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Asbestos burden in lungs of mesothelioma patients with pleural plaques, lung fibrosis and/or ferruginous bodies at histology: a postmortem SEM-EDS study

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Abstract

The causal attribution of asbestos-related diseases to past asbestos exposures is of crucial importance in clinical and legal contexts. Often this evaluation is made based on the history of exposure, but this method presents important limitations. To assess past asbestos exposure, pleural plaques (PP), lung fibrosis and histological evidence of ferruginous bodies (FB) can be used in combination with anamnestic data. However, such markers have never been associated with a threshold value of inhaled asbestos. With this study we attempted to shed light on the dose–response relationship of PP, lung fibrosis and FBs, investigating if their prevalence in exposed individuals who died from malignant mesothelioma (MM) is related to the concentration of asbestos in lungs assessed using scanning electron microscopy equipped with energy dispersive spectroscopy. Moreover, we estimated the values of asbestos concentration in lungs associated with PP, lung fibrosis and FB. Lung fibrosis showed a significant positive relationship with asbestos lung content, whereas PP and FB did not. We identified, for the first time, critical lung concentrations of asbestos related to the presence of PP, lung fibrosis and FB at histology (respectively, 19 800, 26 400 and 27 400 fibers per gram of dry weight), that were all well-below the background levels of asbestos identified in our laboratory. Such data suggest that PP, lung fibrosis and FB at histology should be used with caution in the causal attribution of MM to past asbestos exposures, while evaluation of amphibole lung content using analytical electron microscopy should be preferred.

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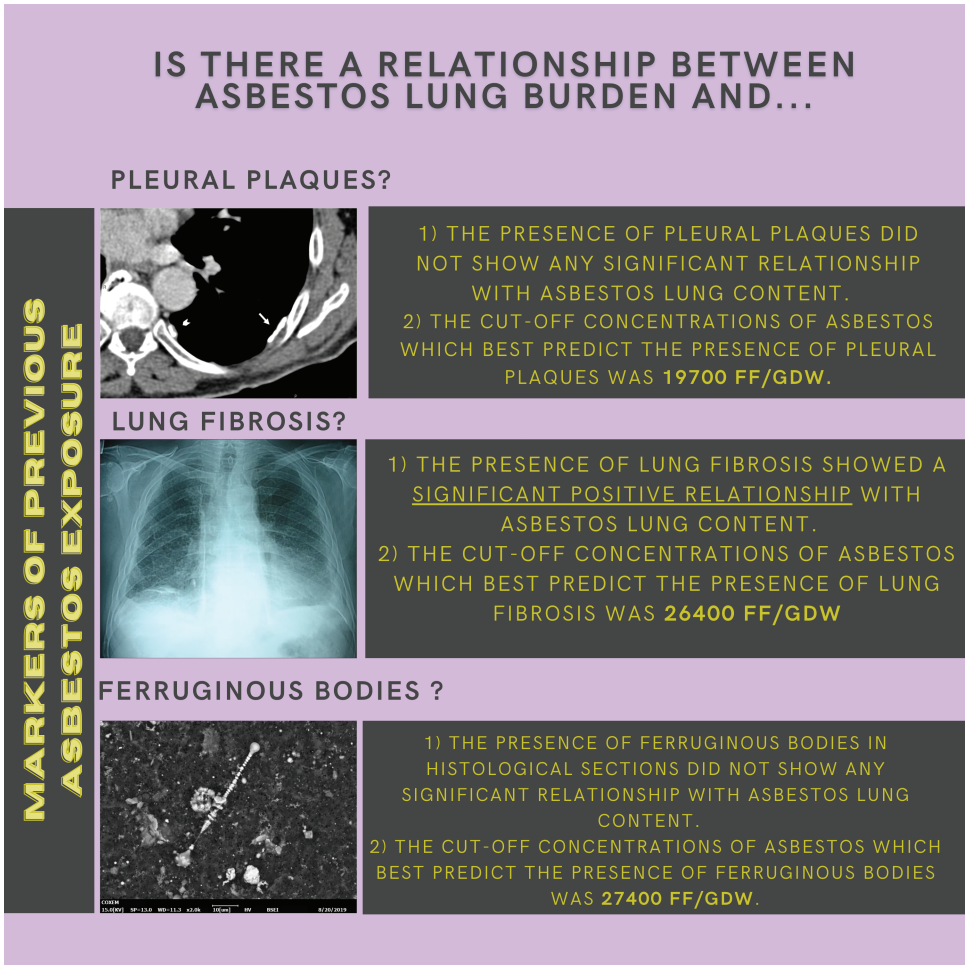
Graphical Abstract



Abbreviations: FB, ferruginous bodies; MM, malignant mesothelioma; PP, pleural plaques, ROC, Receiver operating characteristic.

Introduction

Malignant mesothelioma (MM) is a rare neoplasm, arising from the linings of serosal cavities, well known to be associated with asbestos exposures that occurred 30–40 years before the onset of the disease (1,2). The causal attribution of asbestos-related diseases (especially, MM, lung cancer and asbestosis) to past asbestos exposure is of crucial importance in clinical and medico-legal contexts. Often this evaluation is made on the basis of the exposure history assessed through specific questionnaires, but this method presents important limitations when applied to single individuals and not cohorts, because relevant past asbestos exposures may be unknown or forgotten and, on the other hand, people may report to have been exposed to 'dust' without knowing the nature of such dust; moreover, the awareness that compensation is possible when reporting past asbestos exposure may introduce a bias (3).

In order to assess past asbestos exposure, clinical and radiological markers can be used in combination with anamnestic data. These include pleural plaques (PP), lung fibrosis (which can be evaluated clinically and radiologically, as well as postmortem), and asbestos bodies (ABs) on histological sections or on digested lung tissue. PP are the most frequent benign pleural manifestations related to asbestos exposure, and are described macroscopically as discrete, raised, irregularly shaped, smooth or finely nodular areas of grayish-white to ivory white color located on the parietal pleura (4,5). They are a well-known marker of previous asbestos exposures, and the risk of developing them is positively associated with the time elapsed since the beginning of exposure (6) whilst a dose–response relationship has never been established for these manifestations, and it is still not clear which level of asbestos exposure is necessary to elicit them (7). Asbestosis is defined as a diffuse interstitial lung fibrosis occurring as a consequence of inhalation of 'excessive amounts of asbestos' (8). Even though it is widely accepted that there is a dose–response relationship with asbestosis inhalation (9), there is no agreement on the minimum amount of asbestos necessary to develop asbestosis. In addition, the differential diagnosis between idiopathic lung fibrosis and asbestosis is difficult and based on a history of exposure and the presence of asbestos in tissues (10). Asbestos bodies are inhaled asbestos fibers coated with iron and organic matter mainly composed of ferritin. They have a wide range of shapes and dimensions, and the distribution of the coating is not homogeneous (11). Their formation depends on the ability of macrophages to phagocytose inhaled asbestos fibers. If a fiber is longer than about 20 μm, a single cell is not able to ingest it entirely, and this triggers an inflammatory cascade that promotes the accumulation of iron in the cells. Iron micelles appear in the

macrophage's cytoplasm in proximity of the fiber, and their accumulation, together with homogeneous matrix material, produces a coating around the fiber (12). Asbestos fibers have the intrinsic capacity to form a complex with iron from the surrounding environment (13,14). This initiates a vicious cycle: the more iron the fiber attracts from the tissue, the more inflammation is triggered and, consequently, more iron is accumulated in close proximity of the fiber. Because the observation of AB at microscopy does not allow any chemical characterization, it is more correct to call them ferruginous bodies (FB) unless the core fiber composition is analyzed (12,15). For this reason, in this paper we prefer to use the term FBs rather than ABs, even though the vast majority of FBs are likely ABs. The presence of FB in histological sections can be a sentinel of past asbestos exposures (possibly unknown or forgotten) and, on the other hand, is often regarded as the key element in the differential diagnosis of asbestosis (8,10). However, the presence of FBs has been demonstrated in the general population without asbestos-related diseases and no history of past exposure (16,17). Since the only known cause of FBs is asbestos exposure (if we exclude the rare FB with a core different from asbestos), this finding demonstrates that very low dose exposures unknown to the affected subject might elicit them. Moreover, the amount of FBs not always correlates with the amount of uncoated asbestos fibers in lungs, assessed using electron microscopy, as there is a great individual variability in the process of coating fibers (18,19).

The most reliable tool in the evaluation of past asbestos exposure is the assessment of asbestos lung burden using analytical electron microscopy (20). Yet, the data deriving from this tool must be interpreted taking into account that the time elapsed between the end of the exposure and the subject's death modifies the asbestos lung content, due to the clearance mechanisms that take place in the pulmonary microenvironment (21). This phenomenon mainly concerns chrysotile, which, unlike amphiboles, has a more fragile crystalline structure, which can be fragmented and phagocytosed by alveolar macrophages (22). In fact, previous studies have shown that the inorganic lung content, measured by SEM-EDS, matches well with the retrospective evaluations made by experts if the concentration of amphiboles is taken into consideration (23,24).

In sum, there is a lack of knowledge on the level of asbestos exposure associated with PP, lung fibrosis and FB at histology. On these bases, the aim of the present study are: (i) to understand if the presence of PP, lung fibrosis and histological evidence of FB in MM patients is associated with asbestos lung content (and specifically by asbestos concentration, dimensions and concentration of each type of asbestos) and (ii) find cut-off values of asbestos in lungs that best correlate with each condition (PP, lung fibrosis and FB at histology).

Participants

This is a retrospective observational study conducted on subjects deceased from MM selected from the archive of the Unit of Legal Medicine and Forensic Science of Pavia University—among those who died from asbestos-related diseases between 2005 and 2019. A forensic autopsy, followed by a complete histopathological examination, was performed for each subject. The diagnosis of MM, already known in life, was confirmed postmortem according to the guidelines in effect at the time (10,25–27). During the necropsy, the whole lungs were collected, formalin fixed and stored. Most of the subjects of this study were exposed to asbestos during the activity of Fibronit factory, a large asbestos-cement plant located in Broni (a small town in Pavia Province, northern Italy), which was active between 1932 and 1993. The factory used to manufacture asbestos-cement artifacts using a mixture of chrysotile, crocidolite and smaller amounts of amosite (28).

Sample preparation for SEM-EDS

The technique used here has been described elsewhere (21,29). In summary, for each subject a sample of 0.25 g of formalin-fixed lung, taken from the inferior lobe of the right lung, was chemically digested using 13% sodium hypochlorite, and then filtered through a cellulose-ester membrane (Millipore, Darmstadt, Germany) with a diameter of 25 mm and a pore size of 0.45 µm. The membrane was then coated with graphite, and observed using a scanning electron microscope. Namely, an area of 2 mm^2 was observed at a magnification of 4000 using both secondary and backscattered electrons. The fiber chemical composition was analyzed using an EDS, Oxford Inca Energy 200, equipped with an INCA X-act SDD detector (Oxford Instruments NanoAnalysis, Bucks, UK). The amount of asbestos fibers and FBs observed in an area of 2 mm^2 was normalized to 1 g of dry tissue, reporting concentration in terms of asbestos fibers and FBs per gram of dry weight of lung tissue (ff/gdw), as indicated by international guidelines (28,29). The results have been rounded to three significant digits, considering the accuracy of the methodology. To identify the different types of asbestos fibers, we compared the EDS spectra with a reference database available in the laboratory that performed the tests. SEM-EDS cannot distinguish unequivocally chrysotile from asbestiform antigorite, and tremolite asbestos from actinolite asbestos, since they have similar chemical composition and analogous morphology, therefore we used, respectively, the term chrysotile/asbestiform antigorite and tremolite/actinolite asbestos for these minerals.

While the preparation of all samples was carried out in the same laboratory, the SEM-EDS investigation was carried out in two laboratories, and the samples were divided equally between the two labs. In order to avoid the variability deriving from different instruments and microscopists, we defined a detailed, standardized protocol for data collection. A periodic inter-laboratory control was conducted by comparing the images and spectra obtained by each laboratory. In addition, five samples were analyzed in both laboratories, and the ANOVA test for repeated measurements was used to compare the results (Supplementary Table 1). The 'background' concentration of asbestos in lung tissue (that is the concentration of asbestos that can be found in everyone randomly but does not increase significantly the risk of MM) had been identified in our laboratory as below 100 000 ff/gdw. This is threshold currently used by our laboratory to define the asbestos causation according to SEM-EDS analysis.

Variables

The following variables were extracted from the archive of the Unit of Legal Medicine and Forensic Sciences: the exposure

history, defined as occupational, household or anthropogenic environmental. Each subject was classified according to the exposure history, considering occupational exposure as prevalent over the other two, and household exposure as prevalent on anthropogenic environmental one.

The presence of PP at radiological examination performed during life and/or observed during the necropsy, defined dichotomically as present or not.

The presence of lung fibrosis at radiological examination performed during life and/or observed at the postmortem histology, defined dichotomically as present or not. All grades of lung fibrosis, from mild to severe, are considered as positive if clearly evident at radiological imaging and/or postmortem histology.

The presence of FB at histology was re-evaluated on five histologic lung sections for each subject (at least one for each lobe) stained with H&E and Perls (for trivalent iron). Also this variable was defined as dichotomic (yes or no). In this paper we did not attempt a quantification of FBs and chose to use 'yes or no' to describe this variable because all the patients with FBs in lung sections had large clusters of FBs in each section (well above the 2 FB/cm^2 requested by the asbestosis criteria (8).

The following endpoints were assessed through SEM-EDS: the concentration of asbestos fibers, expressed as number of fibers per gram of dry weight (ff/gdw); the mean length and width of detected asbestos fibers (in µm); the concentration of each type of asbestos (ff/gdw), classified as chrysotile/asbestiform antigorite, crocidolite, amosite, tremolite/actinolite asbestos, and anthophyllite asbestos; the concentration of FBs, expressed as FBs/gdw.

In the present work only asbestos fibers longer than 5 µm, thinner than 3 µm, and with an aspect ratio greater than or equal to 3:1, according to the WHO definition of 'biologically critical fiber' (30) were counted, measured and classified according to the EDS spectrum. This criterium also fits the concept of 'regulated' asbestos fiber according to the Italian law.

Statistical analysis

Samples with presence and absence of PP, presence and absence of lung fibrosis, and presence and absence of FB were compared with regards to concentrations and characteristics of asbestos fibers. Unadjusted *P* values were obtained using *t*-tests, and *P* values adjusted for age and sex were obtained from logistic regression models. Cut-point analyses were performed to obtain optimal values of asbestos concentration for determining presence of PP, lung fibrosis, and FB. Logistic models were created to assess the effect of asbestos concentration on presence of PP, lung fibrosis, and FB. Receiver operating characteristic (ROC) curves were created to assess the significance and accuracy of the predictive model. From the ROC curves, the Youden Index was calculated to determine optimal cut-point of asbestos concentration at which both specificity and sensitivity are maximized. All statistical analyses were performed using SAS, version 9.4. Statistical significance was defined as $P < 0.05$.

Ethics approval and consent

Since this is a retrospective study on autopsy samples, it is not possible to obtain informed consent, which is therefore not necessary according to the Provision containing the prescriptions regarding to the treatment of special categories of data (art. 21, paragraph 1 of Legislative Decree 10 August 2018, n. 101). The study has been approved by the Ethical Committee of Policlinic San Matteo of Pavia.

Results

The study includes 95 subjects who died from MM (93 pleural, 2 peritoneal) between 2005 and 2018; 52% of them were males and 48% were females. The mean age at death was 70 years (SD 11). The past asbestos exposure (according to the medical history and the forensic records) was occupational in 40% of cases, household in 18%, anthropogenic environmental in 41%. One subject had no known history of exposure. Concerning the histological type of MM, 74% had epithelial MM, 10% had sarcomatoid MM, 13% had biphasic MM and 3% had desmoplastic MM. The mean duration of exposure was 25 years (SD = 15.6), the mean latency between the beginning of exposure and the MM diagnosis was 50 years (SD = 12.98), while the mean time elapsed between the cessation of exposure and death was 26 years (SD = 10.64). The mean survival since MM diagnosis was 17 months (SD 15).

Asbestos concentration ranged between 0 and 7 570 000 ff/gdw (mean = 158 000 ff/gdw, SD = 240 000), whereas FBs concentration ranged between 0 and 3 000 000 FBs/gdw (mean = 75 000 FBs/gdw, SD = 333 000) where 0 means below the detection limit. The concentration of asbestos fibers was below the detection limit in 26.3% of subjects, 1–9999 ff/gdw in 15.8%, 10 000–99 999 ff/gd in 43.2%, 100 000–999 999 ff/gdw in 13.7% and above 1 million ff/gdw in 1%. The mean length of asbestos fibers ranged between 6 and 55 µm (mean = 23.7 µm, SD = 12.42), while the width ranged between 0.21 and 1.9 µm (mean = 0.68 µm, SD = 0.28). Considering asbestos as a whole, 0.54% of fibers were classified as chrysotile/asbestiform antigorite, 40.58 % as crocidolite, 48.33% as amosite, 0.85% as anthophyllite asbestos and 9.70% as tremolite/actinolite asbestos.

Among the 95 MM cases investigated, PP was identified in 53.68% of them; the information was missing in 5 (5.26%) cases (*n* for PP = 90). No statistically significant difference was observed in the asbestos lung content in subjects with and without PP (adjusting for age and sex), (Table 1). Namely, The asbestos' concentration, the concentration of each type of asbestos and the dimensional characteristics of asbestos (mean fibers length and width) were not different between subjects with and without PP. The cut-off concentration of asbestos which best predicts the presence of PP (with a sensitivity of 0.6471 and a specificity of 0.6667) was 19 800 ff/gdw (Figure 1a, table 4).

As shown in Table 2, 28.42% of the analyzed individuals presented with lung fibrosis. The concentration of asbestos fibers, as well as the concentration of crocidolite, amosite and tremolite/actinolite (adjusted for age and sex), were higher in subjects with lung fibrosis compared to those without it ($P = 0.0043$, 0.0124, 0.0184, respectively). Instead, the concentration of FBs and the dimensional characteristics of fibers (adjusted for age and sex) showed no statistical difference according to the presence of lung fibrosis. The cut-off asbestos concentration which best predicts the presence of lung fibrosis was 26 400 (with a sensitivity of 0.70 and a specificity o 0.72) (Figure 1b, Table 4).

Table 1. Asbestos burden in MM patients according to the presence of pleural plaques (PP); $n = 90$

	PP $n = 51$ (56.67%) Mean (SD)	No PP $n = 39$ (43.33%) Mean (SD)	*P*-value	*P*-value*
Asbestos concentration (ff/gdw)	11400 (311 000)	27600 (51 300)	0.0539	0.0764
Concentration of asbestos bodies (FB/gdw)	124100 (449 000)	12900 (36 400)	0.0843	0.3901
Concentration of chrysotile asbestiform antigorite fibers (ff/gdw)	406 (1780)	525 (2150)	0.7741	0.7673
Concentration of crocidolite fibers (ff/gdw)	47900 (130 000)	8600 (24 300)	0.0398	0.1216
Concentration of amosite fibers (ff/gdw)	57800 (189 000)	113000 (23 800)	0.0880	0.0676
Concentration of anthophyllite asbestos fibers (ff/gdw)	918 (4080)	446 (2790)	0.5160	0.7269
Concentration of tremolite actinolite asbestos fibers (ff/gdw)	7950 (121 000)	6790 (11 600)	0.6481	0.8303
Asbestos fibers length, (μm)	23.97 (13.17)	23.37 (11.53)	0.8484	0.7134
Asbestos fibers width, (μm)	0.69 (0.17)	0.69 (0.40)	0.9633	0.1820

ff/gdw, fibers × gram of dry weight.
*Analysis adjusted for age and sex.

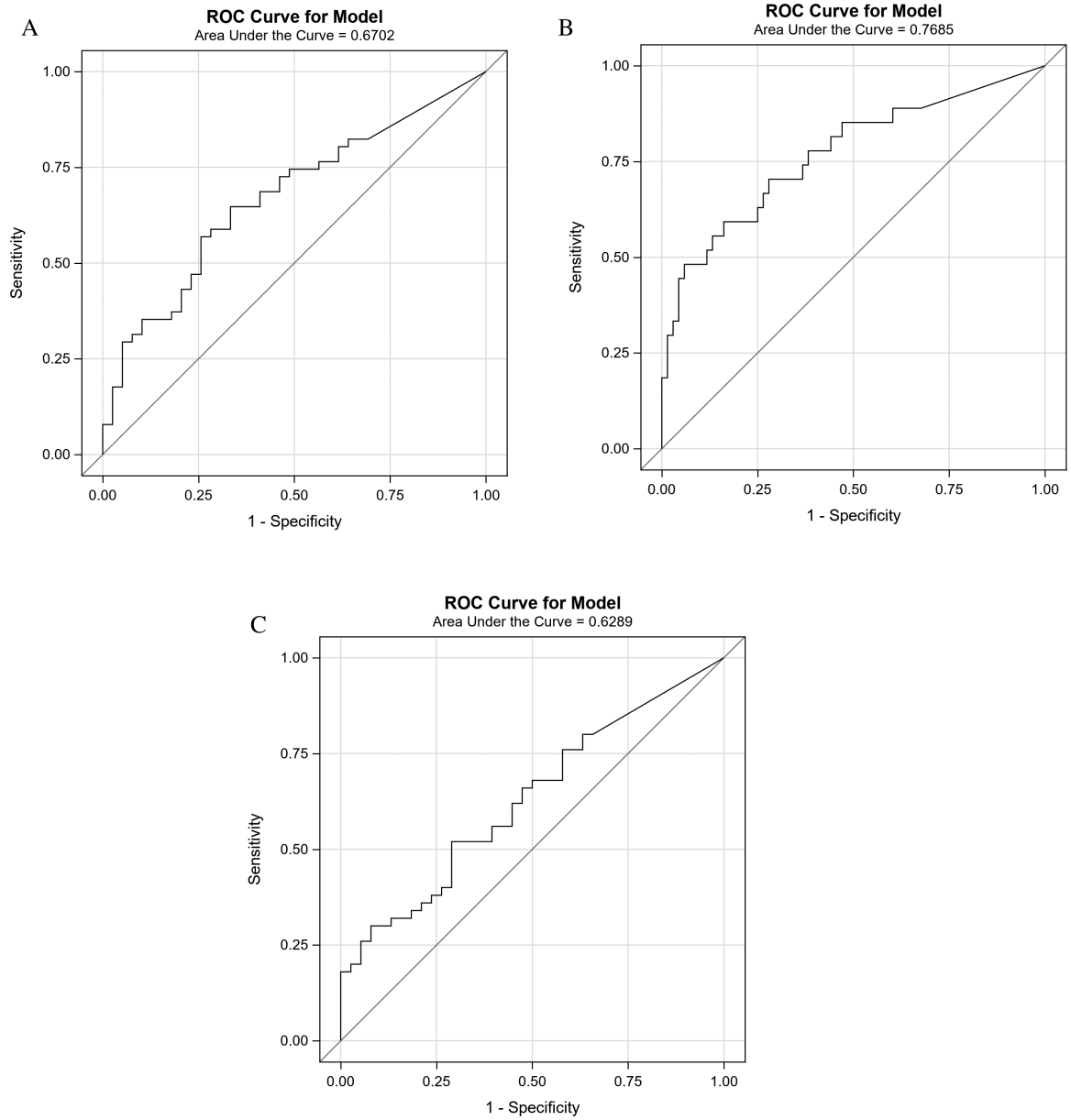


Figure 1. The ROC curves and 'critical' concentrations of asbestos for developing PP, lung fibrosis and FB at histology (a–c).

Table 2. Asbestos burden in MM patients according to the presence of lung fibrosis (n = 95)

	Lung fibrosis n = 27 (28.42%) Mean (SD)	No lung fibrosis n = 68 (71.58%) Mean (SD)	*P*-value	*P*-value*
Asbestos concentration (ff/gdw)	212 000 (421 000)	27 100 (47 000)	0.0313	**0.0043**
Concentration of asbestos bodies (FB/gdw)	206 000 (595 000)	22 400 (97 000)	0.1210	0.3643
Concentration of Chrysotile asbestiform antigorite fibers (ff/gdw)	432 (1750)	434 (1950)	0.9968	0.2013
Concentration of crocidolite fibers (ff/gdw)	92 000 (177 000)	8600 (21 000)	0.0217	**0.0124**
Concentration of amosite fibers (ff/gdw)	105 000 (257 000)	12 000 (21 600)	0.0712	**0.0086**
Concentration of anthophyllite asbestos fibers (ff/gdw)	1730 (5520)	255 (2110)	0.1863	0.1852
Concentration of tremolite actinolite asbestos fibers (ff/gdw)	12 700 (15 500)	5760 (11 400)	0.0417	**0.0184**
Asbestos fibers length (μm)	24.86 (14.23)	23.33 (11.47)	0.6294	0.4885
Asbestos fibers width (μm)	0.73 (0.18)	0.66 (0.32)	0.2864	0.4719

ff/gdw, fibers × gram of dry weight.
*Analysis adjusted for age and sex. In bold are significant p values.

Table 3. Asbestos burden in MM patients according to the presence of ferruginous bodies (FB); n = 88

	FB n = 50 (56.82%) Mean (SD)	No FB n = 38 (43.18%) Mean (SD)	*P*-value	*P*-value*
Asbestos concentration (ff/gdw)	127 000 (323 000)	29 000 (43 800)	0.0393	0.0890
Concentration of asbestos bodies (FB/gdw)	137 000 (452 000)	6390 (15 100)	0.0466	0.1040
Concentration of Chrysotile asbestiform antigorite fibers (ff/gdw)	265 (1380)	735 (2520)	0.3049	0.5008
Concentration of crocidolite fibers (ff/gdw)	53 500 (136 300)	10 000 (23 800)	0.0313	0.1415
Concentration of amosite fibers (ff/gdw)	63 300 (194 000)	11 500 (16 400)	0.0655	0.0749
Concentration of anthophyllite asbestos fibers (ff/gdw)	937 (4110)	458 (2820)	0.5193	0.5515
Concentration of tremolite actinolite asbestos fibers (ff/gdw)	8950 (13 200)	6480 (13 500)	0.3918	0.7451
Asbestos fibers length (μm)	23.99 (13.05)	22.60 (11.37)	0.6658	0.7774
Asbestos fibers width (μm)	0.68 (0.26)	0.72 (0.32)	0.6032	0.1499

ff/gdw, fibers × gram of dry weight.
*Analysis adjusted for age and sex.

Table 4. The asbestos cutoffs with sensitivity, specificity and Youden index for each variable

	Asbestos cut-off (ff/gdw)	Sensitivity	Specificity	Youden index
Pleural plaques	19 800	0.6471	0.6667	0.3137
Lung fibrosis	26 400	0.7037	0.7206	0.4243
Ferruginous bodies	27 400	0.5200	0.7105	0.2305

Furthermore, FB were present at histology in 52.63% of cases, whereas in seven cases (7.37%) the sections could not be retrieved from the archive and the information was not reported clearly in the autopsy report (n for FB = 88). The asbestos lung content in subjects with FB at histology (adjusted for age and sex) did not show any statistically significant difference compared to those without FB (Table 3). The cut-off asbestos concentration which best predicts the presence of FB at histology was 27 400 (with a sensitivity of 0.52 and a specificity of 0.71) (Figure 1c, Table 4).

Discussion

In this study we found no association between the presence of PP or of FB at histology and the amount, the dimensions and the types of asbestos in lungs. Instead, the onset of lung fibrosis seems to be related to higher amounts of asbestos in lungs, and specifically of crocidolite, amosite and tremolite/actinolite. We also observed that the critical amount of asbestos necessary to develop, respectively, PP, lung fibrosis and FB observable at histology is 19 800, 26 400 and 27 400 ff/gdw,

The main limitation of this study is that we did not analyze FBs in digested lung tissue at light microscopy, which is the most suitable technique to count FBs in lung tissue (31). Instead, we identified and counted them at SEM-EDS in the same sample we used to detect uncoated asbestos fibers. Therefore, the concentration of FBs might be underestimated, as we counted them in a smaller lung sample than those usually examined at light microscopy. A second possible limitation regards the choice to analyze only fibers longer than 5 μm, thinner than 3 μm, and with an aspect ratio greater than or equal to 3:1 (30), according to the WHO definition of 'biologically critical fiber', even though in literature a relationship

between fibers with a low aspect ratio and MM has been reported (18,32). Notwithstanding, the current, shared opinion is that the pathogenicity of asbestos is mostly determine by fibers longer than 10 μm (33).

In our series, PP have been observed in 53.68% of MM patients, a lower incidence compared to other studies on PP prevalence in asbestos exposed individuals. For example, Kato *et al.* (34) found PP in 89.4% among 2132 subjects previously exposed to asbestos and Barbieri *et al.* (35) found PP in 89.51%. Furthermore, we found that asbestos concentration did not correlate with the presence of PP. In contrast, Paris *et al.* (36) pointed out a relationship between PP and both cumulative dose of exposure and time since the first exposure. However, in the study by Paris *et al.* the cumulative dose of asbestos exposure was evaluated through questionnaires and not demonstrated using analytical electron microscopy. Recently, a strong positive relationship between the presence of PP and asbestos lung burden has been described (35): for example, Sichletidis *et al.* (37) found PP in dentists as a consequence of very low exposures (measured in air using contrast phase microscopy) repeated daily. The hypothesis that very low doses of inhaled asbestos can provoke the onset of PP seem to be in line with our data, as eight of our cases with PP had no asbestos at SEM-EDS investigation. However, as chrysotile is subjected to clearance in the lung, we cannot draw conclusions about the actual amount of inhaled asbestos and its relationship with PP in those cases where the analysis of lung content was negative for amphiboles (22,38). Hourihane and Mc Caughey (39) suggested that the dose of asbestos necessary to develop PP is intermediate between the dose necessary to develop MM and asbestosis, while Whitwell found that all people with PP had more than 20 000 ff/gdw (40), identifying for the first time a 'critical dose' of asbestos related to the development of PP. This result is in line with our estimation of the cut-off value of asbestos concentration in lungs that present the best relationship with the presence of PP (19 800 ff/gdw), that is considered a low level of exposure compared to the typical asbestos burdens in lungs of asbestos workers (17,18), and even lower than background exposure observed in some studies on general population (16). In our laboratory, the observed 'baseline' level of amphiboles in lungs of individuals without a known exposure to asbestos is below 100 000 ff/gdw. This means that in our experience, most individuals never exposed to asbestos (occupationally, familiarly or environmentally) have a lung burden below 100 000 ff/gdw. However, we observed many cases of MM with well-documented exposure with a lung content below that cut-off. Therefore, in a legal context, each case must be evaluated in the light of all the available data and a lung burden below 100 000 amphibole ff/gdw should not exclude compensation if a compelling history of exposure or the presence of the other markers strongly suggest previous asbestos exposure.

Warnock *et al.* (41) identified significantly higher median concentrations of amosite and crocidolite in subjects with PP, while we did not find any relationship between the concentration of any specific type of asbestos and the risk of developing PP. Kraynie *et al.* (42) found that the occurrence of PP in patients with mesothelioma indicated a probability of having an elevated tissue asbestos content of around 99%, even though asbestos is not the only cause of PP. In addition, Roggli *et al.* (43) found that around 50–55% of mesothelioma patients had PP, similar to what reported in the present study. These findings indicate that the presence of PP in mesothelioma patients strongly point at an asbestos etiology, but the absence of plaques does not rule out that possibility.

We found lung fibrosis in 28.42% of our MM patients, a prevalence that is in line with a previous study on asbestos exposed individuals (34). Instead, Dodson (18) found lung fibrosis in 29 out of 55 cases of MM, in a cohort of cases mainly exposed occupationally. Moreover, lung fibrosis appeared to be related to significantly higher lung concentrations of asbestos, but not with higher FBs concentrations, confirming what was previously stated by Roggli and Shelburne (44). Dodson *et al.* (18) found a higher concentration of both asbestos fibers and ABs in MM patients with asbestosis, even though they pointed out that the dose–response relationship for asbestosis is not constant, given the wide range of asbestos and FBs concentrations reported in asbestosis patients. Bellis *et al.* (45) found low asbestos fibers in some asbestosis patients. Our results are in line with these data, as our estimation of the critical concentration of asbestos in lung that show the best relationship with lung fibrosis (26 400 ff/gdw) is, indeed, lower that the value typically observed in patients with asbestosis (several million fibers per gdw) (46). Furthermore, in the present study, 15 cases with lung fibrosis showed less than 100 000 ff/gdw, and in two cases no asbestos fibers at all; on the other hand, two cases with more than 100 000 ff/gdw had no lung fibrosis. Such data confirm the known dose–response relationship for asbestosis, but also the impossibility to predict the onset of this disease based on asbestos lung content, as previously stated by Dodson *et al.* (18). In addition, the present data suggest that the presence of lung fibrosis is associated with higher concentration of amosite, crocidolite and tremolite/actinolite. This correlation is likely to be due to the longer persistence of amphiboles in lungs compared to chrysotile, rather than to an intrinsic more intense fibrogenicity of amphiboles, and is in line with previous studies (46,47).

Finally, despite the finding of FBs at histology sections is generally regarded as a pillar in the diagnosis of asbestos-related diseases, especially asbestosis (8,48) we did not find any correlation between the asbestos concentration in lungs and the presence of FBs at histological sections. This may be due to the fact that MM and asbestosis correlates better with uncoated asbestos fibers than FBs (49). Indeed, the tendency to cover asbestos fibers is related to each individual response, and some individuals are 'poor coaters' (18), as demonstrated by the wide range of ratios between uncoated fibers and FBs (21,49). A different coating efficiency has been described even in different areas of the same individual's lung (50). Interestingly, the 'critical' concentration of asbestos in lungs necessary to develop FB at histology was estimated as 27 400 ff/gdw, a value well-below the threshold considered indicative of past exposures to asbestos and currently used for causal attribution of asbestos-related diseases (100 000 ff/gdw) (10). Therefore, even though the presence of FBs is suggestive of asbestos exposure, the present data suggest that FB in histological sections, as well as in digested tissue, should not be regarded as a compulsory criterion for causal attribution of a disease to past asbestos exposure, and they cannot provide any reliable quantitative information about asbestos lung content. On the other hand, the low value of asbestos concentration we estimated as 'cut-off' for FB suggests that FB can be identified in people with background exposure (sometimes higher than 4000 ff/gdw) in the general population (16).

In conclusion, especially considering the cut-off values of asbestos concentration in lungs we estimated for each condition examined, these data corroborate the recommendation of the quantification of uncoated asbestos fibers at electron microscopy for the causal attribution of MM to past asbestos exposure.

Conclusions

This study investigated the relationship between the asbestos lung content, assessed using SEM-EDS on digested lung tissue, and the presence of PP, asbestosis and FB at histological section. Only the development of asbestosis resulted to be associated with asbestos lung burden, while PP and FB did not show any significant correlation to the amount of asbestos in lungs. Moreover, we were able to identify, for the first time, critical values of asbestos concentration in lungs that showed the best relationship with the onset of each condition (PP, lung fibrosis and FB at histology), that were all well-below the expected values. Such data suggest that PP, lung fibrosis and FB at histology should be used with caution (and combined with history of exposure) in the causal attribution of MM to past asbestos exposures, while evaluation of amphibole lung content using analytical electron microscopy should be preferred, especially in the legal context. However, lung content analysis should be carefully interpreted, and each case should be evaluated thoroughly, considering all the available data.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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