosis in patients with non-small cell lung cancer (NSCLC) (13); however, to the best of our knowledge no clinical studies have examined YKL-40 levels in patients with pleural MM.

Endothelins and vascular endothelial growth factor (VEGF) are well-known biomarkers that have many potential roles in tumours, including modulating angiogenesis, inducing mitogenesis and the invasion of tumour cells, and protecting cells from apoptosis (14-16). Pass *et al.* have recently showed that plasma levels of fibulin-3, an extracellular glycoprotein that is normally expressed in small quantities and inversely correlates with cell growth, can distinguish healthy individuals exposed to asbestos from patients with mesothelioma (17).

Interleukin-8 (IL-8), a pro-inflammatory and angiogenic cytokine, has an important role in tumour-related neovascularisation (16).

The aim of this cross-sectional study was to investigate a panel of independent biomarkers that could improve for the specificity and sensitivity of mesothelin as a biomarker of MM in patients with disorders after extensive exposure to asbestos.

Patients and Methods

Patients. The study consisted of four groups of prospectively recruited individuals. The first had non-malignant asbestos-related lung and pleural disorders (pleural thickening and/or asbestosis) acknowledged by the Italian Workers'Compensation Authority (INAIL) on the basis of their documented occupational exposure to asbestos accompanied by a positive (HRCT) chest X-ray. The patients with asbestosis were recruited during a clinically stable phase of the disease when they were undergoing thermal treatment supported by the INAIL. The other three groups were: patients with biopsy-proven MM; patients with lung cancer, recruited at the University Hospital of Parma at the time of diagnosis before receiving any treatment; and a control group of patients referred to the Thoracic Endoscopy Service of the same hospital for diagnostic purposes who showed no signs of malignancy and had not been occupationally exposed to asbestos. Those with undefined tumour stage were those who did not complete the diagnostic characterization, despite histological evidence. Therefore, they were considered in the overall sample, but were excluded from stratification due to lack of data.

Patients suffering from MM and those with NSCLC were staged according to the TNM classification (18, 19).

A venous blood sample was obtained from all participants. The study was approved by our local Ethics Committee (approval number 9509).

Blood biomarkers. The serum/plasma concentrations of all of the biomarkers (mesothelin, YKL-40, VEGF, endothelin-1, IL-8, fibulin-3) were measured using commercially available, specific sandwich enzyme-linked immunosorbent assays (ELISAs).

The presence of soluble mesothelin-related peptides (SMRPs) was evaluated using the Mesomark™ (Fujirebio Diagnostic Inc., Malven, PA, USA), an immunoassay based on two different monoclonal antibodies with calibrators (2-32 nM) referenced to a standard prepared by the manufacturer. The lowest antigen concentration that can be distinguished from zero is 0.3 nM.

The working range of the YKL-40 immunoassay (Quidel Corporation, San Diego, CA, USA) is from 20 (the minimum detection limit) to 300 ng/ml.

VEGF and endothelin-1 were both measured using quantitative sandwich enzyme immunoassays (R&D Systems, Minneapolis, MN, USA). The VEGF Quantikine Assay provides accurate measurements within the range of 31.2-2,000 pg/ml, and has a detection limit of 9 pg/ml. The highest assayable concentration of the human endothelin-1 kit is 120 pg/ml, and the detection limit is ~1 pg/ml. The samples were prepared in accordance with the extraction protocol suggested by the manufacturer, and the concentrations read from the standard curve were corrected/divided by the reconstitution factor.

The ultra-sensitive sandwich ELISA for human IL-8 (Invitrogen Corporation, Camarillo, CA, USA) has a detection limit of <100 fg/mL and the highest assayable concentration is 25 pg/ml. The intensity of the coloured product was read by means of a temperature-controlled Multiskan Ascent photometer (Thermo Labsystems, Helsinki, Finland). The intra- and inter-assay coefficients of variation (CVs) of all of the assays were <10% at the different concentrations.

Plasma concentrations of fibulin-3 were quantified using the sandwich enzyme immunoassay of USCN Life Science Inc. (Wuhan, P.R. China) according to the manufacturer's protocol. The kit provides accurate measurements within the range of 100-1.56 ng/ml, and has a detection limit of 0.55 ng/ml.

Statistical analysis. Data distribution was assessed by means of the Kolmogorov-Smirnov test. As the distribution was neither normal nor log-normal, and values were below the detection limits, the between-group differences were assessed using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Non-normal data are given as median values (interquartile range). Spearman's correlation was used to test the relationships between pairs of variables. Crude or unadjusted (non-nomalised) odds ratios (ORs) were calculated using a multinomial (with the controls as the group with an OR=1) or binary logistic regression model with a single covariate, whereas adjusted ORs were calculated by adding other covariates to the model as indicated.

ROC curves were used to test the sensitivity and specificity of a marker, and to find the cut-off values, with the area under the curve (AUC) being given with its 95% confidence interval (CI). The cut-off value was the value at which the sum of sensitivity and specificity was greatest. The cut-off values were further used to distinguish the multi-positive individuals (*i.e.* those with more than one test with a value that was equal to or greater than the cut-off value). In the case that more than one marker was used to build up the ROC curve (marker pattern), the probability of being in a given group, as calculated by means of logistic regression was used instead of marker values.

The data were statistically analysed using IBM SPSS 20.0 (IBM, Armonk, NY, USA) and a *p*-value of 0.05 was considered significant.

Table I. *Characteristics of the studied groups.*

	Controls	NSCLC	Asbestosis	MM
Number	66	77	16	50
Gender	46 M; 20 F	56 M; 21 F	9 M; 7 F	26 M; 24 F
Age, years	61.6 (SD: 10.0)	68.1 (SD: 8.1)	72.2 (SD: 8.7)	70.2 (SD: 7.8)
Smokey status (Non smoker- Ex smoker- Current smoker)	33-22-11	13-27-37	8-5-3	24-22-4
Diagnosis	34 No RD	52 ADK	-----	9 Generic MM
	13 Benign nodules	24 SCC		32 Epithelioid MM
	4 Bronchiectasis	1 Other		3 Sarcomatoid MM
	15 Other RD			6 Biphasic MM
Stage	----	32 IA	-----	10 IA
		15 IB		4 IB
		5 IIA		13 II
		6 IIB		13 III
		13 IIIA-B		10 IV
		6 IV		

Results

Table I shows the characteristics of the studied groups, which were not perfectly homogeneous in terms of gender ($p=0.08$), age ($p<0.01$; the controls were significantly younger than the other three groups), or smoking habit ($p<0.01$; the non-smokers ranged from 13.5% in the NSCLC group to nearly 50% in the other three groups). The results were, therefore, controlled for these possible confounding factors in the adjusted models.

The influence of diagnosis [(adenocarcinoma (ADC) *vs.* squamous cell cancer (SCC)] and stage (I *vs.* II-IV) was assessed in patients with the NSCLC; in those with MM , stages I-IV were considered separately and the diagnosis of epithelioid MM was compared with the other forms.

Mesothelin. Figure 1 shows the scattergram of serum mesothelin values. Patients with MM had significantly higher levels than the other three groups, with the median value being more than that of the control values; there was no difference between the values for the other three groups.

In the NSCLC group, neither histotype nor stage (I *vs.* II-III-IV) significantly influenced serum mesothelin values. There was a trend, albeit non-significant, towards higher values in the MM group going from stage I to stage IV ($p=0.08$, data not shown), whereas histology (epithelioid *vs.* other forms) had no effect. Gender and smoking habits did not have a significant effect on mesothelin levels whether considering the whole cohort or the individual groups, but were included as factors in the multivariate models. Age weakly correlated with mesothelin level ($r=0.24$, $p<0.01$) in the samples as a whole, and the correlation was as high as $r=0.30$ in the MM group; age may be considered a weak confounding factor.

Table II shows the unadjusted and adjusted ORs (for 1 nM increase in mesothelin level) in the four groups and in the MM group *vs.* the others; in both cases, the ORs did not change when the analysis was adjusted for confounding factors.

A ROC curve (Figure 2) was used to assess the statistical diagnostic power of mesothelin alone in the MM group against the other three groups together. The AUC was 0.85 (0.79-0.91), which is significantly higher than 0.5. The cut-off point (*e.g.* when sensitivity and specificity was at its greatest) was 1.26 nM, with a sensitivity of 74% and a specificity of 86.1%. Figure 3 shows the results when this value was applied to the MM group by stage and histology (epithelioid *vs.* other forms): sensitivity was greatest in the patients with epithelioid MM stage III-IV (88.8%), and least in patients with epithelioid MM stage I-II (57.1%).

YKL-40. Figure 4 shows the trend of serum YKL-40 levels, which were highest in the MM group. Patients with NSCLC had higher levels than did the controls, but these did not significantly correlate with histotype or stage (I *vs.* II-III-IV). There was no significant trend in the MM groupings (I-II-III-IV and histology). Gender and smoking habits had no significant effect on YKL-40 levels in the cohort as a whole, nor in the individual groups, but were included as factors in the multivariate models. Age moderately correlated with YKL-40 levels ($r=0.40$, $p<0.01$) in the cohort as a whole, and the correlation reached $r=0.49$ in the control group; age can therefore be considered a possible confounding factor.

Table III shows the unadjusted and adjusted ORs (for 1 pg/ml increase in YKL-40 levels) in the four groups and in the MM group *vs.* the others.

The ROC curve (Figure 5) used to assess the statistical diagnostic power of YKL-40 alone in the MM group against the other three groups together showed an AUC of 0.78 (0.71-0.85), which is significantly higher than 0.5. The cut-off point was 167 pg/ml, with a sensitivity of 68% and a specificity of 74.3%.
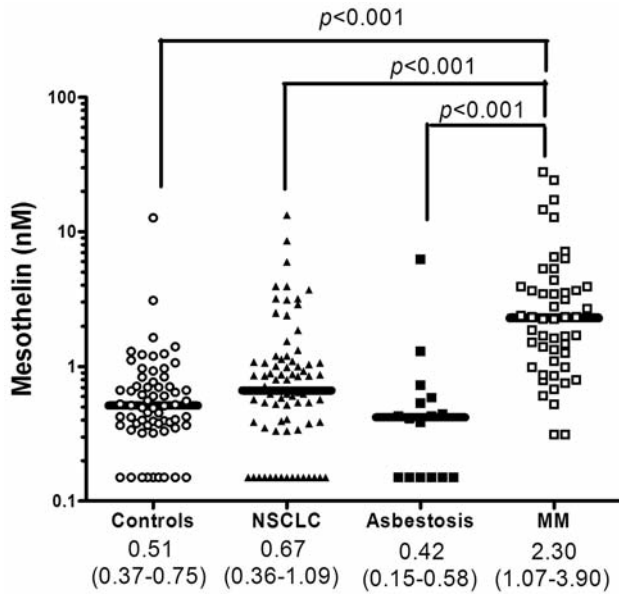
Figure 1. *Mesothelin levels in the studied Groups. Median values (25°-75°percentiles) are reported.*
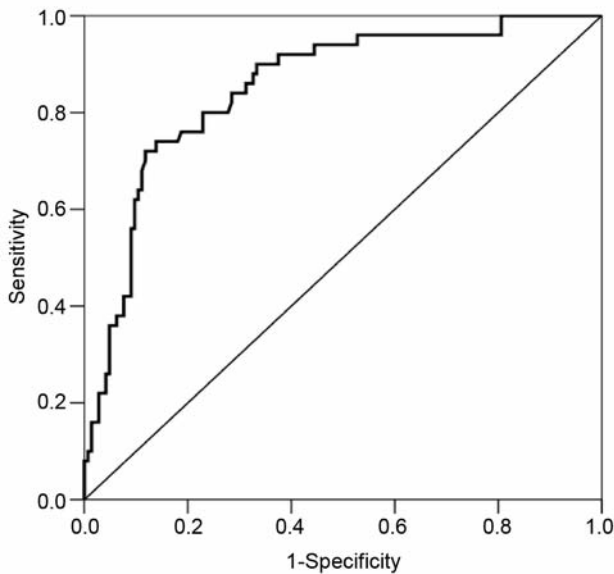

Figure 2. *Receiver Operating Characteristic curve for Mesothelin.*

Figure 3. *Patients affected by Malignant Mesothelioma (%) with a Mesothelin cut-off of 1.26 nM, distinguished by histology (epithelioid vs. other) and stage (I-II vs. III-IV).*

Table II. *Unadjusted and adjusted Odd Ratio (95% Confidence Interval) for increase in mesothelin values of 1 nM. aConfounders: age, gender, smoking habits. MM significant in all scenarios at $p<0.001$.*

Model 1	Unadjusted OR	Model 2	Unadjusted OR
Controls (ref)	1	Non-MM	1
NSCLC	1.43 (0.99-2.04)		
Asbestosis	0.95 (0.47-1.90)		
MM	1.93 (1.35-2.76)	MM	1.54 (1.24-1.92)
	Adjusted OR[a]		Adjusted OR[a]
Controls (ref)	1	Non-MM	1
NSCLC	1.19 (0.88-1.61)		
Asbestosis	0.87 (0.44-1.72)		
MM	1.66 (1.21-2.29)	MM	1.54 (1.22-1.94)

[a]Confounders: age, gender, smoking habits. MM significant in all scenarios at $p<0.001$.

IL-8, VEGF and endothelin-1. The trend for the three proteins is shown in Figures 6A-C. IL-8 and VEGF levels were significantly higher in the MM group than in the controls ($p<0.001$ and $p<0.01$, respectively), but they were not significantly higher than those observed in the NSCLC and asbestosis groups, which were both significantly higher than in controls ($p<0.001$ and $p<0.01$, respectively). Endothelin-1 levels were never significantly different from those in the controls. On the basis of these data,
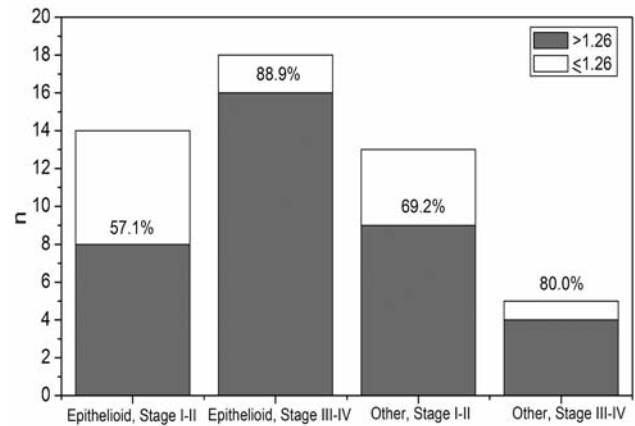
regardless of strata and confounding factors, these biomarkers were insufficiently specific for diagnostic purposes and no further description of the analyses will be given.

Fibulin 3. Figure 7 shows the distribution of serum fibulin-3 levels, which were higher in the MM group than in the NSCLC ($p<0.01$) and control groups ($p<0.05$), but not significantly different from those of the asbestosis group. However, it should be noted that serum fibulin-3 was assayed in a limited number of patients because we started to analyze it on the samples collected after its indication in the literature as an important marker of MM (17), in order to validate this finding.

Multivariate analysis. On the basis of the findings described above, mesothelin and YKL-40 were entered together in a multivariate logistic regression model in order to assess

Table III. *Unadjusted and adjusted Odd Ratio (95% Confidence Interval) for increase in Chitinase-3-like protein 1 values of 1 pg/ml. aConfounders: age, gender, smoking habits. Significant at p<0.05 (*), p<0.001 (**).*

Model 1	Unadjusted OR	Model 2	Unadjusted OR
Controls (ref)	1	Non-MM	1
NSCLC	1.007 (1.003-1.012)**		
Asbestosis	1.004 (0.997-1.011)		
MM	1.014 (1.009-1.019)**	MM	1.009 (1.006-1.012)**
	Adjusted ORa		Adjusted ORa
Controls (ref)	1	Non-MM	1
NSCLC	1.005 (1.000-1.010)*		
Asbestosis	1.002 (0.994-1.009)		
MM	1.011 (1.005-1.016)**	MM	1.008 (1.004-1.012)**

[a]Confounders: Age, gender, smoking habits. Significant at $p<0.05$ (*), $p<0.001$ (**).

Figure 4. *Chitinase-3-like protein 1 levels in the studied groups. Median values (25°-75° percentiles) are reported.*

whether their combination had more diagnostic power than that of the individual proteins. To do this, we used an unadjusted model (MM *vs.* the other groups) but, at the same time, we also tested the unadjusted multinomial model and the adjusted binary/multinomial models in order to strengthen the significance of the differences. In both the unadjusted and adjusted models, the two variables were always significant for the MM group (mesothelin: *p*-values between 0.001 and 0.003; YKL-40 *p*-values between <0.001 and 0.018), but the use of these calculated probabilities to create the ROC curve showed that there was no improvement over the diagnostic power of mesothelin alone: AUC=0.86 (0.80-0.91), with a sensitivity of 72% and a specificity of 83.8% at the cut-off point (data not shown).

Combining the data of the two biomarkers using a logistic regression model also failed to improve sensitivity. The logistic model gave no problems of multicollinearity, but mesothelin and YKL-40 were sufficiently correlated to explain this statistical result and did not ensure sufficient inter-independence for their combined use (r=0.45, *p*<0.01) in the sample as a whole (Figure 8). However, the figure clearly suggests that the use of both assays in series for those individuals positive for the first test, and in parallel for those negative for the first test, may be clinically useful as only five patients with MM (10%) had both values under the cut-off points calculated using the ROC curves.

Discussion

The present study study examined the usefulness of combining serum levels of mesothelin and some other biomarkers that have not been previously studied in MM in order to screen patients with asbestos-related lung diseases. The median mesothelin levels in our cases and controls are in line with previous data reviewed by Hollevoet *et al.* (6),
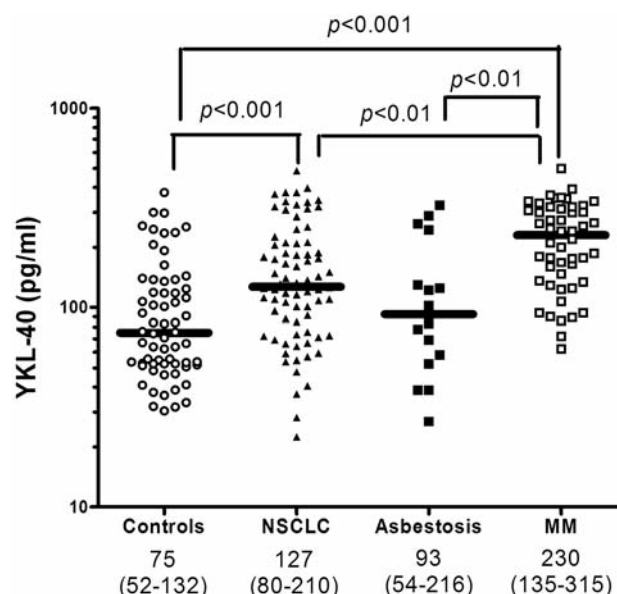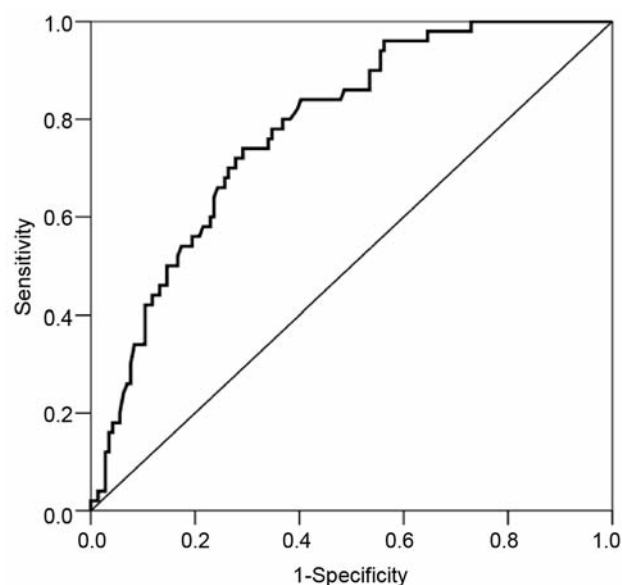

Figure 5. *Receiver Operating Characteristic curve for Chitinase-3-like protein 1.*

and were significantly high in the patients with histologically-confirmed MM. The cut-off point of 1.26 nM, which had a sensitivity of 74% and a specificity of 86.1%, was practically identical to that previously reported by Van den Heuvel *et al.* (1.3 nM) (20) and very similar to those reported by Cristaudo *et al.* (1 nM) (21), Beyer *et al.* (1.5 nmol/L) (22), and Di Serio *et al.* (1.5 nM) (23).
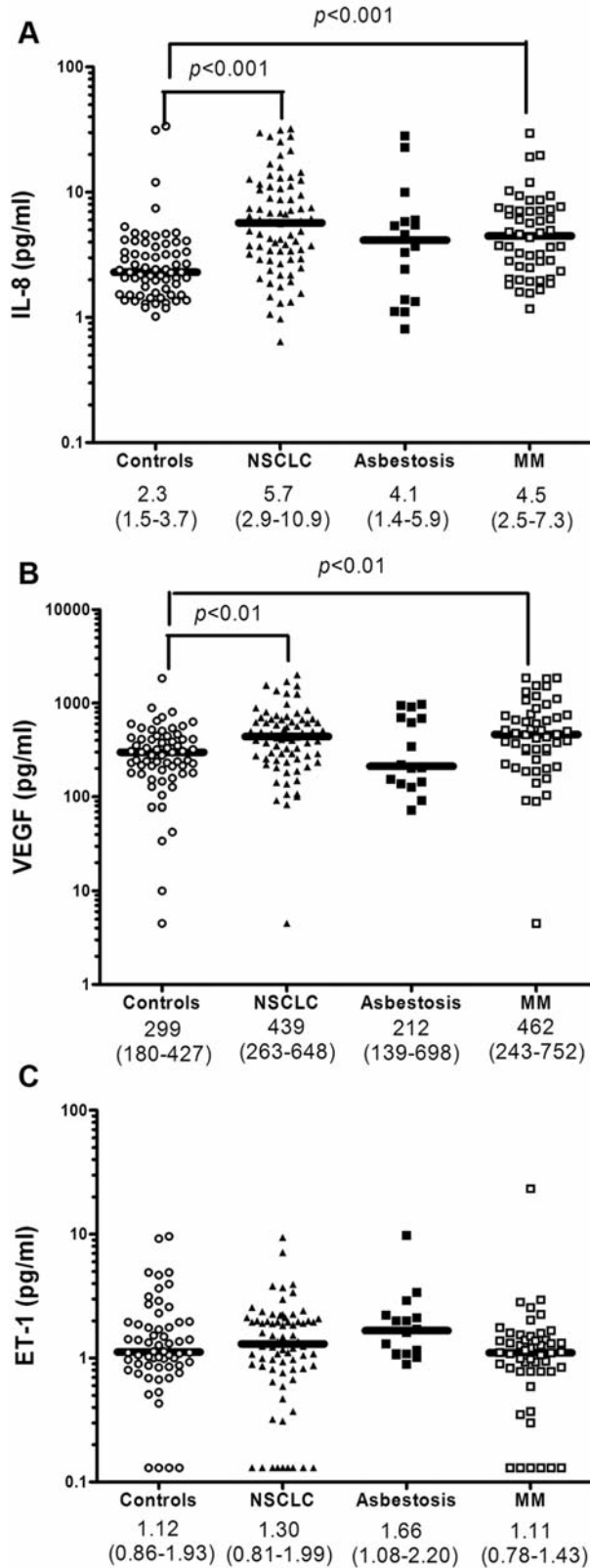
Figure 6. (A) Interleukin-8 levels; (B) Vascular Endothelial Growth Factor levels; (C) Endothelin-1 levels in the studied groups. Median values (25°-75° percentiles) are always reported.
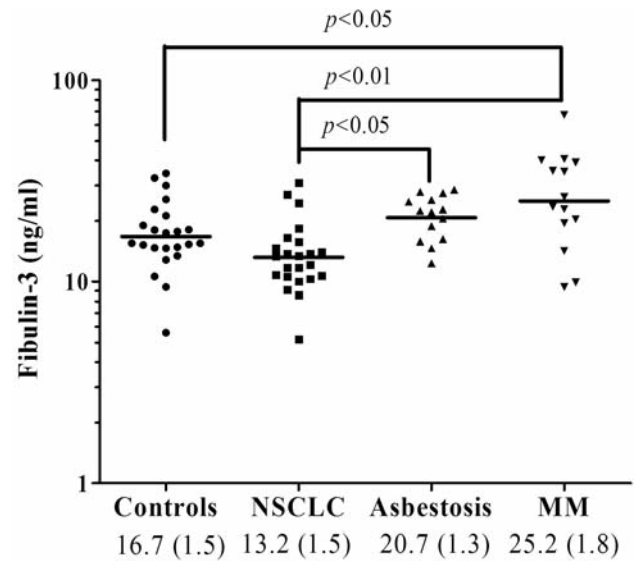

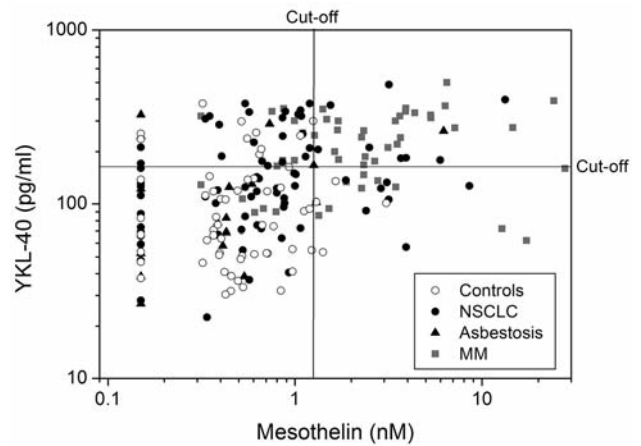Figure 7. Fibulin-3 levels in the studied groups. Geometric Mean (Geometric SD) are reported.

Figure 8. Correlation between Mesothelin and Chitinase-3-like protein 1 in detecting mesothelioma.

This is the first study, to our knowledge, to show that patients with MM have high serum YKL-40 levels but, although this distinguishes patients with MM from healthy controls, the specificity and sensitivity is less than that of mesothelin. Kim *et al.* found high serum YKL-40 levels in the pleural effusions of patients with lung diseases (with the highest levels being observed in those with exudative effusions), and levels were also high in patients with early-stage lung cancer (12). Furthermore, Thom *et al.* identified serum YKL-40 levels to be an independent prognostic biomarker in patients with metastatic NSCLC (13).

As can be seen from data shown in Table I, the prevalence of MM is about the same for men and women:

this appears to contrast with epidemiological data (24), but correspondes of petrochemical sites, industrial areas, *etc.*, relative rate of mortality for MM appears to be even greater for women (25).

Stratification of our MM patients by histology and tumour stage revealed a somewhat borderline significant correlation between the latter and mesothelin concentration whereas neither correlated with YKL-40 concentration. Although the number of patients with sarcomatoid MM in our series was limited, our finding that they did not have high biomarker levels is in line with those of previous reports and with the absence of mesothelin overexpression in this histological subtype (26). Only mesothelin levels were significantly higher in patients with advanced MM compared to those with early-stage disease, but this may be explained by the limited number of patients in the different stages and the current challenges of MM staging.

However, although the YKL-40 concentration alone is unlikely to be clinically valuable in diagnosing MM, it may improve the diagnostic specificity of mesothelin. In a simulation involving 1,000 patients with MM and 1,000 patients in the other groups (prevalence 50%), 503/539 patients with MM were positive for mesothelin and positive for YKL-40 (prevalence 93.3%), and 83/723 were negative for mesothelin and negative for YKL-40 (prevalence 11.5%), whereas the prevalence rate in the positive/negative and negative/positive patients was overall 56%. Despite the initial simulated prevalence, it is quite clear that false-positives were rare in the double-positive patients (only 36 out of the initial 1000 controls) and false-negatives were rare in the double-negative group (only 83 out of the initial 1,000 MM patients). This clearly indicates the two groups as being at high and low risk respectively of MM, with a possible differentiation in the diagnostic course. On the other hand, the prevalence of MM remained substantially the same in the single positive groups, thus indicating that they should both be treated as being at high risk.

The patients with MM also had higher IL-8, VEGF and fibulin-3 levels than did the controls, but there was no difference in these levels between the patients with lung cancer and those with asbestosis, except for fibulin-3. We did not confirm the results of Pass *et al*. since in our study there was no difference in levels of fibulin-3 between the patients with MM and those with asbestosis (17). On the contrary, endothelin-1 levels overlapped in the four groups.

In our study, the prevalence of asbestosis was lower than that of MM: this could be explained by the fact that asbestosis has a shorter period of latency than MM and asbestosis, being asbestosis an occupational disease, our cases are extrapolated from the archives of occupational diseases reported and compensated by INAIL, and this could create underestimation (27).

The strength of this study lies in the fact that we recruited unselected controls from patients referred to our bronchoscopy unit for diagnostic purposes, which gave us a similarly aged 'real world' sample.

Conclusion

The addition of YKL-40 may improve the specificity of mesothelin measurements alone for detecting patients with MM. Further studies on larger groups of patients are necesessary to validate the present finding and to assess for the validity of fibulin 3.

Clinical practice point. No tumor marker has demonstrated sufficient specifity and sensitivity for malignant mesothelioma: published data on mesothelin as a biomarker show a high rate of false-positives in healthy individuals. Currently no clinical studies have examined the YKL-40 levels in patients affected by MM. In our cross-sectional study, including other asbestos-related disorders (lung cancer and asbestosis), we validated two independent biomarkers, YKL-40 and mesothelin, in order to improve the specificity and sensitivity of mesothelin as a biomarker of MM.

The use of these two markers in asbestos-exposed individuals could predict the development of MM at an early stage, when therapy would be more effective.

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