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First Identification of Pulmonary Asbestos Fibres in a Spanish Population

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Abstract
Introduction This study aimed to characterize, for the first time in Spain, the type of asbestos fibres (AF) in the lungs of exposed and non-exposed populations.
Materials and Methods Lung samples from 38 subjects living in Barcelona and Ferrol, Spain, were studied, which were divided into three groups: Group A—five subjects without known respiratory disease; Group B—20 ex-shipyard workers and Group C—13 patients with lung cancer. After eliminating the organic material, the inorganic residue was analysed using electronic microscopy (EM). To identify the type of fibre, the samples were analysed by scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX).
Results All the fibres identified corresponded to amphiboles (crocidolite 45%, anthophyllite 22%, tremolite 16%, amosite 15% and actinolite 3%). In 14 patients (37%), a single type of asbestos was found in the lungs (amosite in two, actinolite in one, anthophyllite in four, crocidolite in five and tremolite in two). Forty-six percent of the AF analysed had a length > 5 μm and a diameter < 0.2 μm.
Conclusions The results of this study provide the first data on the type of asbestos retained in the lung of Spanish population. A particularly striking finding is the exclusive retention of amphiboles, which suggests that chrysotile is eliminated after inhalation. Our findings support estimations considering Spain and other southern European countries with similar asbestos imports and consumption at a high risk to develop asbestos-related diseases in the years to come.

Keywords Asbestos bodies · Lung cancer · Asbestos fibres

Introduction

Asbestos is the generic name given to a group of silicate minerals. The most common are amphiboles: amosite, crocidolite, tremolite, actinolite and anthophyllite, and the serpentine chrysotile. These forms differ in terms of their chemical structure, their biopersistence in humans and their toxicity. Inhaled asbestos fibres are deposited in the respiratory system where they interact with epithelial cells and alveolar macrophages to produce an immune response. As described extensively in the scientific literature, exposure to asbestos has been associated with asbestosis, mesothelioma, lung cancer and benign pleural lesions [1–3].

In Spain, asbestos was widely used in industry for many years. Its use in this country reached its peak between 1970 and 1990. In 1992, Spain was the second largest European importer with 25,428 tons [4], and the total ban on the use of the mineral did not come until 2002. This means that a large number of workers have been exposed to asbestos and will remain exposed in the future, given its

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incorporation in a great number of structures and buildings [5]. According to the voluntary registry of respiratory diseases in Catalonia, diseases resulting from exposure to asbestos fibres are the second largest group [6].

The diagnosis of diseases caused by the inhalation of asbestos is based on three factors: knowledge of exposure, a compatible clinical picture and exclusion of other diseases. In some cases, there is a mismatch between the exposure and the clinical picture, which makes diagnosis difficult and often poses medical-legal problems. In these cases, it is necessary to analyse the amount of asbestos retained in lung tissue [7, 8]. Light microscopy is useful to detect asbestos bodies from tissue samples, but detection of asbestos fibres requires observation by electron microscopy. Electron microscopy equipped with devices like EDX (Energy-dispersive X-ray spectroscopy) also allows determination of the chemical composition and the crystal structure of the fibre, thus permitting a precise asbestos identification.

Although chrysotile accounted for nearly 90% of all asbestos consumption worldwide, some former north American studies showed that most of the fibres retained in the lung of patients with asbestos-related diseases corresponded to amphiboles [9, 10]. In the past years, more information has been available from northern and central Europe countries, but only a few studies have provided data on lung fibre deposition in urban populations from southern Europe [11–14]. In Spain, up to the present, information on asbestos lung retention is limited to asbestos bodies detected by light microscopy [15–17]. However, a characterization of the asbestos lung fibre burden in exposed and non-exposed subjects has not been done yet.

The aim of this study was to characterize, for the first time in Spain, the type and dimensions (length and diameter) of asbestos fibres in exposed and unexposed populations.

Materials and Methods

Study Population

We studied lung samples from 38 subjects, which were divided into three groups: five residing in the city of Barcelona without known respiratory disease (Group A), 20 who had worked in the Ferrol shipyards and had been exposed to asbestos (Group B) and 13 lung cancer patients living in the city of Barcelona (Group C).

Sampling Criteria

Lung samples from patients in Group A were collected prospectively from June 2004 to June 2005 at the Institute of Legal Medicine of Catalonia. In this group, the following sampling criteria were applied: absence of pulmonary disease and residence in the city of Barcelona for at least 10 years. Lung samples from Ferrol shipyard workers (Group B) were consecutively sent to our laboratory between 2008 and 2010 from the Pathology Department of Hospital Arquitecto Marcide in El Ferrol. The lung cancer group (Group C) comprised 13 patients diagnosed with lung cancer and operated consecutively at the Vall d'Hebron Hospital, from whom resected specimens were obtained.

Exposure Determination

Information on the potential exposure of the patient was obtained by interview with the next of kin in autopsy cases (Groups A and B) and through a personal interview with the patient in cases of pulmonary resection (Group C).

After verifying that they met the inclusion criteria, patients were invited to participate in the study. In the case of autopsies, the proposal was made to the next of kin. All interviews were conducted by two of the researchers, one in each of the cities. In all cases, informed consent was requested. Consent forms were signed by the affected individual or by a close relative, depending on whether the samples had been obtained by resection or autopsy. The local Ethics Committee approved the study.

Protocol for Obtaining Samples

In Group A, lung tissue specimens of 2 cm^3 in size were obtained from three regions of the right lung: apex of the upper lobe (zone 1), apex of the lower lobe (zone 2) and base of the lower lobe (zone 3). In Group B, in each autopsy lung tissue specimens of 2 cm^3 in size were obtained from an area in the right lung and another area in the left lung whenever possible. As regards the resections in Group C, samples were obtained from the lung area where the cancer had appeared and whenever possible from more than one area.

All samples obtained were fixed in formaldehyde and sent to our hospital laboratory for analysis. All the lung specimens were examined by a pathologist from our hospital. The presence of chronic pulmonary disease was ruled out in all cases.

Preparation of Lung Samples

Two 0.5-g fragments of lung tissue that did not contain pleura or vessels were obtained from each specimen. One of these fragments was frozen, lyophilized and weighed to determine the dry tissue weight, in keeping with an international agreement to express asbestos body (AB) results in

terms of grams of dry lung tissue. Once the dry weight was known, the lyophilized sample was discarded, because this technique may cause changes in the concentration and size of the fibres [8]. Thirty cc of filtered sodium hypochlorite was added to the non-lyophilized tissue section, and the sample was shaken for 24 h to facilitate digestion of the tissue and to eliminate other organic material. The sample was then centrifuged at 3,700 rpm for 20 min, the sodium hypochlorite was eliminated, and the sample was re-suspended in filtered distilled water. To dissolve the AB present in this liquid medium, the sample was sonicated for 10 min using an ultrasonic water bath (UCI-50 Raypa SL; 300 W, 50/60 Hz), and was then washed and re-suspended in 20 cc of filtered distilled water. The solution obtained was passed through a 0.45-mm pore diameter filter (millipore membrane filters, HAWP02500). The filter was dried overnight at 378C, placed on a microscope slide and made transparent using acetone vapour (JS Holdings vaporizer; 240/110 v) for subsequent reading. In all the samples, an adequate filter loading for counting was obtained, and it was not necessary to dilute the sample.

Asbestos Body Counting by Optic Microscopy

The filters were examined with an optic microscope (CX21FS2; Olympus Life Science Europe GMBH, Hamburg, Germany) at ×400 magnification. All filters were examined by a single experienced reader in accordance with a protocol previously described by our group [16]. All the samples obtained from each individual were assessed: in Group A, the values obtained for each of the three lung zones were evaluated, and in Groups B and C all the zones from which a sample could be obtained were evaluated. Applying the criteria established by the working group of the European Respiratory Society in 1998, AB levels exceeding 1000 AB/g dry tissue were considered significant [8]. For the analysis, the highest value of the different samples analysed in each patient was considered.

Asbestos Fibre Identification by Electron Microscopy

After completing the reading by optic microscopy, the filter was cleaned by immersion in serial baths of ethylene glycol and carbon tetrachloride. The filter was degassed overnight and was then covered again with carbon (Emitech CC7650, Quorum Technologies; Sussex, UK). Once the carbon layer was deposited on the filter, the asbestos was determined using scanning electron microscopy (SEM) (Quanta-200, FEI, Hillsboro, Oregon, USA). The type of AF in each subject was assessed at an accelerating voltage of 15 kV, screen magnification of ×2.000 and a scan rate of 10 s per frame. Chemical analysis of the fibres observed was performed with electron microscopy including electron diffraction and energy-dispersive X-ray spectral analysis (EDX). Fibre analysis was performed at a magnification of ×20.000.

Statistical Analysis

The data are expressed as the median and the range unless otherwise stated. Analysed with the Kolmogorov–Smirnov test, the values obtained did not follow a normal distribution; therefore, the Wilcoxon test was used to determine the differences between groups. The statistical analysis was carried out with GraphPad software (2002–2005).

Results

Clinical Characteristics of the Study Population

The baseline characteristics of the 38 individuals included in the study are shown in Table 1. In the five patients in Group A, the main causes of death were heart disease and traffic accidents. In the 20 patients in Group B, the diagnosis was lung cancer in eight patients, mesothelioma in four, pleural plaques in four, asbestosis in three and

Table 1 Baseline characteristics of the 38 individuals included in the study

	Group A $n = 5$	Group B $n = 20$	Group C $n = 13$
Age, years[a]	66 (48–78)	69 (56–86)	66 (51–77)
Sex	4 M/1 F	20 M	12 M/1 F
Smoking habit, *n* (%)[b]			
Smoker	1 (20%)	7 (44%)	2 (15%)
Ex-smoker	2 (40%)	6 (37%)	10 (78%)
Non-smoker	2 (40%)	3 (19%)	1 (7%)
Exposed/non-exposed (%)	0/100	100/0	23/77
Years of exposure[a]	0	27 (13–44)	ND
Disease, *n*			
No respiratory disease	5	–	–
Asbestosis	–	3	–
Lung cancer	–	8	13
Mesothelioma	–	4	–
Pleural plaques	–	4	–
Pulmonary embolism	–	1	–

M male, *F* female, *ND* no data recorded in the clinical history

[a] Data expressed as median (range)

[b] In four patients smoking habit was not specified in the medical record

pulmonary thromboembolism in one. The 13 patients in Group C were all diagnosed with lung cancer.

Types and Dimensions of Asbestos Fibres

Figure 1a summarizes the types of asbestos found in the different samples. Amphibole fibres accounted for 100% of fibres recovered in lung samples. No chrysotile fibres were found in the samples analysed. Crocidolite was the most common type of fibre observed (45% of samples analysed). In 14 patients (37%), a single type of asbestos was found in the lungs (amosite in two, actinolite in one, anthophyllite in four, crocidolite in five and tremolite in two).

Patients in Group A had a higher percentage of actinolite than their Group B and C peers ($p = 0.012$ and 0.023, respectively). Patients in Group B had a higher percentage of amosite than those in Groups A and C ($p = 0.022$ and 0.031, respectively). Finally, Group C patients had a higher percentage of tremolite than those in Groups A and B ($p = 0.011$ and 0.017, respectively) (Fig. 1b).

Table 2 shows the levels of asbestos bodies measured by optical microscopy and the mean dimensions and length distribution of the fibres obtained from the different samples. Group B had higher levels than Groups A and C ($p = 0.001$ and 0.02, respectively). Group C also had higher levels of AB than Group A ($p = 0.032$). The length of the fibres exceeded 5 μm in over 80% of the samples

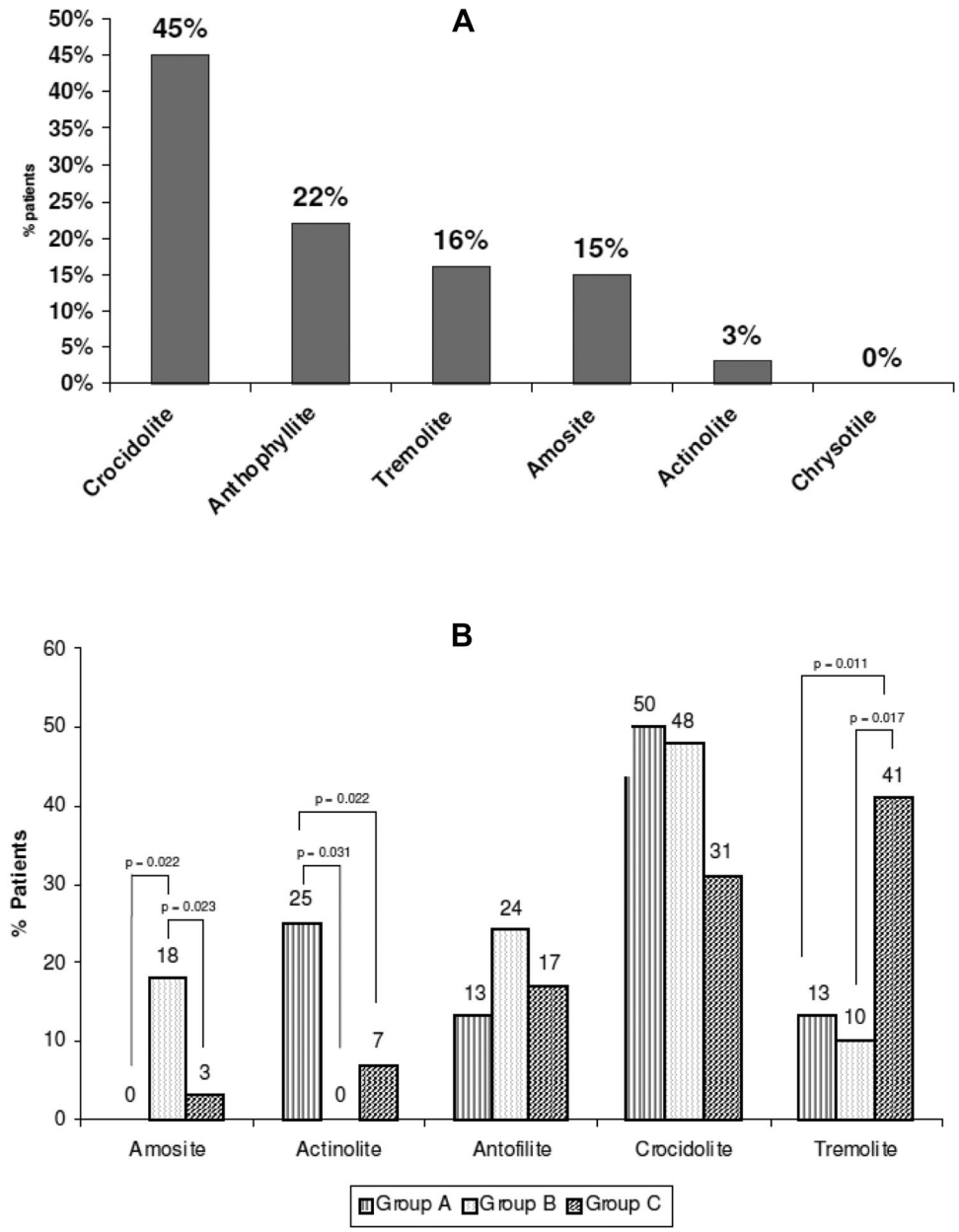

Fig. 1 Types of asbestos fibres found in the different samples analysed

Table 2 Length (L) and diameter (ø) of the asbestos fibres analysed by SEM

	Group A $n = 5$	Group B $n = 20$	Group C $n = 13$
Number of AB*	0	14,647**,¶	326&
	(0–599)	(1327–4,660,059)	(0–26,280)
AB > 1000; *n* (% patients)	0 (0%)	20 (100%)	6 (46%)
$L > 5$ µm	90%	88%	84%
ø < 0,2 µm	10%	56%***	21%
$L > 5$ µm and ø < 0.2 µm	9%	49%***	18%

AB asbestos bodies measured by optical microscopy

* Data expressed as median (range)

** $p < 0.001$ compared with Group A

¶ $p = 0.02$ compared with Group C

*** $p < 0.001$ compared with Groups A and C

& $p = 0.032$ compared with Group A

analysed in the three groups. In Group B patients, 49% of the fibres tested had a length > 5 µM and a diameter < 0.2 ($p = 0.001$). In all groups, the fibres with a length > 5 µM and a diameter < 0.2 µm corresponded to amosite in 97% of cases.

Discussion

This study is the first to characterize the type of asbestos retained in the lung in a Spanish population. We found an exclusive presence of amphiboles, suggesting clearance of inhaled chrysotile. Moreover, the physical characteristics of the fibres detected in the samples of workers in the shipyards of Ferrol differed from those of our other two study populations. The shipyard workers presented the longest and thinnest fibres, which are considered the most likely to cause asbestos-related diseases.

Electron microscopy analysis showed that the most common fibre found in our study population was crocidolite, despite the fact that it has been prohibited since 1984. Furthermore, 72% of individuals in the Ferrol shipyard group predominantly presented crocidolite in their lung samples. Similarly, crocidolite was the most common fibre type in reference population. Explanations for this high content may be exposure previous to 1984 and subsequent exposures occurring during repair activities carried out in recent years in asbestos-containing buildings and structures.

One remarkable result was the absolute predominance of amphiboles and the absence of chrysotile in the samples analysed. It concurs with previous data showing that although chrysotile is the most commonly used type of asbestos, crocidolite is the fibre most commonly found in the lungs of patients with mesothelioma. Indeed, the 1974 study by Desbordes and Fondimare [11] already suggested this idea: those authors reported that in the lung tissue of patients with high exposure to both amphiboles and chrysotile, the fibres present were almost exclusively large amphiboles. This is thought to be due to the fact that, after inhalation, asbestos undergoes a drainage process that minimizes its final deposit. Consequently, chrysotile levels detected years afterwards may not reflect the intensity of the previous exposure [18]. Contrarily, amphibole clearance is much lower. Churg and Vedal [19], in a series of 144 shipyard workers, found that time since last exposure was correlated with decreasing amosite concentration, and calculated a clearance half-time of about 20 years. The idea of a higher lung persistence of amphiboles over chrysotile is supported by animal studies. Thus, in animal studies, pulmonary accumulation of crocidolite has been shown to be three or four times greater than that of chrysotile [20]. In contrast, some authors argue that the difficulty of observing chrysotile is due to the technical procedure. In their study of asbestos in 110 cases, Roggli et al. [21] observed a loss of a substantial proportion of small chrysotile fibres during the centrifugation step in the ethanol–chloroform interface.

Interestingly, 16% of asbestos corresponded to tremolite, a percentage that raised to 41% in lung cancer patients. Despite the fact that tremolite is associated with environmental asbestos-related diseases in the Mediterranean area (in Turkey, Greece and Corsica, for instance), its commercial importance is relatively low and it is a frequent natural contaminant (as a geological component) of other minerals as chrysotile or talc. In American shipyard workers, tremolite and chrysotile concentrations were significantly correlated, indicating that the tremolite originated from chrysotile products. Therefore, one possible explanation of our results, particularly among cancer patients, is that the presence of tremolite may be an indicator of former chrysotile inhalation [22].

With regard to the other types of fibres found, amosite was only found in Groups B and C. The most notorious amosite burden was observed in workers in the shipyards of Ferrol. These results reflect the widespread use of amosite in shipyards as an effective pipe insulator. In this sense, Kishimoto and coworkers [23], analysing the characteristics of 32 patients exposed to asbestos in a former Japanese naval shipyard, found that 14 patients had been exposed to crocidolite and ten to amosite. Langer and coworkers [24] found that amosite was present in all the lungs of the insulation workers studied, most of whom were shipyard workers, and that its highest concentrations were found in this exposure category. Probably the type of fibres retained in the lung of workers in Spain reflects the type of work performed in shipyards in repair and construction, and suggests a similar pattern of use of asbestos types in this sector worldwide. Import data in Spain show that asbestos was mainly imported from producing countries like Canada (41%), South Africa (24%) and Italy (8.3%), while lower percentages corresponded to manufacturing countries like Germany (9%) and England (3.4%) [4].

In our opinion, the results of the present study may be representative of imports and consumption patterns in southern European countries. However, data related to type of asbestos fibres retained in the lungs of individuals of neighbouring countries are scarce. Sebastien et al. analysed the lungs of French asbestos-exposed workers with several related diseases. Amphiboles were mostly found in lung fibrotic areas, while chrysotile predominated as ultimate fibrils in pleura [25]. Some more data are available from Italy, where it was found that amphiboles clearly predominated in the lungs of general population in Milano [12], thus concurring with our results in the general population of Barcelona. Conversely, chrysotile accounted for two-thirds of the asbestos detected in urban population in Rome [13]. In Italian exposed patients, amphiboles were exclusively present in the vast majority of lungs from subjects in asbestos cement manufacturing areas [14, 26], although in these studies a distinction between crocidolite and the rest of amphibole fibres was not accounted for. Apart from that data, a differential pattern of asbestos lung retention has been described in other southern European countries, in subjects environmentally exposed to erionite in Cappadocia, Turquey [27], and tremolite in Metsovo, a rural area in the northwest of Greece [28].

Regarding the physical characteristics of the fibres detected, 46% of the AF analysed had a length > 5 μm and a diameter < 0.2 μm, and these fibres were mainly found in the samples analysed from shipyard workers. There is evidence that these long thin fibres are the most likely to cause asbestos-related diseases. Fibres below 5 μm in length show greater penetration into the respiratory system, but have a shorter retention as they are easily removed. In a case control study of patients with mesothelioma, McDonald and coworkers [29] found that the concentration of amphiboles (amosite, crocidolite, tremolite) longer than 8 μm was the best predictor of mesothelioma, and that shorter fibres did not contribute to its development. Nevertheless, further experimental and epidemiological studies suggest that the toxicity of small asbestos fibres cannot be ignored. Recent data in humans [30–35] indicate that exposure to longer fibres was associated with higher rates of lung cancer, but no definite conclusions can be ascertained for the other size classes. Nevertheless, the authors of these studies noted that exposure to short, thin fibres was associated with lung cancer risk, and that these fibres represented the majority of those counted in the lung samples of the patients. We cannot yet determine whether the association of these short fibres with lung cancer is a spurious effect due to correlations between fibre-size categories, or evidence that short fibres do indeed play a specific role in carcinogenesis.

In conclusion, the results of this study provide the first data on the type of asbestos retained in the lungs of Spanish populations. Amphiboles appear to be almost exclusively retained in Spanish patients and this finding might be extrapolated to other southern European countries. It also reinforces estimations of Spain as one of the European countries with a higher risk to develop asbestos-related diseases in the years to come.

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Compliance with Ethical Standards

Conflict of interest None.

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