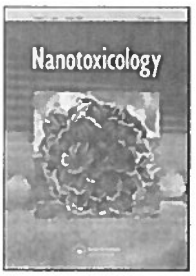
Nanotoxicology



Elemental analysis of histological archival tissues: a method to unmask (nano)asbestos fibers.

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	(i.e. with a diameter of less than 100 nanometers) were unequivocally detected in 2 out of the 5 lung cancer patients with uncertain occupational exposure, whereas no fibers were detected in controls. We feel that the proposed technique can represent a formidable tool for linking the disease to previous workplace exposure in uncertaincases. Furthermore,Formalin-Fixed, Paraffin-Embedded (FFPE) tissues stored in the pathology departments might be re-evaluatedin cases of negative response and possible asbestos exposure. Since diseases acquired owing to occupational exposure to asbestos are generally covered by workers' insurance in most countries, the application of the protocol used in this study may have also relevant social and economic implications.

Elemental analysis of histological archival tissues: a method to unmask (nano)asbestos fibers.

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Keywords

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Abstract

There is epidemiological evidence that the diagnosis of asbestos-related lung cancer is under-estimated. This may be due, at least in part, to the fact that currently used diagnostic tools may miss asbestos fibers in the nanometric range, which have been recently suggested to be strongly associated with lung cancer.

In this work, we utilized Energy Dispersive X-ray (EDX) microanalysis through transmission electron microscopy (TEM) in paraffin blocks taken from 5 patients with lung cancer and uncertain occupational exposure to asbestos, in whom conventional diagnostic techniques failed to detect asbestos fibers. Morphometric and elemental analysis were used to confirm the chemical composition of the fibers.

The same protocol was applied to 10 randomly selected lung cancer patients with no history of previous asbestos exposure. Technicians were blind in respect to the association sample/patient.Several asbestos fibers (i.e. with a diameter of less than 100 μm) were unequivocally detected in 2 out of the5 lung cancer patients with uncertain occupational exposure, whereas no fibers were detected in controls.

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Introduction

Despite the dramatic decline in asbestos exposure in industrialized countries from the 1970s, asbestos-induced lung and pleura diseases remain a major issue in public health (Ashford, 2002). In fact,although asbestos has been banned in most countries more than 20 years ago, it is still an important health problem, giventhe long-lasting latency (about 30 years) between exposure and the development of clinically evident disease,making asbestosis an actual illness yet (Cugell, 2004; Robinson, 2005; Prazakova, 2014); indeed, the peak of asbestos-related disorders is expected within the next decade (Brims FJ, 2009).

Worldwide, asbestos accounts for 100,000–140,000 lung cancer deaths per year and contributes to nearly 5% to 7% of all lung cancers (LaDou, 2004).

It is estimated that the total number of asbestos-related deaths in the United States may exceed 200,000 by the year 2030 (Nicholson, 1982).

A correct diagnosis of asbestos-related (or unrelated) disorders has relevant social and economic implications, since diseases acquired owing to occupational exposure to asbestos are generally covered by workers' insurance in most countries (Van der Bij, 2013). The presence of asbestos in occupational settingsis generally performed by means of phase-contrast light microscopy (PCM), and the standard protocol includes only fibers with a length higher than 5 micron (Dement, 1990). Furthermore, fibers having a diameter less than 0.25 μm diameter are missed because of the insufficient resolution power of most light microscopes. In other words, the standardmethods used in the workplace do not detect fibers shorter than 5 micron and with a diameter of less than 0.25 μm. Of note, fibers shorter than 5 μm account for the majority of fibers released in the workplace in several plants (Dement, 2008).

PCM-based studies indicate that long asbestos fibers have the highest carcinogenic potential (Berman,2008), and two recent recent transmission electron microscopy (TEM)-based survey confirmed this finding (Loomis, 2010; Stayner, 2008); however, both TEM-based studies found a strong association with fibers thinner than 0.25 micron (i.e. very close or in the range of

nanofibers). As discussed above, exposure to such fibers is missed with conventional methods, and in the case of development of diseases possibly related to asbestos, such as lung cancer, the causal relationship may remain uncertain.

In these case, the detection of asbestos fibers in the context of lung cancer may be crucial for a correct etiologic diagnosis. Once again, the search for asbestos fibers is generally associated with the histopathological examination of tissue specimens, which isperformed with optical microscopy, adiagnostic procedure which may miss very small asbestos fibers, especially in the nanometric range. In the light of the above-reported epidemiological data, this fact may have relevance both in term of correct etiologic diagnosis and also in terms of socio-economic compensation in occupational settings.

In this histopathological study, we show that Energy dispersive X-ray (EDX) microanalysis through TEM by paraffin blocks allows the detection of asbestos fibers smaller than 0,1μm in patients with lung cancer and history of possible asbestos exposure,in whom the conventional histological report was negative for asbestos fibers.

Materials and Methods

Patients

In this retrospective study, we re-evaluated5 lung biopsiesoflung cancer patients with a history of possible exposure toasbestos, in which the conventional search for asbestos fibers yielded negative results. Moreover, we analyzed 10 randomly selected lung cancer patients with no history of previous asbestos exposure.

Histological analysis

All biopsies were formalin-fixed and paraffin embedded; four μm-thick sections were hematoxylin and eosin (H&E) stained and the morphological study was blindly verified by two pathologists (Fox, 1985) (Fig.1 A-B).

Immunohistochemistry

The phenotype of lung cancer was characterized by the presence of the thyroid transcription factor 1 (TTF-1) and cytokeratin 7 (typically expressed by adeno-carcinomas) (Fig.1 C-D).

Briefly,3-μm-thick sections were pre-treated with EDTA citrate pH 7.8 for 30 min at 95°C and then incubated respectively with rabbit monoclonal anti-Cytokeratin 7 for 30 min (1:100 clone OV-TL12/30, Novus Biologicals) and rabbit monoclonal anti-TTF-1 for 30 min (1:100 clone SP141, Spring Bioscience). Washing were performed with PBS 4% + Tween20 pH 7.6 produced by UCS diagnostic; reactions were revealed by HRP - DAB Detection Kit (UCS diagnostic) (Scimeca 2014).

Transmission Electron Microscopy (TEM)

a) Formalin-Fixed, Paraffin-Embedded (FFPE) tissues retrieval forultrastructuraland elemental analysis: roll slice embedding (RSE).

For each sample, a 10μm paraffin section was collected in 1 ml eppendorf vial. Sections were de-paraffinized, 3x15 min in noxyl, hydrated by a series of incubations in 100%, 95%, 70%, 30% ethanol and phosphate buffer 0.1 M, and embedded in 200 μl of 2% agarose. After 20 min, they were osmium tetroxide post-fixed, washed with phosphate buffer 0.1 M, and dehydrated by a series of incubations in 30%, 50%, 70%, ethanol. Dehydration was continued by incubations in 95% ethanol, absolute ethanol and propylene oxide.After infiltration with 1:1 epon-propylene oxide solution for 30 min., 3:1 epon-propylene oxide solution for 30 min., and epon absolute for 24 hours, sampleswere embedded in epon (Agar Scientific, Stansted Essex CM24 8GF United Kingdom)by incubation for 24 hours at 60°C (Hayat, 2001).

b) FFPE tissues retrieval forultrastructuraland elemental analysis: flat slice embedding (FSE).

H&E sections were used in order to identify areas suspected to harbor pollutant fibers (Fig.2A). Sixμm serial sectionswerecollected on histologysuper-frost plus slides (Fig.2B).These sections were de-paraffinized, 3x15 min. in noxyl and hydrated by a series of incubations in 100%, 95%, 70%, 30% ethanol and phosphate buffer 0.1 M. Then, sections were osmium tetroxide post-fixed, washed with phosphate buffer 0.1 M, and dehydrated by a series of incubations in 30%, 50%, 70%, ethanol. Dehydration was continued by incubations in 95% ethanol, absolute ethanol and propylene oxide.Tissues were incubated with 1:1 epon-propylene oxide solution for 30 min., 3:1 epon-propylene oxide solution for 30 min., and epon absolute for 3 hours(Fig.2C). Embedding beam capsules were placed over areas previously identified on H&E section (Fig.2D) and incubated for 24 hours at 60°C.Finally, beam capsules were detached from the slide (Fig.2E).

Both RSE and FSE epon embedded tissues were cut (Reid, 1975; Dykstra, 1992) and stained with heavy metals solutions (uranium acetate and lead citrate) as described by Reynolds (Fig.1F) (Reynolds, 1963).

All samples were studied by TEM Hitachi H-7100.

EDX microanalysis

The EDX microanalysis is a technology that performs the elemental and chemical analysis of a sample in a transmission electron microscope. When the electron beam in an electron microscope hits a thin sample, some atoms of the sample will be excited or ionized. When they return into their ground state, they will emit characteristic x-rays. The x-ray emission at different wavelengths may then be measured by a photon-energy-sensitive detector.

The EDX detector system performs a simultaneous display of all mid-energy (1-20 keV) x-rays collected during any individual analysis period. Therefore it is possible to detect those elements with N.A.>10. The minimal detectable elemental concentration, which requires some signal

averaging, is approximately 0.1mmol per kg of dry specimen (i.e., 10 ppm), whereas spatial resolution ranges from about 10 nm to a few micrometers (Scimeca, 2014).

For the EDX microanalysis 100 nm-thick unstained ultrathin sections were placed on specific copper grids. The EDX spectra were acquired by a Hitachi 7100FA transmission electron microscope and an EDX detector (Thermo Scientific, Waltham, MA USA) at an acceleration voltage of 75 KeV and 12000 magnification. Spectra were semi-quantitatively analyzed by the Noram System Six software (Thermo Scientific, Waltham, MA USA) using the standardless Cliff-Lorimer k-factor method.20 The EDX microanalysis system was calibrated using the x-ray microanalysis standard (Micro-Analysis Consultants Ltd, Cambridgeshire UK) (Scimeca, 2014). The sample standards used as controls were: Dural (Al, Cu, Mg), Apatite (Ca5(PO4)3(OH)), Lead Sulphide (PbS), Chromite (FeCr2O4), Chromium (Cr), Iron (Fe), Manganese (Mn), Silicon (Si), Tungsten (W), Zinc (Zn), Cadmium sulfide (CdS) and Cobalt (Co).This semi-quantitative approach pointed out the presence of asbestos fibers in the tissues.

Results

All lung biopsieswere classified as lung adeno-carcinomas, according to the criteria released in 2012 from the World Health Organization (WHO, 2012).The observation of tissues by optical microscopy did not allow the identification of fibers related to contamination of asbestos.

Ultrastructural analysis of tissues reaped and processed according to RSE method showed a consistent loss of morphology as compared normal tissues (Fig.3A-D). The observation of tissues by TEM did not point out fibers of asbestos.

Even though the samples processed byFSE method (fig.3E-F) presented a reduction of structural details, for lower magnifications (‹10,000x) it was still possible tocarry out morphological analysis. Actually, a strong morphological correlation was foundbetween the selected area on H&E slice and the corresponding area on the FSE embedded. In some of the FSE section we demonstrated nano-particles unnoticeable by optical microscopy.

In fact, in 2 out of 5adeno-carcinoma tissues of patients with a history of possible exposure to asbestos, we observedseveralfiberswith a length variable from 0,1 to 0,6μm and a diameter below 0.1 micron (Fig.4). Conversely, we did not found asbestos fibers in adeno-carcinoma tissues of patients with no history of previous asbestos exposure.

The EDX microanalysis allowed us to identify these elements as asbestos fibers, mainly composed of silicon and iron (Fig.4E-F). Comparing the morphometric data of the fibers to the microanalytical ones, we classified these fibersas a specific asbestos iso-type.

In particular, fibers detected into lung adeno-carcinoma were compatible with asbestiform variety amosite (Fig.4).

Noteworthy, corrosive substances used during tissues processing, such as paraformaldehyde, osmium tetroxide and propylene oxide do not damage sections and/or extract particles or fibers from them.

Discussion

The use of asbestos in many products surged during the 20th century, and asbestos exposure continues despite a sharp reduction in production since the 1980s (LaDou). Asbestos is an established cause of mesothelioma and of lung cancer. Although many uses had been discontinued, asbestos is still used in some limited applications.(Ullrich, 2004).

The two most relevant asbestos related disorders are pleural mesothelioma and lung cancer. Whereas the causal relationship between asbestos exposure and mesothelioma is so specific to be considered as an index of societies' past usage of asbestos, the association between asbestos and lung cancer is much more problematic, given the typically multi-factorial etiology of this cancer. Most epidemiological modeling analyses estimate that the ratio of mesothelioma and lung cancer caused by asbestos should be between 1:2 or 1:1 (Henderson, 2004). By comparison with these estimates, the lung cancer casesattributed to asbestos exposure are much less than they should: For example, according to the number of deaths from asbestos in the United Kingdom for the years 1929-1996, the ratio of mesothelioma to lung cancer was 1:0.1 (Howie, 1999), i.e. ten to twenty times higher than that expected on the basis of the epidemiological estimates. Similar under-recognition occurs in other western countries (Mollo, 2002; Kishimoto, 2003; Teschke, 1992). This fact may be explained by the natural history of lung cancer caused by asbestos exposure. It in fact develops several decades after exposure to asbestos, so it may be difficult to obtain reliable documentation about the presence and extent of past exposure (and, when available, it may miss very small fibers, as discussed above);the duration of assumed past exposure is often used as a surrogate,however, its predictive power for the future development of lung cancer has been recently questioned (Villeneuve,2012). To circumvent these limitations, the search for asbestos fibers associated with lung cancer is used to demonstrate a causal relationship, on the basis of the well known bio-persistence of asbestos fibers within the human body: once again, the techniques currently used for this purpose may not have sufficient power to detect asbestos nanofibers.Therefore, a technique allowing a reliable identification of these fibers in the context or

in the proximity of lung cancer in the case of suspected exposure to asbestos would be very welcome, since epidemiological data suggest that a high proportion of "suspected" cases are indeed "true" cases.

In this work, we evaluated the ability of EDX in un masking asbestos fibers in the nanometric range, missed by conventional diagnostic techniques. For this purpose, we tested two different protocols: the RSE method and the FSE method.

RSE did not allow us to obtain much more information compared to the optical analysis. In fact, we were unable to detect fibrillar material in 5 tissues in lung cancer patients with possible asbestos exposure. It was maybe the consequence of fibres extraction from tissues during various steps into the eppendorf vials. Moreover, samples completely lose their morphological architecture through the embedding procedures.

On the other hand, the FSE method allowed us to unmask hidden asbestos fibers thanks to the correlation between H&E areas and those for TEM and EDX microanalysis and to the partially preserved cellular morphology.

A potential limitation of FSE method is represented by the loss of ultrastructural details in paraffin-embedded tissues, which does not allow a magnification higher than 10.000x. However, we were ableto perform asatisfactory morphological analysis and to identify fibers deposits. Indeed, it is important to verify the presence offibers in cluepathological tissue areas. The spotting of asbestos fibers in patients previously classified as asbestos-free demonstrates that histological analysis is not able to detect asbestosis in the case of exposure to asbestos nano-fibers.

The pathogenetic role of asbestos fibers in the nanometric range in inducing lung cancer has been highlighted by one study showed the strongest association for long fibers (> 10 micron) with a diameter below 0.25 micron (Stainer, 2008). It should be noted that these dangerous dimensions are the same suggested for the possible potential carcinogenicity of carbon nanotubes (Kostarelos, 2008), which is in turn considered to have an injuring potential similar to that of asbestos.

Furthermore, recent data show that asbestos nanofibers cause susbstantial oxidative stress to lung cell lines (Turci, 2012).

The asbestos fibers evidenced with our technique had not only a diameter in the nanometric range, but were also very short, with a length lower than 0.6 μm. It is of interest that a recent epidemiologic study detected a strong association between lung cancer and asbestos fibers shorter than 1.5μm (Loomis, 2010).

Ultrastructural studies and EDX microanalysis on archival FFPE could therefore offer exposed workers the chance to ask for a more sensible and specific re-evaluation of their case. The social implications of this refined diagnostic method are self-evident.

Morphometric and elemental analysis of fibers allowed us to identify the specific asbestos iso-type found in tissues. In particular, amosite asbestos nanofibers were detected. This variety of asbestos is associated with excess mortality from lung cancer and mesothelioma (Acheson, 1984) and is considered more harmful than other varieties such as chrysotile, given its tendencyto stay in the lung for a longer period of time (Agency for Toxic Substances and Disease Registry, 2001). These obtained indisputable identification of asbestos fibers can represent a formidable tool for linking the disease to previous workplace exposure in cases in which the correlation remain uncertain.

Conclusion

In this work, we proposed a new analyticalprotocol that can bea helpful diagnostic test to detect asbestos nanofibers also inretrospective analysis, using archival lung FFPE tissues. This method allows to unmask exposure to asbestos nano-fibers undetected with conventional methods.

In the light of the wide underestimation of the causal link between asbestos and lung cancer, FFPE tissues represent a treasure stored in the anatomic pathology departments allowing to nail the "culprit", absolved in previous trial. Furthermore, the revaluation of these biopsies can be a valid support instrument for medico-legal cases aimed at the resolution of work services.

Acknowledgments

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Declaration of Interest

Authors declare no conflict of interest.

References

Acheson ED, Gardner MJ, Winter PD, Bennet C. 1984.Cancer in a factory using amosite asbestos.Int J Epidemiol 13: 3-10.

Agency for Toxic Substances and Disease Registry. 2001. Toxicological Profile for Asbestos. Available at: http://www.atsdr.cdc.gov/toxprofiles/tp61.pdf.

Ashford NA, Castleman B, Frank AL, Giannasi F, Goldman LR, Greenberg M, Huff J, Joshi KT, LaDou J, Lemen RA, et al. 2002. The International Commission on Occupational Health (ICOH) and its influence on international organizations. Int J Occup Environ Health. Apr-Jun;8(2):156–162.

Berman DW, Crump KS. 2008. A meta-analysis of asbestos-related cancer risk that addresses fiber size and mineral type. Crit Rev Toxicol 38(Suppl 1):49e73.

Brims FJ. 2009. Asbestos–a legacy and a persistent problem. J R Nav Med Serv, 95:4-11.

Cugell DW, Kamp DW. 2004. Asbestos and the pleura: a review. Chest. 125:1103–17.

Dement JM, Kuempel ED, Zumwalde RD, et al. 2008. Development of a fibre size-specific job-exposure matrix for airborne asbestos fibres.Occup Environ Med 65:605-12.

Dement JM, Wallingford KM. 1990. Comparison of phase contrast and electron microscopic methods for evaluation of occupational asbestos exposures.ApplOccup Environ Hyg 5:242-7.

Dykstra MJ. (1992). Biological electron microscopy: theory, techniques and troubleshooting. New York: Plenum Press.

Fox CH, Johnson FB, Whiting J, Roller PP. 1985. Formaldehyde fixation. J HistochemCytochem. 33:845-853.

Hayat MA. (2001). Fixation for electron microscopy. New York: Academic.

Henderson DW, Rodelsperger K, Woitowitz H-J, Leigh J. 2004. After Helsinki: a multidisciplinary review of the relationship between asbestos exposure and lung cancer, with emphasis on studies published during 1997–2004. Pathology 36(6). pp. 517–550.

Howie R. 1999. Asbestos-induced deaths in the United Kingdom.In Sourcebooks on asbestos diseases. Peters GA, Peters BJ EdsCharlottville Lexis 19. 219-238.

Kishimoto T, Ohnishi K, Saito Y. 2003. Clinical study of asbestos-related lung cancer.Ind Health 41, 94-100.

Kostarelos K. 2008. The long and short of carbon nanotube toxicity. Nat Biotechnol 26, 774-776.

LaDou FJ. 2004. The asbestos cancer epidemic. Environ Health Perspect. 112:285–90.

Lakhani SR, Ellis IO, Schnitt SJ. et al. 2012. WHO Classification of Tumours.IARC Press; Lyon, France.

Loomis D, Dement J, Richardson D, Wolf S. 2010. Asbestos fibre dimensions and lung cancer mortality among workers exposed to chrysotile. Occup Environ Med 67:580-584

Luft JH. (1961). Improvements in epoxy resin embedding methods. J BiophysBiochemCytol. 9:409-414.

Mollo F, Magnani C, Bo P, Burlo P, Cravello M. 2002. The attribution of lung cancers to asbestos exposure: A pathologic study of 924 unselected cases. Am J ClinPathol 117, 90-95 .

Nicholson WJ, Perkel G, Selikoff IJ. 1982. Occupational exposure to asbestos: population at risk and projected mortality; 1980–2030. Am J Ind Med. 3:259–311.

Prazakova S, Thomas PS, Sandrini A, Yates DH. 2014. Asbestos and the lung in the 21st century: an update. ClinRespir J. Jan;8(1):1-10.

Reid N. (1975). Ultramicrotomy. In Practical methods in electron microscopy. Volume 3. Amsterdam: North Holland; Part 2.

Reynolds ES. 1963. The use of lead citrate at high pH as an electron opaque stain based on metal chelation. J Cell Biol. 17:208-212.

Robinson BWS, Lake RA. 2005. Advances in malignant mesothelioma. N Engl J Med. 353:1591–603.

Scimeca M, Giannini E, Antonacci C, Pistolese CA, Spagnoli LG, Bonanno E. 2014. Microcalcifications in breast cancer: an active phenomenon mediated by epithelial cells with mesenchymal characteristics. BMC Cancer. Apr 23;14:286.

Scimeca M, Orlandi A, Terrenato I, Bischetti S, Bonanno E. 2014. Eur J Histochem. 12;58(3):2403.

Stainer R, Kuempel E, Gilbert S et al. 2008.An epidemiological study of the role of chrysotile asbestos fibre dimensions in determining respiratory disease risk in exposed workers.Occup Environ 65, 613-19.

Stayner L, Kuempel E, Gilbert S, et al. 2008. An epidemiological study oif the role of chrysotile asbestos fibre dimensions in determining respiratory disease risk in exposed workers. Occup Environ Med 65:613-19.

Teschke K, Barroetavena MC. 1992. Occupational cancer in Canada: What do we know? Can Med Assoc J 147: 1501-1507.

Turci F, Colonna M, Mantegna S, Cravotto G, Gulino G, Aldieri E, Ghigo D, Fubini B. 2012. Surface reactivity and cell responses to chrysotile asbestos nanofibers. Chem Res Toxicol 25: 884-894.

Ullrich RL. (2004). Etiology of cancer: Physical factors. In: DeVita VT Jr., Hellman S, Rosenberg SA, editors. Cancer: Principles and Practice of Oncology. Vol. 1 and 2. 7th ed. Philadelphia: Lippincott Williams and Wilkins.

Van der Bij S, Baas P, van de Vijver MJ, de Mol BA, Burgers JA. 2013. Legal claims for malignant mesothelioma: dealing with all cases. Lung Cancer. 80(2):153-8.

Villeneuve PJ, Parent ME, Harris SA, et al. 2012.Occupational exposure to asbestos and lung cancer in men: evidence from a population-based case-control study in eight Canadian provinces.MC Cancer 12:595.

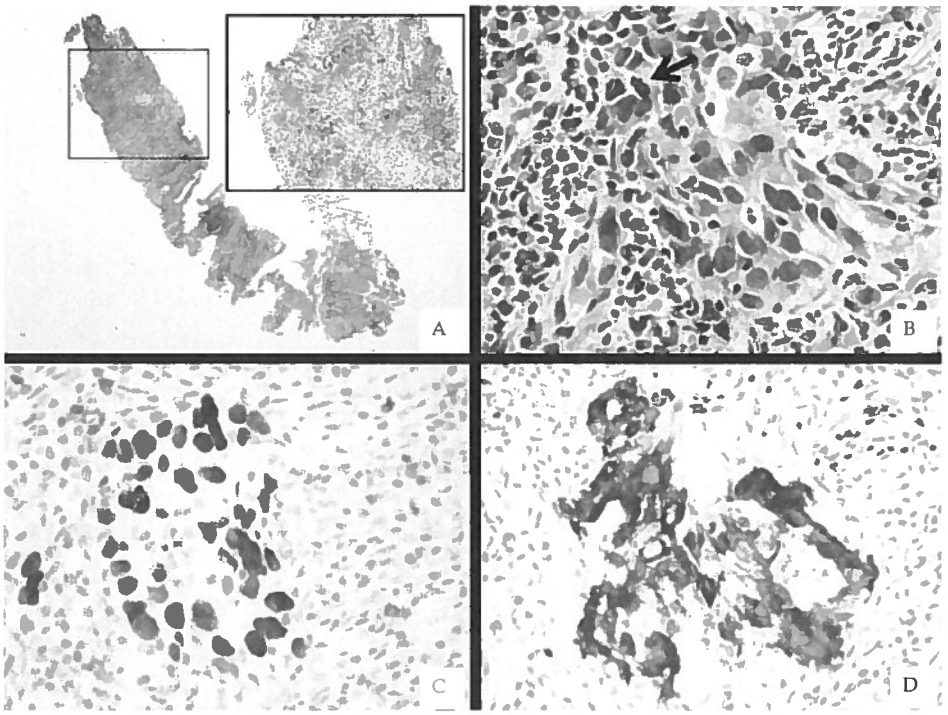
Figures

Fig.1 Lung histological classifications. A) H&E of lung biopsy (2x). Square show main lung lesion (10x). B) High magnification display cohesive malignant cells with abundant cytoplasm, large nuclei and atypical mitosis (arrow) (40x). Neoplastic cells were characterized by nuclear expression of TTF-1 antigen (C) and CK7 positivity (D) (40x).

Fig.2 FSE method. A) H&E sections were used in order to identify areas suspected to harbor pollutant fibers (circle). B) 6 µm serial sections were collected on histology super-frost plus slides and processed for Epon embedding. C) Embedding beam capsules were placed over areas previously identified. D) After incubation for 24h at 60°C, beam capsules were detached from the slide. E) Epon embedded tissue were cut and stained with heavy metals solutions as uranium acetate and lead citrate.

Fig.3 Ultrastructural preservations of RSE and FSE Epon embedded tissues. A-B) Lung tissues processed by standard transmission electron microscope protocol (5.000x). C-D) Ultrastructural analysis of tissues reaped and processed according to RSE method showed a consistent loss of morphology as compared to standard tecnique that hampered the morphologycal analysis (5.000x). E-F) Lung samples processed by FSE method showed a satisfactory preservations of ultrastructural details. Indeed, for lower magnifications (‹10,000x), it was still possible carry out morphological analysis (5000x).

Fig.4 EDX microanalysis of asbestos fibers.TEM electron micrographs of lung adeno-carcinoma processed by FSE method show numerous fibers with variable length, of 0,2-0,6 µm (case #2 panel A-B; case #4 panel C,D). EDX Microanalysis spectrum allowed us to identify these electrondense bodies as asbestos fibers, mainly composed of Si, Fe, Mg and Al (E-F).



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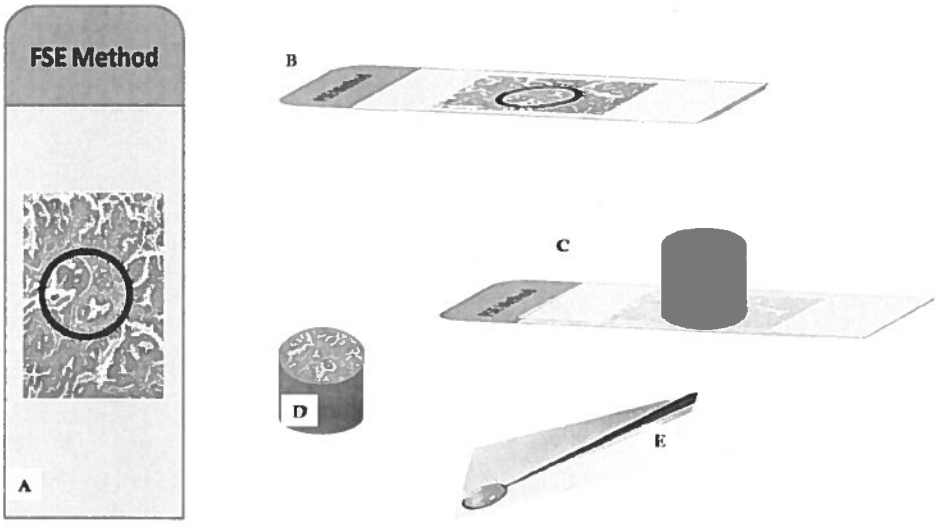

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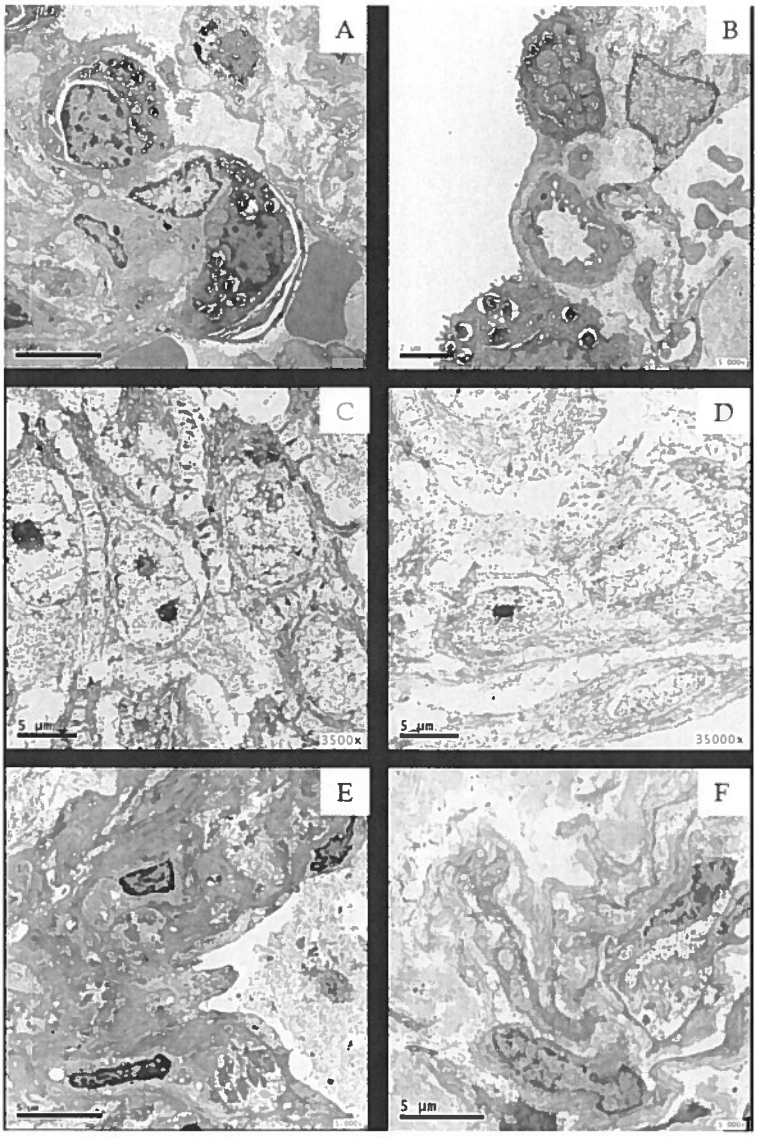

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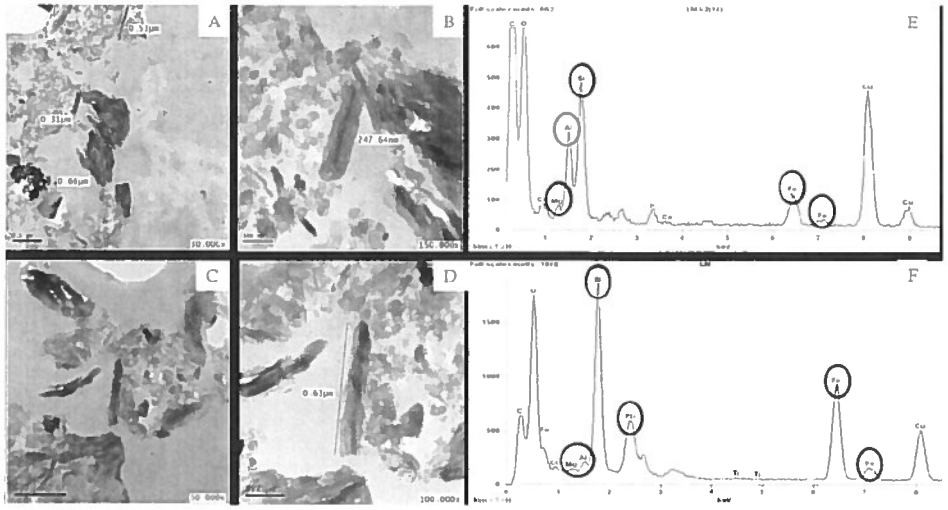

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