

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

The wild rat as sentinel animal in the environmental risk assessment of asbestos pollution: A pilot study

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/142448> since

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:

Questa è la versione dell'autore dell'opera:

Science of the Total Environment, 479-480, 2014

<http://dx.doi.org/10.1016/j.scitotenv.2014.01.108>

The definitive version is available at:

La versione definitiva è disponibile alla URL:

<http://ees.elsevier.com/stoten/>

The wild rat as sentinel animal in the environmental risk assessment of asbestos pollution: a pilot study

Michele Ardizzone^a, Carlotta Vizio^b, Elena Bozzetta^c, Marzia Pezzolato^c, Serena Meister^c, Alessandro Dondo^d, Ilaria Giorgi^d, Angelo Seghesio^e, Dario Mirabelli^{f,g}, Silvana Capella^{g,h}, Ruggero Vigliaturo^h, Elena Belluso^{g,h,i}

- a) Via Bligny 4, 15033, Casale Monferrato (AL), Italy
- b) Regione Bocca 16/A, 15034, Cellamonte (AL), Italy
- c) Histopathology Laboratory, Veterinary Medical Research Institute for Piemonte, Liguria and Valle d'Aosta (IZSPLVA), Torino, Italy
- d) Animal Diagnostic Department, Veterinary Medical Research Institute for Piemonte, Liguria and Valle d'Aosta (IZSPLVA), Torino, Italy
- e) Local Health Authority of Alessandria and Asti, Italy
- f) Cancer Epidemiology Unit, University of Torino and CPO Piemonte, Torino, Italy
- g) Interdepartmental Centre for Studies on Asbestos and other Toxic Particulates "G. Scansetti", University of Torino, Italy
- h) Department of Earth Sciences, University of Torino, Italy
- i) Institute of Geosciences and Earth Resources, CNR, Unit of Torino, Italy

Corresponding author:

Michele Ardizzone, DVM, PhD

Email: mikeardi@libero.it

Mobile: +39 335 7957522 or +39 340 1729651

Via Bligny, 4

15033 Casale Monferrato (AL)

Italy

ABSTRACT

Asbestos has been banned in many countries, including Italy. However, sources of exposure may still exist, due to asbestos in-situ or past disposal of asbestos-containing wastes. In an urban area with past high environmental exposure, like Casale Monferrato, the lung fiber burden in sentinel animals may be useful to identify such sources. A pilot study was conducted to assess the feasibility of its determination in wild rats, a suitable sentinel species never used before for environmental lung asbestos fiber burden studies.

Within the framework of pest control campaigns, 11 adult animals from 3 sites in the urban area of Casale Monferrato and 3 control rats from a different, unexposed town were captured. Further, 3 positive and 3 negative control lung samples were obtained from laboratories involved in breeding programs and conducting experimental studies on rats. Tissue fiber concentration was measured by Scanning electron microscopy with energy dispersive spectrometry.

Asbestos (chrysotile and crocidolite) was identified in the lungs from rats from Casale Monferrato, but not in control rats and in negative control lung samples. Asbestos grunerite at high concentration was found in positive control lung samples.

Measurement of the lung fiber burden in wild rats has proved feasible: it was possible not only to detect, but also to characterize asbestos fibers both qualitatively and quantitatively. The pilot study provides the rationale for using wild rats as sentinels of the soil contamination level in Casale Monferrato, to identify areas with the possible presence of previously unrecognised asbestos sources.

KEYWORDS

Asbestos pollution

Sentinel animals

SEM-EDS

Environmental Risk Assessment

Information on funding sources

1. *Lega Italiana per la Lotta contro i Tumori (LILT) – sezione provinciale di Alessandria*
 - covered the financial costs required for the conduct of the various operational phases of the pilot study reported here
2. *Azienda Sanitaria Locale – Alessandria (ASL-AL)*
 - covered the financial costs required for the purchase of materials needed for the conduct of the pilot study reported here
3. *Fraunhofer Institute of Hanover*
 - kindly offered samples of lung tissue, used as a positive control, from a sub-chronic inhalation toxicology study previously conducted in accordance with the GLP (Good Laboratory Practice) guidelines, **in full compliance with national and international legislation (EU Directive 86/609) regarding animal welfare.**
4. *Charles River Italy*
 - kindly offered samples of lung tissue, used as a negative control, from animals bred in their facility **in full compliance with national and international legislation (D.Lvo 116/92 and EU Directive 2010/63).**

1. Introduction

Asbestos is one of the most widely used industrial materials. Despite its long-recognized link to chronic pulmonary related diseases such as fibrosis and asbestosis (Cooke, 1924; Cooke, 1927; Oliver, 1925; Bartrip, 2004) and carcinogenicity (Doll, 1955; Wagner et al, 1960), its world-wide consumption peaked at about 5 million tons in the mid-1970s and has recently levelled off to about half that value (Nishikawa et al, 2008) as, while banned in some countries, it is still mined and used throughout the world, mainly in emerging economies like Brazil, Russia, India and China (The Mesothelioma Center, 2012). Italy was one of the major producers and users of asbestos until the end of the 1980s. Between the end of the Second World War and 1992, when a legal ban was imposed, almost 4 million tons of raw asbestos were produced and a further 50 thousand tons were imported every year. The single most important use was in asbestos-cement production. In Casale Monferrato (Alessandria district), a small city of 35 thousand inhabitants situated in the eastern part of Piedmont, the oldest and largest Italian factory producing asbestos-cement materials (Eternit) was active from 1907 to 1986, with serious consequences for the health of workers, their relatives and members of the general population as well as for the environment due to contamination over such a long period (Magnani et al, 2008; Ferrante et al, 2007; Maule et al, 2007). Currently about 35 new cases of malignant mesothelioma occur every year among city residents, corresponding to crude incidence rates of 86 and 89 cases per 100,000 person/years in men and women, respectively, according to 2005-2009 statistics from the Piedmont Malignant Mesothelioma Registry (Mirabelli D., personal communication).

Today, about 25 years after the factory was closed, there is still environmental pollution in Casale Monferrato and surrounding towns caused by the presence of waste materials, from crushing scrap asbestos-cement products – so-called “*paving*” – and from grinding the extremities of pipes and pressure pipes, so-called “*dust*”. Such materials were freely available to the population and have been used to fill and level the ground in courtyards, pavements, roads and even sportsgrounds, or to provide inexpensive thermal insulation under roofing. The memory of their presence may have been lost, as current users and dwellers of contaminated sites and buildings may no longer be the same who installed the asbestos-containing materials. How can contaminated sites be identified? Can we pinpoint areas to concentrate the search for such sites by studying spatial differences in exposure indices?

Animals have long been used as monitors of environmental hazards (Glickman et al, 1983; van der Schalie et al, 1999). Rats are suitable sentinels for asbestos soil pollution, sharing the environment with humans in metropolitan areas (Ceruti et al, 2002; Doungchawee et al, 2002). Rats have been used in inhalation studies on asbestos toxicity, showing similarity in the lower respiratory tract function and anatomy with humans.

They live in colonies covering territories generally between 15 and 50 meters of radius around the nest and even if they can travel up to 400 meters or more daily to search for food and water, they always return home. A project for systematically seeking latent sources of asbestos in Casale Monferrato by using the lung fiber burden in wild rats as sentinel animals was set up. The idea was to identify the neighbourhoods of the city with the greatest potential for human exposure by measuring the asbestos fiber content in the lungs of rats and to concentrate the search for potential sources of exposure, in particular asbestos deposits, in these areas. To translate the idea into a study design and test its feasibility, we conducted a pilot study consisting of four steps schematically shown in [Figure 1](#), based on the lung burden analysis of breathed inorganic fibers in both wild and laboratory rats. We wanted to assess the presence of asbestos fibers and to assure the harmonization of all the tasks, including rodent capture, transport to the laboratories, lung tissue sampling, and specimen preservation during the procedure. Validation of the preparation method for the assessment of biological material by scanning electron microscopy (SEM-EDS) in rats was another important endpoint of the study, since never tested previously in these animals.

2. Methods and Materials

2.1. Source of animal lung tissue for examination

To investigate the burden of breathed inorganic and organic fibers, lung samples from twenty wild or laboratory rats were collected and examined. A list of the samples and their origins are given in [Table 1](#). Fourteen lung samples were obtained from wild rats captured by the authors in the urban areas of Casale Monferrato (Alessandria district, Piedmont region, NW Italy) and Asti (Piedmont region, NW Italy), in full compliance with the rodent control program operated by the two municipalities. Eleven rats from three different areas of Casale Monferrato town were the group of interest for our study and three rats from one area of Asti town were used as an anthropogenic environmental control, since no major asbestos company or asbestos-using company has ever operated in Asti. As negative controls, three lung samples from Sprague Dawley rats kindly provided by Charles River Italy, where the animals had been bred and grown, were investigated.

As positive controls, three lung samples from Fischer 344 rats kindly provided by the Hannover Fraunhofer Institute, where animals breathed asbestos grunerite for 3 months in a subchronic inhalation study on man-made vitreous fibers ([Brown et al, 2002](#); [Bellmann et al, 2002](#)) were investigated.

2.2. Wild rat capture

Wild rats in the urban areas of Casale Monferrato were captured using a multi-capture electromechanical environmental-friendly device (Ekomille®), selected due to its reliability and safety both for men and non-

target animals. This product, covered by two international patents, has been certified and recognised by the Italian Ministry of Health and other public institutions, with special appreciation of the ecological principles adopted (<http://www.derattizzazione-disinfestazione.it/ekomille/ekomille/>). Rats in the urban areas of Asti were captured using spring traps.

2.3. Transportation of wild rat carcasses to the Veterinary Medical Research Institute for Piemonte, Liguria and the Valle d'Aosta (IZSPLVA)

The wild rat carcasses collected in the urban areas of Casale Monferrato and Asti were transported to the IZSPLVA under the responsibility of the Local Health Authority of Alessandria and Asti, in accordance with the procedures set up by the local health monitoring plan, duly implemented by the IZSPLVA, with the purpose of monitoring the potential human risk for zoonoses.

2.4. Necropsy of wild rats

Necropsy of the fourteen wild rats collected in the urban areas of Casale Monferrato and Asti was performed in an appropriate bio-safety level laboratory at the Animal Diagnostic Department of IZSPLVA, according to internal standard protocols. All wild rats were sexed and weighed ([Table 1](#)), and any macroscopic changes in organs and tissues evaluated to exclude the presence of lesions referable to potential zoonoses (e.g.: granulomas, necrosis, splenomegaly and nephropathies). Spleen, liver and kidneys were collected and tested by Polymerase Chain Reaction (PCR) test for *Francisella tularensis* and *Leptospira* spp., etiologic agents of Tularaemia and Leptospirosis. Lungs were removed, weighed and split into two parts (left and right) before being fixed in 4 % buffered formaldehyde solution. The left lungs of eleven out of the fourteen wild rats were sent to the histopathology laboratory of IZSPLVA while the corresponding right lungs were sent to the Department of Earth Sciences of Turin University. Both the right and left lungs of the three remaining rats were sent directly to the Turin University Department of Earth Sciences ([Table 1](#)).

2.5. Histopathological evaluation of lungs

Histological investigations were performed at the Histopathology Laboratory of the IZSPLVA on twelve only out of twenty left lungs available ([Table 1](#)); eleven lung samples from wild rats captured in the urban areas of Casale Monferrato and Asti and one from a Fischer 344 rat kindly provided by the Hannover Fraunhofer Institute, used as positive control.

Lungs were routinely processed, embedded in paraffin wax, serially sectioned at 3-5 µm and stained with haematoxylin and eosin (HE) and Perls staining. Histological observation by light microscopy was performed on HE-stained paraffin sections to highlight any possible microscopic change induced in lung tissue by asbestos fibers, and on Perls-stained sections to reveal any possible asbestos bodies.

2.6. Scanning electron microscopy with energy dispersive spectrometry (SEM-EDS) investigation

Samples were prepared for SEM-EDS and examined at the Department of Earth Sciences of the University of Torino, to assess the presence, kind and burden of asbestos fibers and other inorganic fibers in the lung tissue of rats.

The lung portion used is shown in [Table 1](#).

For each animal 250 mg of tissue was chemically digested by sodium hypochlorite (NaClO) at room temperature, to eliminate the organic fraction. The inorganic material was then recovered by filtering the suspension on cellulose mixed ester filters having a diameter of 25 mm and porosity of 0.45 μm . Each membrane was investigated by SEM (StereoScan 360 Cambridge Instruments) using back-scattered electron (BSE) and secondary electron (SE) images. We observed a number of selected microscopic fields (MF's) large enough to obtain a statistic sampling. We observed 800 MF's at 2000 M to cover an area of 1.85 mm^2 of the filter, larger than 1 mm^2 , area indicated in the official methods for analyzing airborne particles (ISO 14966, AIA-RTM 2, DM 6/9/94) taking into account that aren't official methods for fiber counting in biological samples. The MF's are distributed along 5 parallel strips. Each strip is 16 mm long and is sampled at steps of 100 μm , thus obtaining 160 MF's per strip. The MF's along the same strip are spaced of 40 μm . The length of the steps (100 μm) and the separation of 2.5 mm between two adjacent strips are such that overlapping between different MF's cannot occur. The detailed and standardized overall protocol had previously been described in another work by two authors of this paper ([Belluso et al., 2006](#)). Only particles corresponding to the mineralogical definition of fiber (i.e. having length/width ratio ≥ 3) were considered. For each one, the dimensions were measured and chemical composition by EDS (Link-Oxford Pentafet ATW2) collected.

For each animal 2.5 g of tissue was also dehydrated in an oven to obtain the dry weight useful for normalizing the fiber amounts to 1 gram of dry weight (ff/gdw) according to the international standard ([De Vuyst et al., 1998](#)). If in the examined area none fiber was detected, we assumed that any fiber was not in the sample and therefore the number of ff/gdw is supposed equal to 0. Anyway **it is possible that the fibers very little have been lost during the sample preparation.**

The detected fibers were identified by comparing their EDS spectrum with those in the database built by two authors of this paper (i.e. Capella S. and Belluso E.) According to their dimensions, the identified fibers were distinguished as being asbestos or not asbestos and classified as breathable (Bff) or shorter fibers (sff): the former having a length $> 5 \mu\text{m}$ and width $< 3 \mu\text{m}$ (size of asbestos fibers: Directive 2003/18/EC; WHO, 1986)

and the latter having a length < 5 µm and width < 3 µm. For both, the length/width ratio of the fibers considered was ≥ 3 .

To obtain the burden of asbestos and other inorganic fibers, the amount (number) of each kind of fiber detected in the area examined by SEM-EDS was related to a gram of wet lung. Finally the number of fibers was normalized to 1 gram of the dry tissue weight (ff/gdw) as indicated by the international guidelines (De Vuyst et al., 1998).

3. Results

3.1. Necropsy and PCR

At necropsy, no macroscopic lesions relevant for potential zoonoses (e.g.: granulomas, necrosis, splenomegaly and nephropathies) were found in any of the wild rats captured in the urban areas of Casale Monferrato and Asti and also PCR tests performed on their spleen, liver and kidneys were negative for Francisella and Leptospira.

3.2. Histopathological evaluation of lungs

Microscopic analyses of lungs provided by the Hannover Fraunhofer Institute, used as positive control, highlighted a low degree of interstitial fibrosis localized on the walls of alveoli as well as yellow-brown asbestos bodies embedded within fibrous tissue but also within alveolar walls and spaces. These bodies were easier to distinguish using Perl's staining (Fig.2) than by HE staining. No similar lesions were seen in any lung samples from rats captured in the urban area of Casale Monferrato where only focal to diffuse inflammatory lesions characterized by lymphocytes, plasma cells and macrophage cells were highlighted. The three samples of lung examined from wild rats captured in the urban area of Asti were characterized by severe autolytic processes. No asbestos bodies were detected by Perl's staining in any of the samples examined.

3.3. Evaluation of inorganic fiber burden by SEM/EDS

Investigations by SEM-EDS allowed the detection of several kinds of inorganic fibers in the rat lungs: asbestos and non-asbestos classified minerals and other inorganic fibers (Fig.3 and Fig.4).

Tables 2 and 3, respectively, report asbestos and non-asbestos fibers detected in lungs from the examined animals as number of fibers per gram of dry tissue weight. Table 2 also gives both literature data and data for a doubtful group (named asbestos/non asbestos group).

Four kinds of asbestos were detected: chrysotile, crocidolite, grunerite asbestos and tremolite/actinolite asbestos. These latter fibrous minerals were grouped together owing to the impossibility of making a firm distinction by the technique used (Fornero et al, 2009).

Crocidolite was detected in three rat lungs related to two sites in Casale Monferrato town.

Asbestos tremolite/actinolite fibers were detected in three samples from three Casale Monferrato town sites. Fibers of this asbestos were also detected in rats from Asti town.

Chrysotile fibers, more frequent and abundant than other asbestos detected in wild rat lungs, were present in five samples related to three town sites.

A fibrous serpentine group was set up for fibers whose morphology was not distinctive enough to discriminate between chrysotile asbestos (splitting and flexible bundle of very thin fibers) and non-asbestos but asbestiform antigorite (splitting thin laths) (Fornero et al., 2009). It is not possible with this technique to classify these fibers as either asbestos or non-asbestos and they were therefore generically named as asbestos/non asbestos fibers (Tab. 2).

As far as the non-asbestos inorganic fibers are concerned, five groups of chemically differentiated fibers were identified: metallic oxides; aluminium silicates (Al-silicates), vitreous silicates, calcium-titanium silicates (Ca, Ti-silicates) and phyllosilicates (excluding chrysotile and asbestiform antigorite). A group of non identified fibers (n.i.) was also found, but related to only one sample (CM116053/1).

Non-asbestos inorganic fibers were more abundant than asbestos ones, being detected in almost all samples. In Casale Monferrato town the samples having the greatest burden of total inorganic fibers are those containing the highest amount of asbestos (CM112961 and CM116053).

As regards the size of fibers from the examined wild rat lungs, Bff prevail clearly over sff in amount and frequency.

In fact they are the only one kind of fibers in the following groups: crocidolite, chrysotile, tremolite/actinolite asbestos, Al-silicates, vitreous silicates, Ca and Ti-silicates.

As expected, a high amount of grunerite asbestos was detected in rat lung from the Fraunhofer Institute sample used as positive control. The grunerite asbestos content was three orders of magnitude higher than that of wild rats. The rat lungs obtained from Charles River Italy did not contain any kind of inorganic fibers, as expected in this negative control case.

Lungs from rats captured in Asti town showed only one kind of asbestos, i.e. tremolite/actinolite asbestos, in an amount similar to that detected in animals from two different Casale Monferrato town sites (CM 107959, CM 116053). The other inorganic fibers detected belong to three non-asbestos group: metallic oxides (both Bff and sff), Ca, Ti-silicates and phyllosilicates. Also here, the amounts are similar to those detected at some Casale Monferrato town sites.

4. Discussion and Conclusions

The overall experimental design of the pilot study emphasized, on the one hand, the efficiency of harmonization achieved in all the steps required, from capture of the rats to fixing of lungs in 4% buffered

formaldehyde solution, and, on the other hand, of validation of the standardized method for the preparation of biological samples and their investigation with SEM-EDS.

Throughout the research, with particular regard to the early stages of collection, transport and necropsy, special attention was paid to the safety of operators directly exposed to contact with animal carcasses, potentially vehicle of diseases. In fact, the prevention and public health local authorities in the area involved by the study have put in place a health monitoring plan for the control of zoonoses (e.g.: leptospirosis and tularaemia).

The use of an appropriate capture system in the urban area of Casale Monferrato able to prevent dispersal of dead animal carcasses and poisonous substances into the environment, contributed to ensuring the safety of persons and non-target animals, indirectly involved in the early stages of the research program, as well as the protection of environment. In the Ekomille® traps, rodents, attracted by the smell of the natural feed in the upper corridor, are captured as soon as they try to eat, falling into a basement tank containing alcohol isopropyl solution which causes loss of consciousness and quick death, without undergoing undue suffering, in line with European Directives 86/609 and the most recent animal welfare recommendations, which take into account the index of humane killing that should be met by the various methods of rodent pest control. This procedure is a good alternative to anticoagulant rodenticides and adhesive systems, still widely used, which are now considered as non-humane methods, inducing late mortality and prolonged suffering and stress in rodents, as well as dispersion of animal carcasses and poisons into the environment, with a potential risk for human or other non-target animal health. In addition, isopropyl alcohol itself does not pose a substantial risk to the environment if used correctly, being less volatile than ethanol and readily biodegradable. Isopropyl alcohol was also found to be a good preservative for biological samples, slowing down the decomposition between the period of time in which the rat died and that in which lung portions were permanently fixed in formalin before paraffin embedding or chemically digested, without interfering with the final results obtained either at histology and with SEM-EDS. Results were well interpretable, in contrast to those obtain from samples of rats captured in Asti, using spring traps and sent to IZSPLVA after freezing within 24 hours of death of the rats, without any contact with isopropyl alcohol.

As far as the inorganic fibers detected in the rat lungs are concerned we can make several considerations concerning the fibers and tissue interactions, the dispersion source kinds, and the lung burden for different situation of exposure. Although asbestos fibers were detected by SEM-EDS in more rats, the histological results on the lungs examined revealed typical lesions (asbestos bodies) only in those that underwent 3 months' exposure to asbestos grunerite fibers, under the experimental conditions of a subchronic inhalation study. It is not possible to establish whether the cause of this is the greater amount of asbestos fibers in the

lung tissue itself or a greater propensity of asbestos grunerite fibers to form corpuscles, compared to other asbestos. However, in the final analysis, these results indicate that asbestos bodies alone do not provide a suitable tool to assess asbestos exposure, and this is in line with previous published works ([Karjalainen et al., 1996](#)).

Crocidolite certainly derived from anthropogenic sources, i.e. asbestos-containing materials (ACM), not being contained in Italian rocks (natural sources). As concerns chrysotile asbestos, in Italy it can be both from natural and anthropogenic sources. In fact it was used, for example, in ACM making in the Casale Monferrato Eternit plant. The tremolite/actinolite asbestos, detected in two out of three sites, could instead, come only from natural sources. The samples from Asti, used as an environmental control, did not show a content of asbestos from anthropogenic sources, but tremolite/actinolite asbestos fibers were detected, as also found in a study on cow lungs ([Belluso et al., 2006](#)). The lung burden of these fibers is similar to that detected in the rats from Casale Monferrato town. Owing to the abundance of non-asbestos inorganic fibers detected in the Asti rat lungs, the total amount of fibers was comparable to that obtained from most of the Casale Monferrato wild rats. The other samples listed in [Table 2](#), shown for comparative purposes are from literature data and refer to asbestos and an uncertain asbestos (fibrous serpentine) group detected in different animals' lungs from various sites: goats from Corsica (France), pets (cats and dogs) from California (USA) and cows from Piedmont Region (Susa Valley). Although it is impossible to compare the concentration of asbestos fibers in the lung tissue of different animal species, due to physiological and anatomical variations and differences in lifespan, however we always confirmed its presence when asbestos was also detected in the environment, both from natural and anthropogenic sources. The data obtained from animals living in areas where rocks with a high amount of asbestos tremolite/actinolite outcrop revealed the content of these kinds of fibers is due to natural environmental exposure. In cases where they were sought, fibers shorter than 5 μm (sff) were also detected but in smaller amounts than others (longer than 5 μm , classified asbestos and breathable named: Bff). In Corsica and Susa Valley chrysotile was also detected, due to natural source dispersion (as likely the generically defined fibrous serpentine) and, for the latter site, also due to release from ACM. Among the fibers detected in cow lungs living in an anthropized area (Susa Valley) also crocidolite and grunerite asbestos were detected, although in low amounts. On the basis of all the data presented, asbestos both from natural and anthropogenic sources is present in the Casale Monferrato rats, there being chrysotile, i.e. asbestos from both sources, in greater amounts than crocidolite (As) and tremolite/actinolite asbestos (Ns). As regards the asbestos from a certain anthropogenic source, i.e. crocidolite, the samples contain higher amounts than those Susa Valley samples, and its presence indicates a likely dispersion source in Casale Monferrato town.

In conclusion, measurement of the lung fiber burden in wild rats has proved to be feasible, allowing not only to detect asbestos fibers, but also to characterize them quantitatively and qualitatively, providing thus the rationale for the use of these animals as sentinels of the environmental asbestos contamination of soil in Casale Monferrato.

As it can be observed in Table 2, asbestos fibers aren't detected in every sample of each area (e.g. in three of the areas only one of three rats had detectable chrysotile fibres). This result highlights the investigation needs of more than one sample for each area to evaluate the presence or absence of the looked for pollutant, inorganic fibers in the present research. Infact one of the target of the pilot study was the comprehension of the amount of samples (rats) useful to obtain data.

References

Abraham, J.L., Case, B.W., Burnett B.R., et al., 2005. Lung-retained fibre in animal lungs confirms environmental exposure to naturally-occurring amphibole asbestos in El Dorado Country, California. Am. Thoracic Soc meeting, San Diego, May 2005.

Bartrip P.W.G., 2004. History of asbestos related disease. *Postgrad Med J* 80:72-76

Bellmann B., Muhle H., Ernst H., et al., 2002. Subchronic studies on man-made vitreous fibres: kinetics of inhaled particles. *Ann. Occup. Hyg.* 46 (Suppl. 1): 166-169

Belluso, E., Bellis, D., Bruni B.M., et al., 2006. Studio preliminare sulla valutazione indiretta delle fibre inorganiche respirabili aerodisperse in Valle di Susa (Piemonte - Alpi Occidentali). *Rend. Soc. Geol. It.*, 3, 41-45

Brown, R.C., Bellmann, B., Muhle, H., et al., 2002. Subchronic studies: toxicity results. *Ann. Occup. Hyg.* 46 (Suppl. 1): 102-104

Ceruti, R., Ghisleni, G., Ferretti, E., et al. 2002. Wild rats as monitors of environmental lead contamination in the urban area of Milan, Italy. *Environmental Pollution* 117: 255–259

- Cooke, W.E., 1924. Fibrosis of the lungs due to the inhalation of asbestos dust. *Br Med J* 2(3317): 140-2, 147
- Cooke, W.E., 1927. Pulmonary asbestosis. *Br Med J.* 2(3491): 1024-1025
- De Vuyst, P., Karjalainen, A., Dumortier, P., et al., 1998. Guidelines for mineral fibre analyses in biological samples: report of the ERS Working Group. *Eur Respir J* 1998; 11: 1416–1426
- Doll, R., 1955. Mortality from lung cancer in asbestos workers. *Brit. J. industr. Med.*, 12, 81-86
- Doungchawee, G., Khoaprasert, Y., Kongtim, S. et al., 2002. Use of Wild Rodents for Environmental Monitoring - Comparison of Rats in Bangkok and Rural Areas of Thailand. *Asian Pacific J Cancer Prevention* 3: 367-368
- Dumortier P., Rey F., Viallant J.R., et al., 2002. Chrysotile and tremolite asbestos fibres in the lungs and parietal pleura of Corsican goats. *Occup. Environ. Med.* 59, 643-646.
- Ferrante, D., Bertolotti, M., Todesco, A. et al., 2007. Cancer mortality and incidence of mesothelioma in a cohort of wives of asbestos workers in Casale Monferrato, Italy. *Environmental Health Perspectives* 115(10): 1401-1405
- Fornero E., Belluso E., Capella S., et al., 2009. Environmental exposure to asbestos and other inorganic fibres using animal lung model. *Science of the Total Environment*, 407, 1010-1018
- Glickman L.T., Domanski L.M., Maguire T.G., et al., 1983. Mesothelioma in pet dogs associated with exposure of their owners to asbestos. *Environ. Res.* 32, 305-313
- Karjalainen, A., Nurminen, M., Vanhala, E. et al., 1996. Pulmonary asbestos bodies and asbestos fibres as indicators of exposure. *Scand. J. Work Environ. Health* 22: 34-38
- Magnani, C., Ferrante, D., Barone-Adesi, F., et al., 2008. Cancer risk after cessation of asbestos exposure: a cohort study of Italian asbestos cement workers. *Occup Environ Med.* 65 (3): 164-70
- Maule, M., Magnani, C., Dalmaso, P., et al., 2007. Modeling Mesothelioma Risk Associated with Environmental Asbestos Exposure. *Environ Health Perspect.*, 115 (7): 1066–1071

Nishikawa, K., Takahashi, K., Karjalainen, A., et al., 2008. Recent Mortality from Pleural Mesothelioma, Historical Patterns of Asbestos Use, and Adoption of Bans: A Global Assessment. *Environ Health Perspect.* 116, 12, 1675-1680

Oliver, T., 1925. Some dusty occupations and their effects upon the lungs. *Journal of the Royal Society of Promotion of Health*, 46 (6): 224-230

The Mesothelioma Center, December 2012, Asbestos and Mesothelioma Around the World. Available at: www.asbestos.com/mesothelioma/related-issues.php

Van der Schalie W.H., Gardner H.S., Bantle J.A., et al., 1999. Animals as sentinels of human health hazards of environmental chemicals. *Environ. Health perspec.*, 107, 4, 309-315

Wagner, J.C., Sleggs, C.A., Marchand, P., 1960. Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. *Br J Ind Med* 17: 260-271

FIGURE CAPTIONS

Figure 1. Schematical phases of the pilot study:

- 1) Rodents capture and transport to the IZSPLVA laboratories;
- 2) PCR investigation
- 3) Histology investigation
- 4) SEM-EDS investigation at the Department of Heart Sciences

Figure 2. Histology of the lung:

asbestos bodies in alveolar walls (Perl's staining).

Figure 3. Electron SEM image:

- A) chrysotile fiber bundle (900 M)
- B) crocidolite fiber (2000 M)
- C) TiO₂ fiber (2000 M)
- D) asbestos tremolite fiber (2000 M)
- E) asbestos grunerite fibers (400 M)

Figure 4. EDS-SEM spectrum:

- A) chrysotile (Na and Cl represent a residue of chemical digestion)
- B) crocidolite
- C) TiO₂ (Na and Cl represent a residue of chemical digestion)
- D) asbestos tremolite (Na and Cl represent a residue of chemical digestion)
- E) asbestos grunerite

Fig 1

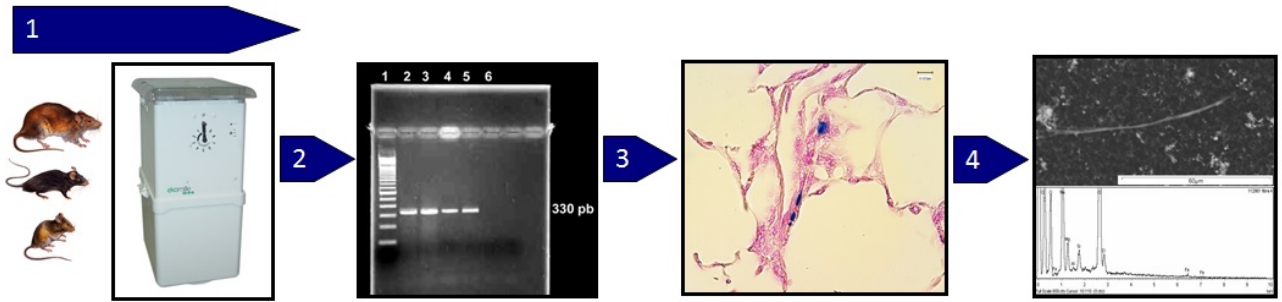


Fig 2

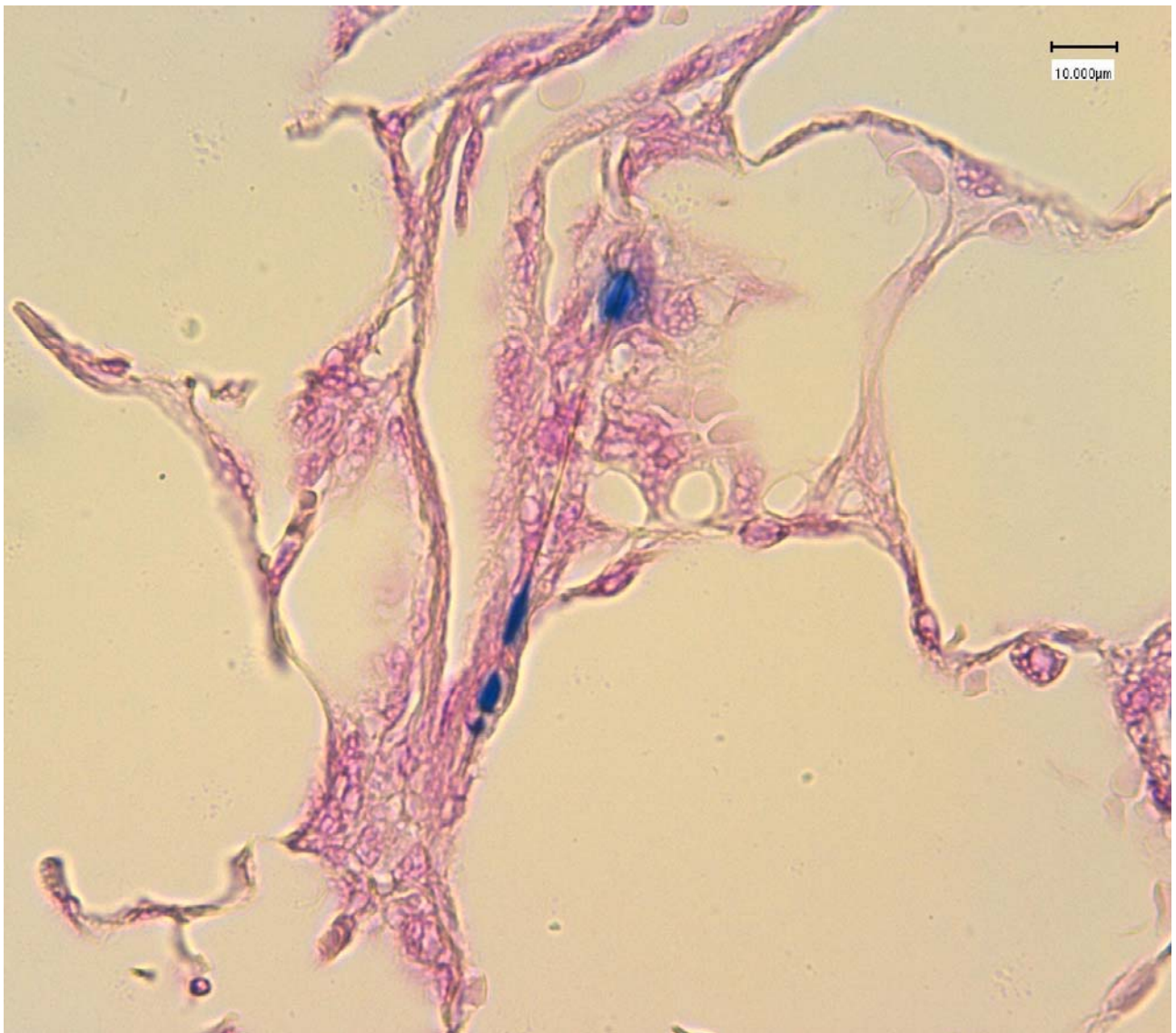
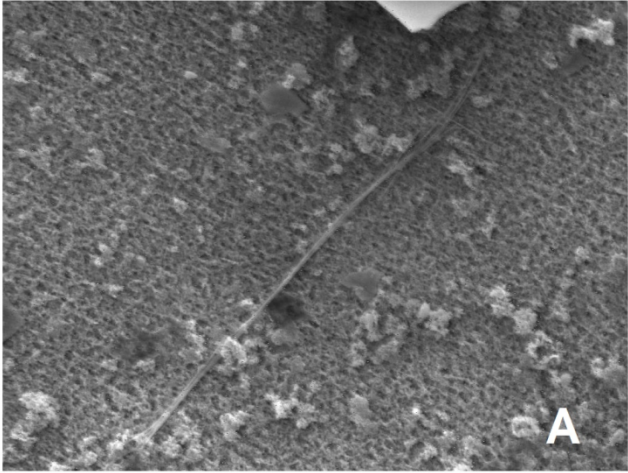
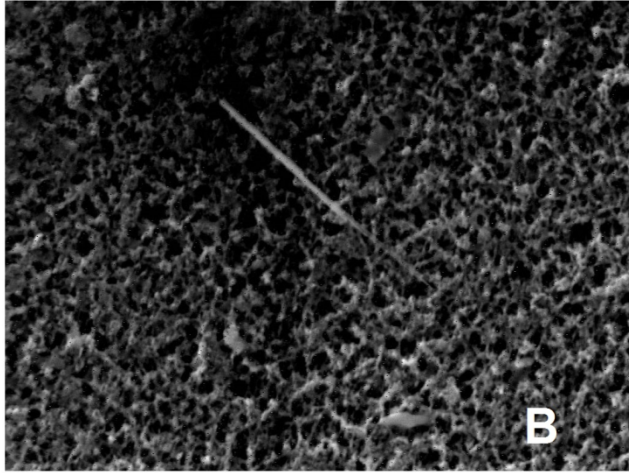


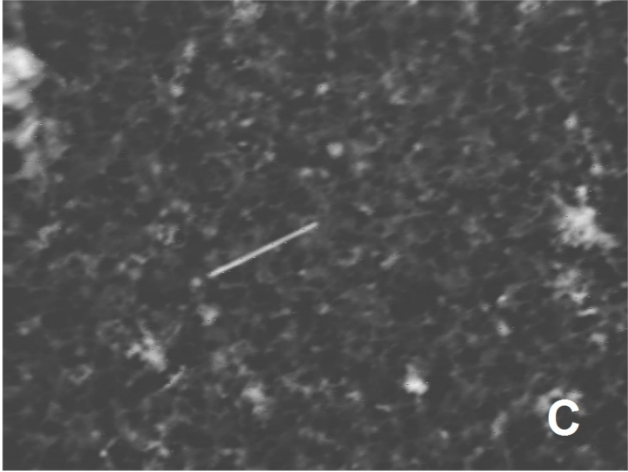
Fig 3



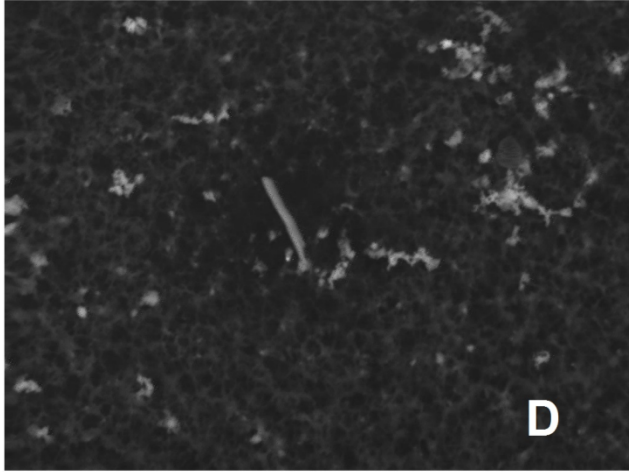
70 micron



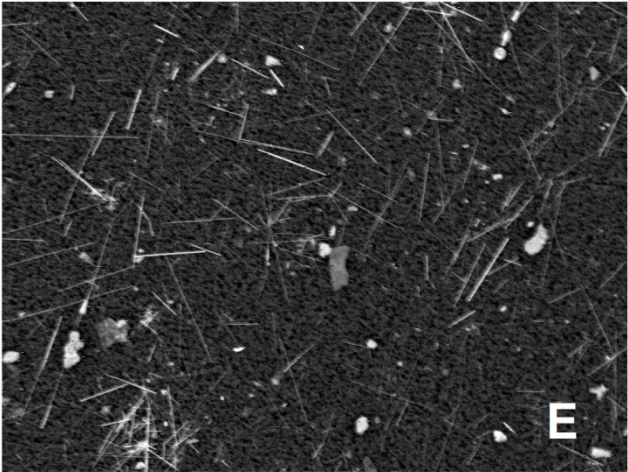
30 micron



30 micron



30 micron



100 micron

Fig 4

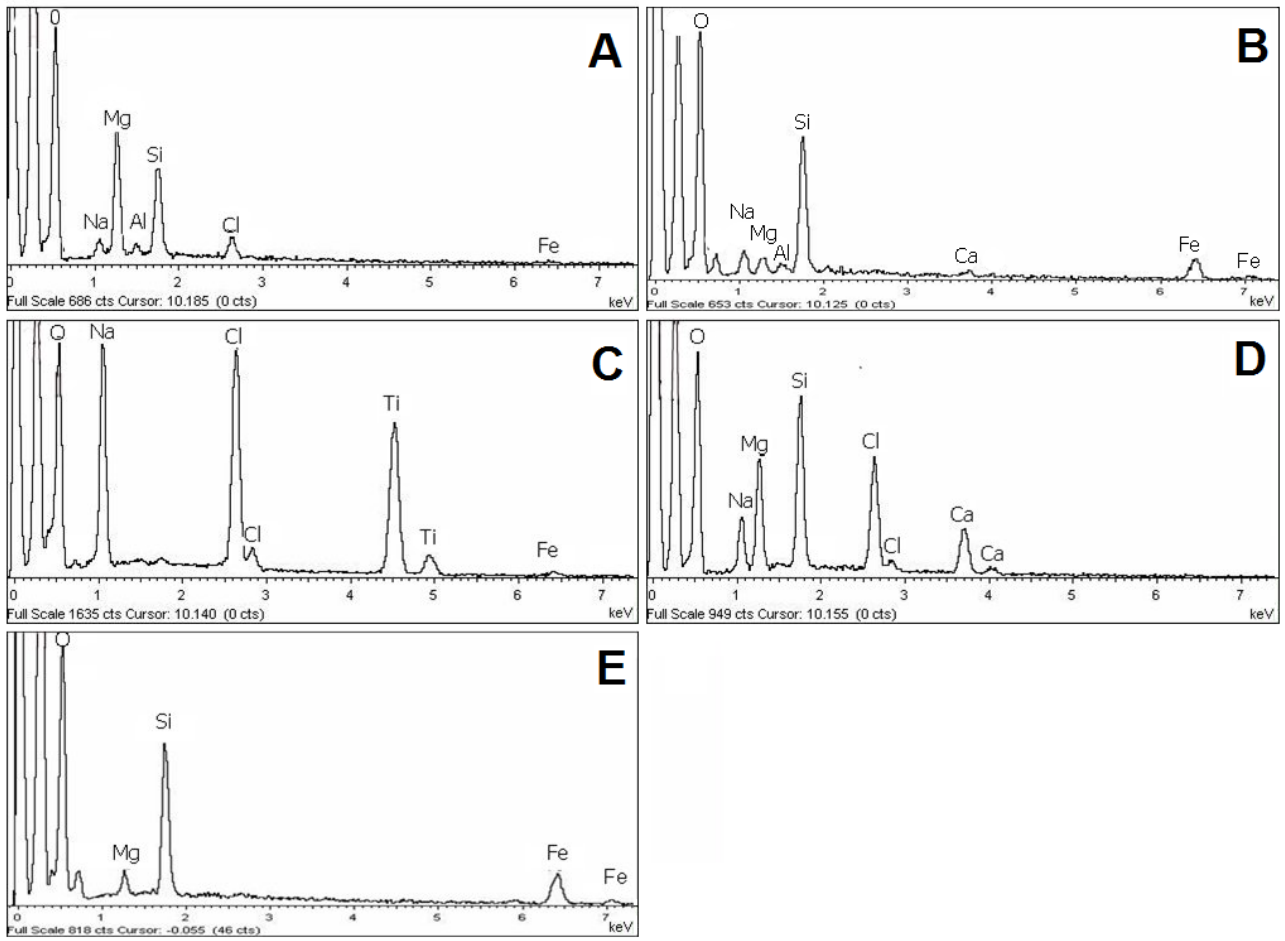


Table 1: synoptic table showing the study design

	Samples	Provenance	Rat Sex	Rat weight (g)	Sample for histology	Sample for SEM-EDS
WILD RATS	CM 107959/1	Casale Monferrato town Site 1	F	110	Left lung	Right lung
	CM 107959/2		F	110	Left lung	Right lung
	CM 107959/3		F	115	Left lung	Right lung
	CM 112966/a	Casale Monferrato town Site 2	M	260	Left lung	Right lung
	CM 112966/b		M	295	Left lung	Right lung
	CM 112966/c		M	301	Left lung	Right lung
	CM 112961/1	Casale Monferrato town Site 3 ^a	F	281	n.e. ^c	Lungs ^c
	CM 112961/2		M	129	Left lung	Right lung
	CM 112961/3		F	202	Left lung	Right lung
	CM 116053/1	Casale Monferrato town Site 3 ^b	M	164	n.e. ^c	Lungs ^c
CM 116053/2	M		187	n.e. ^c	Lungs ^c	
AT/01	Asti	F	337	Left lung*	Right lung	
AT/02		M	82	Left lung*	Right lung	
AT/03		F	208	Left lung*	Right lung	
LABORATORY RATS	FI-GE05/01	Fraunhofer Institute (Germany)	n.a.	n.a.	Residual lungs ^d	Residual lungs ^d
	FI-GE05/02		n.a.	n.a.	n.e. ^e	Residual lungs ^d
	FI-GE05/03		n.a.	n.a.	n.e. ^e	Residual lungs ^d
	CR-I/1	Charles River Laboratories (Italy)	M	300	n.e. ^c	Lungs ^c
	CR-I/2		M	325	n.e. ^c	Lungs ^c
	CR-I/3		F	275	n.e. ^c	Lungs ^c

Legenda:

CM Site 1: private courtyard in Via Lanza, Casale Monferrato

CM Site 2: Trevigi Institute in Via Alessandria, Casale Monferrato

CM Site 3: private courtyard in Strada San Giorgio, Casale Monferrato (^a and ^b refer to two separate shipments over a period of a week)

^c lungs directly undergone preparation for SEM-EDS

^d residual of lungs were examined since these samples gently provided by the Fraunhofer Institute were what was left available by the previous chronic study (Bellmann et al., 2002, Brown et al., 2002)

^e amount of tissue available was not enough for histology evaluation

n.a.: not applicable

n.e.: not examined (see ^c and ^e)

***:** severe autolysis

Table 2: Concentration (ff/gdw) of asbestos and uncertain asbestos (asbestos/non asbestos) inorganic fibres differentiated according to their chemical composition, kind of source, and size

SAMPLES	ASBESTOS								ASBESTOS/NON ASBESTOS	
	asbestos from As				asbestos from As/Ns		asbestos from Ns		fibrous serpentine (ff/gdw)	
	crocidolite (ff/gdw)		asbestos grunerite (ff/gdw)		chrysotile (ff/gdw)		asbestos tremolite/ actinolite (ff/gdw)			
	Bff	sff	Bff	sff	Bff	sff	Bff	sff	Bff	sff
CM 107959/1	0	0	0	0	0	0	0	0	0	0
CM 107959/2	0	0	0	0	0	0	9268	0	0	0
CM 107959/3	0	0	0	0	13902	0	0	0	0	0
CM 112966/a	4541	0	0	0	0	0	0	0	0	4541
CM 112966/b	0	0	0	0	0	0	0	0	18164	0
CM 112966/c	0	0	0	0	9082	0	0	0	9082	0
CM 112961/1	0	0	0	0	0	0	0	0	0	0
CM 112961/2	0	0	0	0	0	0	0	0	4.634	0
CM 112961/3	0	0	0	0	23170	0	4634	0	9268	0
CM 116053/1	4634	0	0	0	4634	0	9268	0	0	0
CM 116053/2	4634	0	0	0	13902	0	0	0	0	0
FI-GE05/01	0	0	110565298	12645298	0	0	0	0	0	0
FI-GE05/02	0	0	107647152	8105960	0	0	0	0	0	0
FI-GE05/04	0	0	100189669	11996821	0	0	0	0	0	0
CR-I /1	0	0	0	0	0	0	0	0	0	0
CR-I /2	0	0	0	0	0	0	0	0	0	0
CR-I /3	0	0	0	0	0	0	0	0	0	0
AT/01	0	0	0	0	0	0	10928	0	0	0
CO ¹	0	0	0	0	109000	13000	101000	40000	<i>n.c.</i>	<i>n.c.</i>
CA ¹	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>	121500	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>
CA ²	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>	2976500	<i>n.c.</i>	<i>n.c.</i>	<i>n.c.</i>
SV ³	605	0	1112	0	2836	408	30741	2448	4317	136

Legenda:

CO¹ Corsica, France: average burden of asbestos tremolite fibers in the lungs and parietal pleura of goats (Dumortier et al., 2002)

CA¹ El Dorado Country, California: average burden of asbestos tremolite/actinolite lung-retained fibres in cats (Abraham et al., 2005).

CA² El Dorado Country, California: average burden of asbestos tremolite/actinolite lung-retained fibers in dogs (Abraham et al., 2005).

SV³ Susa Valley, North-Western Italy: average burden of crocidolite lung-retained fibers in cows (Capella et al., 2013 submitted to Environmental Monitoring and Assessment)

ff/gdw = number of fibers per gram of dry weight

As = Anthropogenic source

Ns = Natural source

n.c. = not considered by Abraham et al. (2005)

Bff = breathable fibers (length > 5 µm; width < 3 µm; length/width ratio > 3)

sff = short fibers (length < 5 µm; width < 3 µm; length/width ratio > 3)

Table 3: Concentration (ff/gdw) of non asbestos inorganic fibers differentiated according to their chemical composition, kind of source, and size

SAMPLES	NON ASBESTOS INORGANIC FIBERS											
	metallic oxides (ff/gdw)		Al-silicates (ff/gdw)		vitreous silicates (ff/gdw)		Ca,Ti-silicates (ff/gdw)		phyllosilicates [§] (ff/gdw)		n.i. (ff/gdw)	
	Bff	sff	Bff	sff	Bff	sff	Bff	sff	Bff	sff	Bff	
CM 107959/1	0	0	18536	0	0	0	0	0	0	9268	0	0
CM 107959/2	4634	0	0	0	4634	0	0	0	13902	0	0	0
CM 107959/3	0	0	4634	0	4634	0	0	0	0	0	0	0
CM 112966/a	0	4541	0	0	9082	0	0	0	13623	9082	0	0
CM 112966/b	18164	0	0	0	0	0	0	0	0	0	0	0
CM 112966/c	0	0	0	0	4541	0	0	0	13623	0	0	0
CM 112961/1	4634	0	9267	0	4634	0	4634	0	0	4634	0	0
CM 112961/2	13902	0	0	0	0	0	0	0	4634	0	0	0
CM 112961/3	50974	4634	0	0	0	0	0	0	0	0	0	0
CM 116053/1	4634	0	0	0	0	0	0	0	4634	0	4634	0
CM 116053/2	0	0	0	0	4634	0	0	0	4634	0	0	0
CR-I /1	0	0	0	0	0	0	0	0	0	0	0	0
CR-I /2	0	0	0	0	0	0	0	0	0	0	0	0
CR-I /3	0	0	0	0	0	0	0	0	0	0	0	0
AT/01	16392	10928	0	0	0	0	5464	0	5464	0	0	0

Legenda:

ff/gdw = number of fibers per gram of dry weight

n.i. = not identified

§ excluding asbestos chrysotile and asbestiform antigorite (see Tab. 2)

Bff = breathable fibers (length > 5 µm; width < 3 µm; length/width ratio > 3)

sff = short fibers (length < 5 µm; width < 3 µm; length/width ratio > 3)