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PRIN 2017 *Fibres - A Multidisciplinary Mineralogical, Crystal-Chemical and Biological Project.* What have we learned after four years of research?

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ABSTRACT

This opening paper introduces the contributions of this special issue on mineral fibres and reports a gallery of the major results accomplished within the multidisciplinary project PRIN (PROGETTI DI RICERCA DI RILEVANTE INTERESSE NAZIONALE) 2017 "*FIBRES: a multidisciplinary mineralogical, crystal-chemical and biological project to amend the paradigm of toxicity and cancerogenicity of mineral fibres*" by the six different Research Units from the Universities of Ancona, Genova, Modena, Rome, Pisa-Parma, and Urbino. The main goal of the project was to increase the knowledge of the mechanisms by which mineral fibres, with special attention to asbestos and fibrous erionite, prompt adverse effects *in vivo*, linking the fibres' crystal-chemical-physical parameters to their toxicity/carcinogenicity potential and recasting the existing mechanistic 'fibre toxicity paradigms'. This special issue contains specific contributions from each Research Unit of the project. The implications of the findings of the project are beyond the advance of the knowledge in the world of mineralogy/crystallography and constitute a remarkable progress in the understanding of the biological activity of mineral fibres *in vivo*.

BACKGROUND

Numerous minerals display a fibrous crystal habit. Among them, the "asbestos" species and fibrous/asbestiform erionite are certainly considered the most hazardous ones (Gualtieri, 2017, 2023). The generic term "asbestos" specifically refers to six silicate mineral species: chrysotile (a member of the serpentine group) and five asbestiform amphibole species: actinolite asbestos, amosite, anthophyllite asbestos, crocidolite and tremolite asbestos (IARC, 2012). Erionite is a natural fibrous zeolite (Gottardi and Galli, 2012). All these fibres are regulated and classified by the International Agency for Research on Cancer (IARC) as "carcinogenic to humans (Group 1)" (IARC, 2012).

The PRIN (PROGETTI DI RICERCA DI RILEVANTE INTERESSE NAZIONALE) 2017 "*FIBRES: a multidisciplinary mineralogical, crystal-chemical and biological project to amend the paradigm of toxicity and cancerogenicity of mineral fibres*" was funded in 2018 and operations began in 2019. The multidisciplinary research team includes six different Italian Research Units from the Universities of Ancona, Genova, Modena, Rome, Pisa-Parma, and Urbino. The aim of the project is to contribute to unravelling the mechanisms by which mineral fibres, with special attention to asbestos and fibrous erionite, promote adverse effects *in vivo*, linking the fibres' crystal-chemical-physical parameters to their toxicity/carcinogenicity potential and recasting the existing mechanistic 'fibre toxicity paradigms'. Specifically, the project is focused to better understanding the role of active surface iron, biodurability, the 'Trojan horse effect' (cellular release of ion toxic cargo from the dissolving fibres), and ion exchange of the erionite zeolite fibres.

The implications of the outcome of the project are far beyond the simple advancement of the knowledge in the world of mineralogy/crystallography. Each single scientific data collected and published on these regulated hazardous fibres may have remarkable health, social, juridical and economic relevance. For this reason, research in this field is subject to strict control, filtering and sometimes even to some sort of surreptitious censorship. Two fronts facing each other are responsible for a conflicting divisive global scenario. One side assumes that all the regulated asbestos fibres are toxic and carcinogenic and asks their ban worldwide (see Ramazzini Collegium, 1999). The other side claims that only the five amphibole asbestos species are toxic and carcinogenic while chrysotile is carcinogenic but with low to null toxicity and low potential for inducing mesothelioma (Bernstein et al., 2013). The latter position enables the countries mining chrysotile and manufacturing chrysotile-containing materials to support the safe ("controlled"; LaDou et al., 2010) use of chrysotile. The result is that only 69 out of 196 countries (35%) in the world have banned all asbestos minerals whereas a "controlled use" of chrysotile is permitted in the remaining (65%) countries (International Ban Asbestos Secretariat, 2022).

To demonstrate the complexity and strife that characterize this field of research, we can say that there is not even agreement on the basic definitions pertaining to mineral fibres. In the years of this project, two authors (AFG and GDV) were involved in the activity of the International Mineralogical Association (IMA) Working Group on "*Asbestos, asbestiform minerals, and other respirable minerals that pose potential negative health risks*". The Working Group was officially nominated during the IMA Congress in Melbourne (Australia) on August 2018 with A.G. Wylie as Chair, AFG as Vice-Chair and B. Bandli as Secretary. After four years of intense work, the group was unable to come to agreement given the polarization of views on several key definitions. Hence, in February 2022 the Chair decided that it was time to vote and a set of definitions that had been the focus of the discussion was formally proposed to the membership. The subsequent vote was 8 in favour, 3 opposed, 1 abstains, and 1 nonvoting. For the benefit of the reader, we quote here the list of definitions in hierarchical order approved by the majority of the IMA Working Group:

Elongate Mineral Particle: Any mineral particle with a length:width ratio (aspect ratio) of 3:1 or greater assuming the width of a particle to be an apparent parameter defined as the longest dimension of the particle in the plane perpendicular to length and the shortest dimension of the two-dimensional outline of a particle.

Mineral fibre: Any elongate mineral particle that attained its shape during formation in nature with an aspect ratio sufficiently large to impart flexibility to the particle.

Fibril: A single crystal of mineral fibre which cannot be further separated longitudinally into smaller components.

Cleavage fragment: Any elongate mineral particle formed by fragmentation. It may have the same chemical formula of the fibrous or asbestiform variety but it is brittle and cannot separate longitudinally into fibres or fibrils.

Fibrous: The crystal habit that describes particles having the appearance of a mineral fibre.

Asbestiform: The crystal habit displayed by elongate mineral particles composed of bundles readily separable into fibres or fibrils which are aligned parallel to their common fibre axis direction but randomly or semi-randomly in the directions perpendicular. Both macroscopically and microscopically, the fibre bundles can display frayed/splayed ends and can be flexible and bent.

Asbestos: A generic term applied to the asbestiform variety of serpentine (chrysotile) and the asbestiform variety of amphibole group minerals (anthophyllite, cummingtonite-grunerite, tremolite-actinolite and riebeckite), which have been exploited, prospected, described in the literature, traded and sold commercially for their unique physical properties resulting from fibril dimension 0.5 µm or smaller in width.

The dissenting members within the Working Group re-mark that a number of issues has not been properly addressed and remains open. For example, the requirement of observable flexibility in the definition of "mineral fibre" is problematic for many reasons and should be better defined or removed. The dissenting members also believe that both fibrous and asbestiform habits should be included in the definition of "asbestos" as during their experience with mineral fibres they observed many "asbestos" fibres apparently with an aspect ratio not great enough to impart flexibility to the particle. Dissenting members of the group doubt the new set of definitions will remove ambiguity, except potentially to eliminate fibres which have until now been called "asbestos". An attempt to define in a more rigorous way the properties of asbestos fibres was made to include a detailed description of the optical, mineralogical, chemical and physical properties. Unfortunately, this revision strategy was not accepted by other members of the group who felt it is important to keep definitions and analytical protocols distinct.

In this conflicting global framework, the contribution of the scientists working in this field should provide solid evidences and models that enable them to deliver objective conclusions free from any subjective interpretation and manipulation. In this way, the discussion remains on the scientific table and legal/political decisions are based only on objective scientific data. Along this line, the project is aimed at understanding the role of key fibres' parameters like iron, biodurability and ion exchange that are still not well understood and prevent to draw a conclusive picture on the toxicity and carcinogenicity of mineral fibres.

All these premises done, this introductory opens this special issue on mineral fibres and reports selected major contribution of each Research Unit to the project and the relevance of these findings to the knowledge for the understanding of the toxicity and carcinogenicity potential of mineral fibres.

CONTRIBUTION OF THE PROJECT TO THE PROGRESS OF KNOWLEDGE OF MINERAL FIBRES - MAJOR RESULTS

The Ancona Research Unit

The Research Unit of Ancona worked on the biological interaction of mineral fibres and tested chrysotile toxicity on two barriers fundamental for survival during foetal and adult life i.e., maternal-foetal barrier and respiratory lung barrier. Human placenta is a transient organ that acts as a barrier regulating the transfer of nutrients and oxygen between the mother and the *fetus* (Fantone et al., 2022). Moreover, the placental barrier ensures the detoxification and efflux of xenobiotics thanks to enzymes and transporters localized in the outer layer of the placenta (Burton and Fowden, 2015). Some studies have shown that environmental chemicals and pollutants, promote pregnancy complications disturbing the establishment of the uteroplacental interface (Deval et al., 2021; Meakin et al., 2023). Among environmental pollutants, asbestos fibres have been found in the human placenta suggesting that these fibres may cross (translocation) the maternal-foetal barrier and reach the *fetus* (Cunningham and Pontefract, 1974) as shown in Figure 1 (modified after msdmanuals.com/).

The respiratory barrier (LeMessurier et al., 2020) is a peculiar structure that can activate physical and cellular strategies in the mucosal surface owing to separate the external environment from the internal soft tissue, and to obstruct microbe and particulate entry. The pulmonary epithelium is composed of two major cell types, Type I and Type II pneumocytes. Type I cells cover the majority of the surface area of the alveolus while Type II cells cover less alveolar surface, are more numerous and are also believed to be the progenitor cells for Type I pulmonary epithelial cells (Foster et al., 1998). In addition, the pleura is lined by mesothelium that is a membrane composed of simple squamous epithelial cells of mesodermal origin. This tissue can become abnormal and divide without control

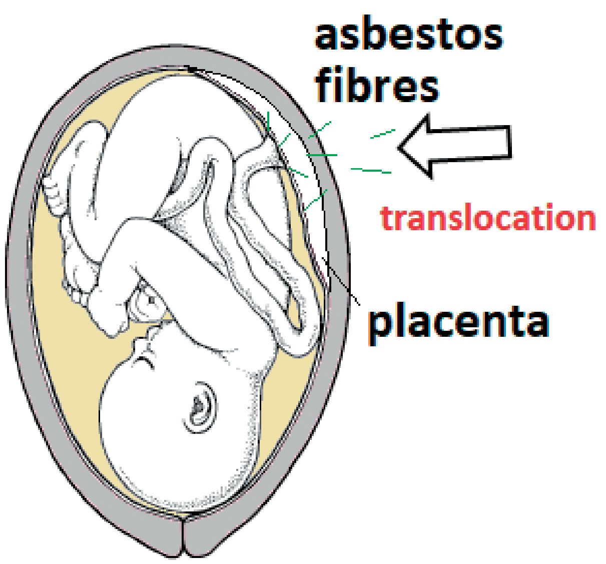

Figure 1. A sketch picture of the translocation of asbestos fibres into the placenta (modified after a picture taken from msdmanuals.com/).

under asbestos exposure and develop mesothelioma which is the most common cancers in people living in areas with a high rate of respirable mineral fibres (Noonan, 2017).

The research unit evaluated the cytotoxicity of mineral fibres in *in vitro* model of syncytiotrophoblast using BeWo cell line, a human choriocarcinoma cell line; of type II pneumocytes using A549 alveolar type 2 epithelial cells that can be considered representative of non-small lung cancer cells (NSLC) and of MeT5A that are a useful model of mesothelioma. These cell lines are actually considered as a good *in vitro* system to investigate asbestos cytotoxic effects.

In vitro cytotoxicity damage was induced by treating BeWo, A549 and MeT5A cells with the different mineral fibres mentioned above at 50 µg/ml that is the concentration considered the best for obtaining reliable data. The cytotoxic effect was evaluated through the MTT colorimetric assay after treating cells with chrysotile, crocidolite, and wollastonite at 24 and 48h. The MTT assay is a spectrophotometric test to evaluate cell viability as verify through mitochondrial succinate dehydrogenase enzyme activity as a consequence of mitochondrial functional state. The results of this work have been submitted for publication.

The Genova Research Unit

The Genova research unit was committed to develop advanced *in vitro* models based on the human alveolar A549 adenocarcinoma, the human THP-1 monocyte and the human HECV endothelial cell lines in single (2D) and organotypic co-culture systems (3D) to gain insight into the mechanisms of toxicity/carcinogenicity of mineral fibres in experimental settings closer to the *in vivo* conditions. The aim was to reproduce the pulmonary alveolar environment. In particular, by the use of these models, the unit focused on the assessment of the perturbation of several biological parameters indicated by the IARC as key factors for the classification of carcinogens (Smith et al., 2016), which were: genotoxicity, oxidative stress, chronic inflammation, alteration of cell proliferation and cytotoxicity. In the first part of the study the following mineral fibres were studied: UICC crocidolite, chrysotile from an Italian mine (Balangero, Italy) and erionite (from Jersey, Nevada).

Initially, to verify the role of naïve human monocytes in early response to fibre exposure, THP-1 monocytic-like cells were submitted to indirect or direct contact, up to 72 h. The indirect exposure was performed by use of a semipermeable porous membrane (0.4 µm) into an insert containing the fibre suspension. In this way, it was possible to investigate the role of metal ions released by the fibres in the cell culture medium. Only chrysotile and erionite revealed a significant release of metal ions, such as Mg, Cr, Ni and Co into the culture media within 24 h in an acellular test (Mirata, 2023). The effects on THP-1 cells during indirect exposure showed that chrysotile affected the cell proliferation rate and promoted the activation of NF-kB and the stress response. However, all the three fibres affected THP-1 viability, increased oxidative stress, and induced genotoxic damage (Mirata, 2023). Similar tests, but in a direct contact experimental setting, were performed with both human naïve THP-1 monocytes and A549 alveolar cells to highlight the contribute to toxicity of membrane-fibre direct interaction. The toxic effect, as well as the oxidative and inflammatory stresses were further exacerbated by the three mineral fibres, with respect to the indirect contact, especially by the chrysotile treatment. Furthermore, direct fibre exposure, as well as stimulation by fibre-treated A549 alveolar cells or THP-1 M0 macrophages conditioned media significantly stimulated naïve THP-1 monocytes towards macrophage maturation, suggesting mechanisms of chronicization of the inflammatory process induced by the mineral fibres in the lung.

To gain insights into the different toxicity mechanisms of mineral fibres, the three M0, M1 and M2 macrophage phenotypes obtained from THP-1 differentiation, were investigated (Mirata et al., 2022). The three mineral fibres apparently acted by different toxicity mechanisms. From these experiments we highlighted that crocidolite seemed to exert its toxicity effects mostly thanks to its biodurability, ROS and cytokine production, and DNA damage. Chrysotile, due to its low biodurability, displayed toxic effects related to the release of toxic metals, ROS and cytokine production. Conversely, in the toxicity of biodurable fibrous erionite, the cation exchange capacity of this mineral, able to alter the intracellular homeostasis of important cations, seemed to play a major role.

During the second part of the project the analyses were focused on the early biological effects of a Russian commercial chrysotile fibre (Yasniy mine), which was compared to UICC crocidolite and NYAD G wollastonite as positive and negative carcinogenic. This type of chrysotile was used at two different lengths (≤5 labelled CHR-S and >5 µm labelled CHR-L) separated through cryogenic milling by the research unit of Modena (Scognamiglio et al., 2021). Figure 2 reports the products of the size separation with the long chrysotile fibres CHR-L (mean L>5 µm) in (a) and the short chrysotile fibres CHR-S (mean L ≤5 µm) in (b).

After 24, 48 h and 7 d direct exposure, the HECV endothelial cells and THP-1 M0 macrophages, separately or in co-culture systems, revealed a significant degree to toxicity and a moderate oxidative stress, with CHR-L showing the highest effects on the two cell types. The toxicity mechanisms were different, with HECV cells

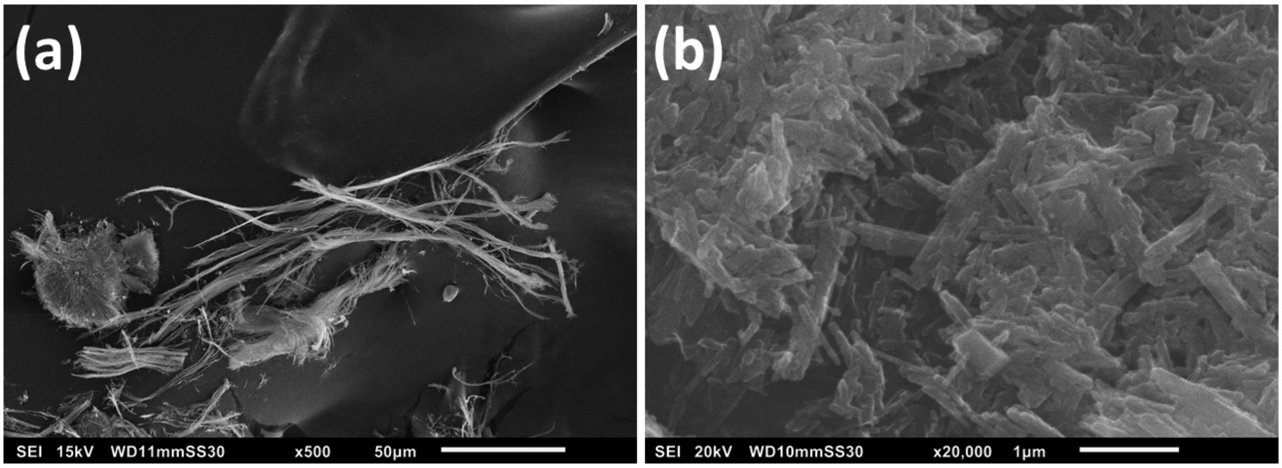

Figure 2. The Russian chrysotile fibres from Yasniy obtained by cryogenic milling (Scognamiglio et al., 2021). (a) the long chrysotile fibres CHR-L (mean L>5 μm); (b) the short chrysotile fibres CHR-S (mean L ≤5 μm).

dying primarily for direct membrane damage and THP-1 M0 macrophages for apoptosis mechanisms and only partially for membrane damage (Mirata, 2023). Furthermore, in both cell lines there was a trigger of the proinflammatory response by increasing several interleukins and chemokines production in the first 24-48 h, the majority going back to basal levels after 7 d in CHR-S, and some still remaining elevated in CHR-L treatment. Moreover, ɣ-H2AX staining, as DNA damage marker, revealed that CHR-L had more dramatic effects on cells as compared to CHR-S (Mirata, 2023). Furthermore, also human HFF2 fibroblasts exposed to conditioned media from fibre treated HECV cells showed a reduction of fibroblast viability index, especially with CHR-L treated HECV medium. The loss in the mitochondria membrane potential was verified by JC-1 dye and the results suggest the onset of apoptosis in fibroblasts treated with conditioned media from fibre treated HECV, and a sustained activity of the respiratory chain (Mirata, 2023).

Finally, the short- and long-term effects of the Russian chrysotile fibre at the two different lengths were analysed on an *in vitro* 3D respiratory epithelial model tissue named EpiAirway™ (MatTek Corporation, Ashland, MA) to better mimic the tracheobronchial tract. The effect of exposure to the fibrous minerals was monitored up to 12 d by viability testing, barrier integrity analysis by TEER measure, pro-inflammatory activation, and genotoxicity. Only short-duration fibre treatments affected cell viability and morphology and an early release of pro-inflammatory cytokines. In contrast, crocidolite, used as a positive control, maintained a high cellular response even for long times, confirming its characteristic as a biodurable fibre. This type of long-term study in an advanced *in vitro* model represents the first pioneering attempt to investigate the initiation and establishment of a chronic condition following inhalation of asbestos fibres. The results of this research line are illustrated in a dedicated paper appearing in this special issue.

The Modena Research Unit

The Research Unit of Modena focussed its activity on chrysotile in the attempt to shed light on its debated toxicity properties and help contributing to solve the global "chrysotile issue". The biodurability and biopersistence of chrysotile fibres has been investigated with a multidisciplinary approach in different environments (*in vitro* acellular media, *in vitro* cell cultures, human *in vivo* tissues) spanning different timeframes (acute, short term, long term/chronic).

In an *in vitro* acellular dissolution study (Gualtieri et al., 2019), it was confirmed that chrysotile is not a biodurable fibre as it reacts in a chemical environment mimicking the macrophage acidic phagolysosomes responsible for the intracellular chemical attack during alveolar macrophage phagocytosis. Dissolution takes place via Mg-leaching of the brucite octahedral sheet, leaving behind an amorphous silica disordered structure (Bales and Morgan, 1985; Gualtieri et al., 2019). Figure 3 (modified after Gualtieri et al., 2019) depicts the structures of undissolved chrysotile and the amorphous silica relict dissolved inside the phagolysosome vacuoles. Acid-leached chrysotile fibres produce free radicals, although the activity less intense with respect to pristine chrysotile. *In vitro* cell tests also show a significant cyto- and geno-toxicity of the dissolution product which releases toxic metals other than iron (Ni, Cr, V …) that prompt detrimental biochemical

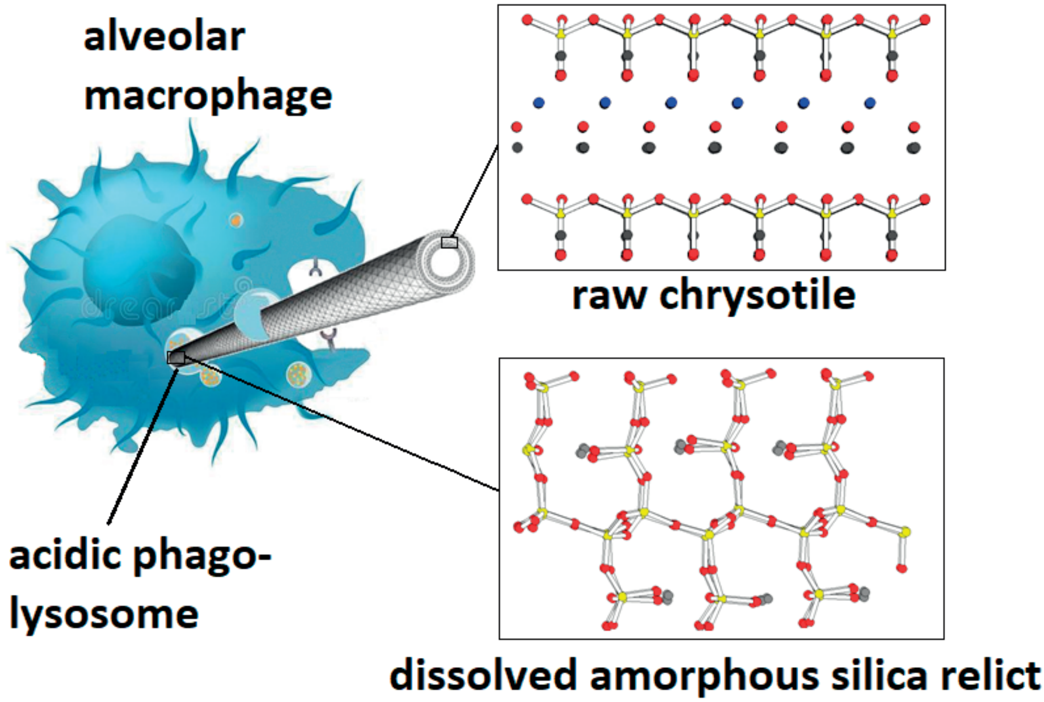

Figure 3. Sketch of the structures of undissolved raw chrysotile (top) and the amorphous silica relict which is the product of dissolution in contact with the phagolysosome sacs (bottom) in the cytosol of alveolar macrophages. Legend: yellow balls = Si atoms; blue balls = Mg atoms; dark balls = hydrogen atoms; red balls = oxygen atoms (modified after Gualtieri et al., 2019).

effects such as DNA damage, ROS production and oxidative stress. Amorphous silica relicts continue to provoke frustrated phagocytosis and cell disturbance and, although to a lower extent, a detrimental biochemical toxic action is also induced by this acid-leached product (Gualtieri et al., 2019).

The toxic effects of the dissolution of chrysotile have been confirmed by an *in vitro* cellular study of the acute toxicity (0 to 8 h) effects on different THP-1 macrophage phenotypes of mineral fibres observed by time-lapse video microscopy (Di Giuseppe et al., 2022; Mirata et al., 2022) conducted with the Research Units of Genova and Parma. In the acute-term study, breaks of chrysotile fibres were not observed but the low durability and the dissolution-induced "Trojan Horse effect" with the release of toxic elements are likely responsible for the acute toxic effects of chrysotile revealed by the TLM and LDH tests (Di Giuseppe et al., 2022).

It was also confirmed that chrysotile is not a biopersistent fibre in the long term by an *ex post* study of human tissues of a patient who died of malignant mesothelioma (Giacobbe et al., 2021). The subject was exposed in the work place to a blend of respirable chrysotile, amosite and crocidolite fibres, residing in his parietal pleura for about 40 y. Only few relicts of chrysotile fibres that were amorphous to diffraction and depleted in Mg (amorphous silica relicts) were found in the lung tissues. Mg-leaching of the chrysotile fibres was already observed in a long-term *ex post* FEG–SEM study of the morphological and chemical characteristics of fibres found in the tissues of Sprague–Dawley rats subjected to intraperitoneal or intrapleural injection of UICC chrysotile (Bursi Gandolfi et al., 2016) and confirmed previous observations (see for example Davis and Jones, 1988). During the same study, intact amphibole fibres were collected and, for first time, the crystal structure refinement of an undamaged amosite fibre was accomplished, demonstrating that the atomic structure of the fibre did not change during 40 y *in vivo* (Giacobbe et al., 2021). These results deliver undisputable evidence that amphibole asbestos fibres can be chemically stable at the atomic scale in the lungs for a lifetime whereas chrysotile asbestos progressively dissolves into an amorphous silica-rich product.

The Modena group in collaboration with the Research Unit of Pisa and Rome studied a representative commercial chrysotile from the Orenburg ore mine (Russia) (Di Giuseppe et al., 2021). The calculated fibre potential toxicity index (FPTI) (Gualtieri, 2018) of this Russian commercial chrysotile is lower than that of the amphibole asbestos but higher than the threshold limit set for "safe" mineral fibres. The same sample has been used to bring new experimental evidence to the *endless* debate on the toxicity of short chrysotile fibres, another open divisive issue where one side claims that short asbestos fibres including chrysotile are toxic and carcinogen (see for example Egilman and Tran, 2016) and the other side argues that epidemiology, toxicology, and *in vitro* studies demonstrate that chrysotile fibres with lengths generally <5 µm do not contribute to the health effects of asbestos (se for example, Bernstein, 2022). To this aim, representative batches of short (length ≤5 mm) and long (length >5 mm) Russian chrysotile fibres were prepared by cryogenic milling (Scognamiglio et al., 2021) and tested *in vitro*. Cryogenic milling allowed us to produce precise size fractions with invariant properties compared to the pristine fibres. Fibre length can be controlled by milling time under cryogenic conditions without inducing structural defects or amorphization; short fibres (95% L ≤5 mm) can be obtained by cryogenic milling for 40 min while a milling time of 10 min is sufficient to yield a sample mainly composed of long chrysotile fibres (90% L>5 mm).

The *in vitro* testing (cell vitality with the MTT assay and cell mortality with the LDH assay) using human monocyte THP1 and human endothelial HECV cell lines (fibre dose was 50 µg/ml; contact times were 24 and 48 h) evidenced a greater cytotoxic activity of the long chrysotile fibres compared to the short ones for both cell lines with a lower viability (or higher mortality) for cell cultures in contact with long (L>5µm) chrysotile fibres with respect to the short (L ≤5 µm) ones (Mirata, 2023). The detrimental biological activity of the long chrysotile fibres is invariably greater than that of the short chrysotile fibres. This result may be explained by the best efficiency of the phagocytic cells to engulf the short fibres compared to the long fibres. Phagocytes in contact with the long fibres undergo frustrated partial phagocytosis resulting in a significantly higher apoptotic cell death rate. Although significantly reduced in intensity, short chrysotile fibres also displayed an *in vitro* toxic potential (Mirata, 2023).

As proposed by Gualtieri (2021, 2023), the outcome of all these experiments under the umbrella of the PRIN 2017 project should contribute to devise a general model aimed at linking the fibres' crystal-chemical and physical parameters to their *in vitro* biological activity, adverse effects *in vivo* and consequently to the ten key characteristics of cancer (Smith et al., 2016).

The Pisa-Parma Research Unit

The Research Unit of Pisa-Parma was involved in the study of impurities in different mineral fibres, considered as asbestos or non-asbestos. The investigation was mainly performed by using micro-Raman spectroscopy as an effective and reliable technique to identify and recognize micrometric crystalline phases - especially Fe-rich phases interspersed within the mineral fibres and possible involved in the production of reactive oxygen species (Figure 4 a,b).

Furthermore, more in-depth analyses finalised to investigate the extent of metal release in *in vitro* cells-fibres systems were also carried out at Italian and European Synchrotron facilities. About the role of iron at the surface of the fibres, particular attention has been focused on detecting Fe-bearing impurities.

A detailed characterization was performed on chrysotile fibres from the Balangero mine (Turin, Italy), the largest asbestos mine in Western Europe, operating until 1990. Iron compounds associated with chrysotile fibres were identified with micro-Raman spectroscopy combined with SEM-EDS analysis (Fornasini et al., 2022). The presence of iron as a constitutive element was also highlighted in balangeroite fibres, detected in Balangero chrysotile. Antigorite lamellae were also observed associated with chrysotile fibres, pointing out the possible presence of Fe by substitution of Mg in the octahedral sheets. Fe-bearing impurities were recognized in micrometric crystals of Fe oxides, carbonates and sulphides. Magnetite was found as the most abundant compound, being known to be present in chrysotile asbestos and formed during the serpentinite evolution. Other Fe-bearing oxides and oxyhydroxides were identified as ilmenite, hematite and lepidocrocite. Besides, Fe-containing carbonates were also observed. The carbonate species does not correspond to pure Fe carbonate (siderite), as the detection of Mg - in addition to Fe - by the EDS analysis suggests the presence of a mixed Fe-Mg carbonate. Among Fe-rich species, Fe sulphides were also identified. The main species corresponds to mackinawite, observed in different forms: nano-mackinawite and partially oxidized mackinawite ($Fe(II)_{1-3x}Fe(III)_{2x}S$). Mackinawite may be considered as an intermediate product in the formation of Fe oxides and oxyhydroxides. The partial oxidation may occur during the transformation into greigite and then into elemental S, Fe(III)-oxyhydroxides and/or magnetite. A rarer sulphide was also observed, containing Ni in addition to Fe. In Balangero chrysotile, the presence of Cr as trace metals was highlighted by the photoluminescence peaks of Cr^{3+} and Cr-Cr pairs, detected in the Raman spectra acquired with the 632.8 nm excitation laser line. The micro-Raman characterization of chrysotile fibres was extended to the commercial chrysotile from the Orenburg ore mine (Russia), which was studied by the Research Units of Modena and Rome. As for the Balangero chrysotile, Cr as trace element was detected in chrysotile fibres and Fe-bearing micrometric phases were identified as Fe oxides and oxyhydroxides: magnetite mainly and akageneite.

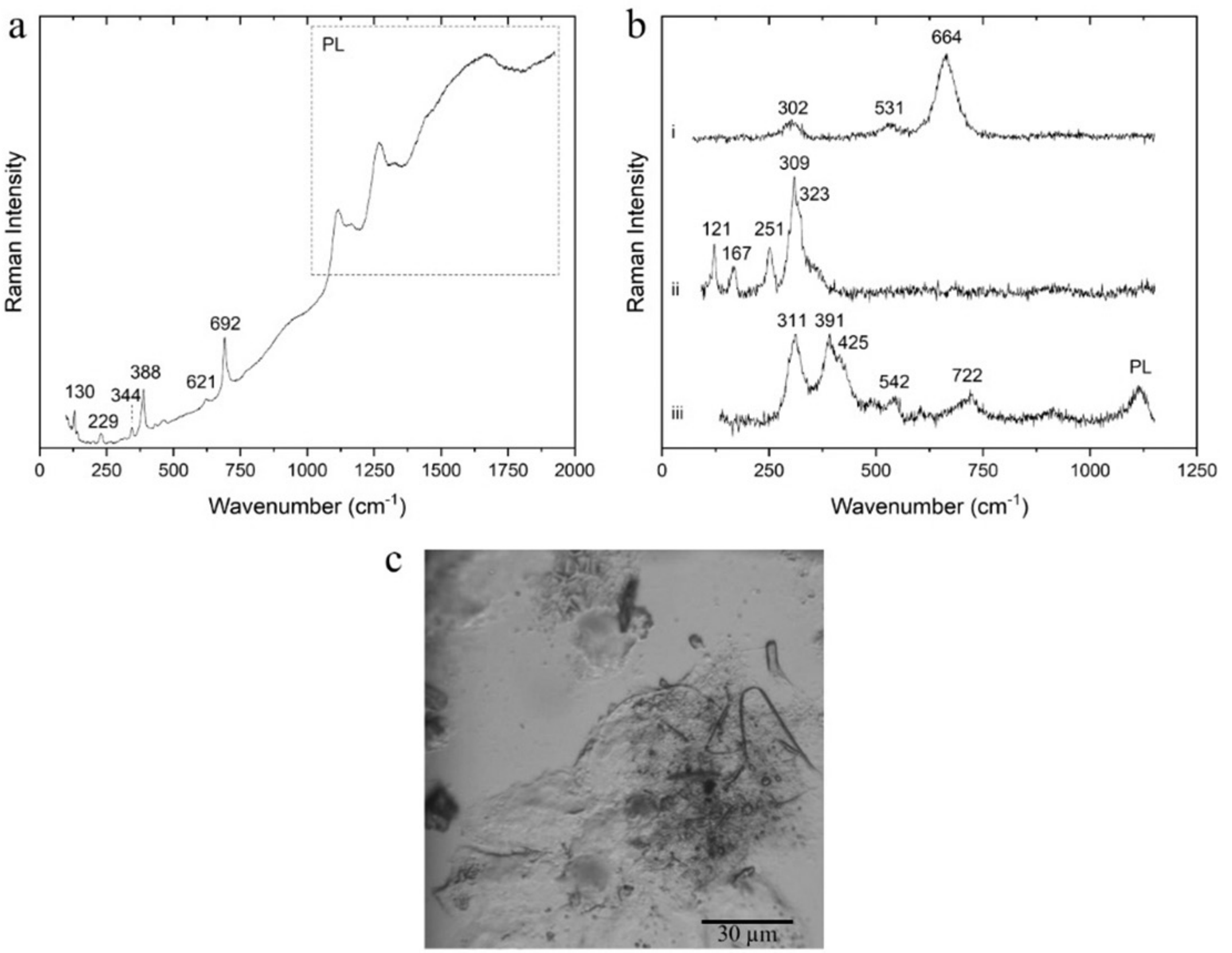

Figure 4. (a) Raman spectrum of chrysotile fibres from Russia acquired with the 632.8 nm laser line. The photoluminescence peaks related to Cr trace element are highlighted (PL); (b) Raman spectra of Fe-bearing compounds detected in mineral fibres: i) magnetite, ii) mackinawite and iii) akaganeite; (c) microscope image of THP-1 cells treated with chrysotile fibres (Balangero, Italy), showing an area of agglomerated materials with fibres and micrometric Fe-containing compounds.

The investigation of mineral fibres with micro-Raman spectroscopy was successfully achieved in human monocytic cell line THP-1 cells exposed to different fibres (chrysotile, erionite and crocidolite: Mirata et al., 2022). Inside the cells, the fibres were identified and micrometric mineral impurities were also detected. Additionally, in chrysotile-treated systems, agglomerations of cellular and mineral materials were observed, containing both fibrous and non-fibrous species, including Fe-bearing phases (as observed in the pure asbestos material) (Figure 4c). At increased treatment time (from 8 h up to 96 h), the size of the agglomerations rose: the Raman signals of fibrous and non-fibrous phases were still recorded there, even on hardly distinguishable fibres/crystals, suggesting that a partial dissolution of the inorganic material may have occurred.

Based on the *in vitro* experiments, an overall investigation of the biological interaction between cells and fibred required not only the identification of metals released in the cellular environment but also the study of their spatial distribution; to this extent, SR-based methods have emerged as effective tool for investigating biological systems at the sub-cellular level (Cammisuli et al., 2018; Pascolo et al., 2011; Pascolo et al., 2013, Pascolo et al., 2016, Kourousias et al., 2021). Thus, a synchrotron-based approach has been applied to study the release of metals in THP1 cell lines exposed to mineral fibres. As expected, synchrotron-based X-ray fluorescence mapping was able to clarify the metals mobilization and the fibres dissolution mechanisms, highlighting both the composition of the dissolution products and their origin, whether related to the fibres and/or to the iron compounds. Micro-XANES at the Fe and Cr K-edge provided additional details about the chemical states of these elements on fibres and mineral-particles within the organic environment. ID21 beamline at ESRF and TwinMic at Elettra have been selected as suitable for mapping the spatial distribution of metals in biological systems; in fact, working at different energies, the combining of data get at the two beamlines guaranteed to scan the majority of chemical elements relevant to understand the metal release in the studied fibres-cells systems, adding also relevant information on metal speciation thank to XANES at ID21. The outcomes of this research are briefly discussed in a dedicate paper within this special issue.

The Rome-Cosenza Research Unit

The Rome-Cosenza Research Unit has investigated several aspects regarding the toxicity of amphibole asbestos and erionite by applying a multi-analytical and multi-disciplinary approach. Moreover, the use of X-ray Synchrotron microtomography (SR-μCT) and portable micro-Raman spectroscopy coupled with portable microscopy (p-μR) and portable X-ray fluorescence (p-XRF) have been tested with the aim to be a complementary technique to other conventional ones in the identification and characterization of asbestos.

Because structural characterization of the different analysed materials, either as pristine or after different treatments was considered as a crucial point, some effort was devoted to optimizing the procedures for X-ray single-crystal Structure REFinement (SREF) and Rietveld analysis of amphiboles (Ballirano et al., 2021). As the result of the study, Ballirano et al. (2021) suggested a refinement procedure for both SREF and Rietveld analysis for tremolite, that was selected as a test case. The procedure for Rietveld analysis was adopted by the Research Unit throughout the structural characterizations of amphiboles that will be discussed in the following.

A second aspect investigated by the Research Unit was the analysis of the morphological and chemical-structural modifications that occur during thermal degradation of amphibole asbestos aimed at inertization (Pacella et al., 2020; Ballirano and Pacella, 2020).

The first study was performed *ex-situ* selecting low-iron tremolite and iron-rich crocidolite as test case (Pacella et al., 2020). Samples were heated at temperatures as high as 1200 °C. During heating, a complex sequence of iron oxidation, migration and/or clustering was observed. Finally, the pristine material converted to a brittle fibrous pseudomorphs consisting of newly formed minerals and amorphous nanophases that were tested for quantifying carbon- and oxygen-centred, namely hydroxyl (•OH), radicals. Heating was found not alter carbon radicals, but significantly affected oxygen-centred radical yields. However, differences were observed as far as the breakdown products of the two amphiboles are referred to. In fact, tremolite reactivity was reduced by migration of reactive iron ions into the more stable TO_4 tetrahedra of the newly formed pyroxene(s). Differently, crocidolite breakdown produced hematite, Fe-rich pyroxene, cristobalite, and abundant amorphous material that were able to restore radical reactivity.

The second study was performed *in situ,* up to the breakdown temperature, on a sample of fibrous tremolite from Maryland (Ballirano and Pacella, 2020). The products of breakdown were identified as subcalcic diopside and calcium-rich clinoenstatite, in a 2:1 ratio, plus traces of hematite and minor silica-rich amorphous material. The onset of Fe^{2+} oxidation/OH^- deprotonation was detected at 723 K from the deviation of the regular trend of thermal expansion. The process is completed at 1023 K. Before completion, a further process starts at 923 K, Fe^{3+} migration towards $M(1)$ and the corresponding counter-migration of Mg to $M(2)$ and $M(3)$. Because in fully oxidised tremolite, Fe^{3+} is prevalently allocated at $M(1)$, and it is well-known that $M(1)$, along with $M(2)$, is the most exposed octahedral site at the surface of amphiboles, this implies that the majority of Fe^{3+} is available for participating to the Fenton-like reactivity potentially suggesting a dangerous behaviour for human health.

A third aspect analysed by the Research Unit was the crystal chemical characterization of Naturally Occurring Asbestos (NOA) and Naturally Occurring Non-Asbestos (NONA) from different Italian and Spain localities. This aspect has been investigated with the help of and supporting other Research Units (see below). In particular, a fibrous tremolite sample from an abandoned serpentine quarry located in Mount Reventino (Calabria, Italy) was fully characterized, by using SEM-EDS analysis, Mössbauer spectroscopy, and X-ray powder diffraction (Pacella et al., 2021a).

Moreover, the Research Unit has performed a full structural and spectroscopic characterization of an UICC amosite (fibrous grunerite) standard sample from Penge mine (South Africa) (Ballirano et al., 2022). This study was aimed at supporting toxicologists during the interpretation of the results of experiments investigating possible correlation between physical-chemical characteristics of the fibres and their chemical reactivity and toxicity as the potential effect(s) of accessory phases may be easily overlooked. Partial agreement with reference data (Koyama et al., 1996; Pollastri et al., 2017) was found, suggesting some degree of inhomogeneity of the standard.

The Cosenza group in collaboration with the University of Salamanca and University of Catania studied the detection of asbestos in serpentinitic rock, in order to understand their possible contribution to the health problems (Bloise et al., 2021). SR-μCT allowed the analysis of asbestos tremolite and chrysotile infill veins in massive serpentinites under high magnification without grinding/milling and without causing morphological damage (Bloise et al., 2020a).

Moreover, the Research Unit has performed a study focused on combining portable micro-Raman Spectroscopy (p-μR) and portable X-ray Fluorescence (p-XRF) with the aim to distinguishing asbestos cement (AC, Eternit) from fibre-reinforced cement (FRC, asbestos-free).

The results of this study (Bloise and Miriello, 2022) showed that the simultaneous use of p-μR and p-XRF may prove useful for: i) the rapid in situ identification of asbestos chrysotile in Eternit; ii) the rapid identification in situ of polyvinyl fibres in FRC used as substitutes for Eternit (asbestos cement).

Another aspect analysed by the Cosenza Research Unit in collaboration with the University of Catania was the chemical characterization conducted on samples of tremolite asbestos from Episcopia and San Severino Lucano villages (Basilicata region, Southern Italy). More specifically, the aim of the research was to determine the potentially toxic elements (PTEs) concentration in tremolite asbestos, using micro X-Ray Fluorescence (μ-XRF) and Inductively Coupled Plasma spectroscopy with Optical Emission Spectrometry (ICP-OES), in order to provide a contribution to asbestos toxicity knowledge. It is worth mentioning that the determination of the PTEs concentration in asbestos minerals may be crucial to test their toxicity (Bloise et al., 2016, 2020b; Wei et al., 2014). The data of the study also have general implications regarding their possible use to calculate fibres potential pathogenicity/toxicity index (FPTI) (Gualtieri, 2018, 2023) which can be a helpful tool for the interpretation of results of *in vitro* testing.

The Cosenza Research Unit in collaboration with the University of Salamanca studied the emission of particles when using a laser in a stone (i.e., serpentinite) cleaning process (Pereira et al., 2023). The quantities of chrysotile detected in the samples after laser ablation were not negligible. The results were very didactic, and the intention is to use them, to build information security sheets that will alert the workers about the need of using masks when working or to use tools with coupled filters as the one we used for the study.

Following on the various studies aimed at describing in detail the exchange properties of erionite (Ballirano and Cametti, 2015; Ballirano et al., 2015; Pacella et al., 2017 a,b; Pacella et al., 2018) for predictive purposes whenever in contact with biological fluids, the Research Unit has performed a crystal chemical and structural characterization of erionite-Na from Agua Prieta, Sonora, México on both pristine and Na, Ca, and Mg exchanged samples (Quiroz et al., 2020).

Finally, the Research Unit has addressed the surface and bulk modifications occurring on amphibole asbestos (crocidolite and tremolite) and erionite during leaching in a mimicked Gamble's solution at acidic pH up to 1 month (Pacella et al., 2021 b,c).

Samples were characterized by using a multi-analytical approach. Results showed that, for amphibole asbestos the fibre dissolution is highly incongruent particularly in the first stages of the process, with the preferential release of cations allocated at the various *M*-sites of the amphibole structure, in agreement with the Madelung site energies. Moreover, the faster alteration observed for crocidolite lead to the promotion of new Fe centres on the fibre surface, especially in the form of Fe(II) of which the bulk is enriched with respect to the oxidized surface. Conversely, for tremolite fibres the slow dissolution rate prevents the exposure of Fe(II) centres from the bulk on the mineral surface, and therefore the Fe(II) centres initially present on the fibre surface are significantly depleted by oxidation.

Concerning erionite, results showed that fibres bind Na from the solution, especially in the first 24 h of sample incubation, following ion exchange with the extra framework cations, in particular Ca. Moreover, the Na binding process caused structural modifications, as the migration of Na toward the Ca2 site coupled with the redistribution of the cations within the erionite cage. TEM investigation pointed out that, similarly to amphibole asbestos, the interaction between erionite and the mimicked Gamble's solution led to the formation of a new surface amorphous layer with an irregular lobate pattern on an earlier surface weathered layer. However, in the case of erionite the silicate framework is not weakened by incubation in the solution at acidic pH. Accordingly, based on Si release normalized to the mineral surface area, fibrous erionite resulted significantly more biodurable than amphibole asbestos.

Considering that reactivity is strictly related to the fibre surface properties, the obtained results are of paramount importance for unravelling possible correlations between physical chemical features of the fibres and their toxicity.

The Urbino Research Unit

The Research Unit of Urbino has focused its activity on two main subjects: (i) the morphological, mineralogical, and physico-chemical features of potentially toxic natural fibrous zeolites and other possibly hazardous mineral fibres; some aspects of this topic benefited from the contribution of the Rome and Modena research units; (ii) the design of a novel method to prepare fibres depositions to be used for toxicological experiments.

As regard the first subject, Giordani et al. (2022a) investigated three samples of natural zeolites: an extremely fibrous erionite (GF2, Mattioli et al., 2016a; Pacella et al., 2018), a highly fibrous mesolite (GF3a) and a prismatic thomsonite (GF3b). The GF2 erionite sample comprises fibrils in the range of inhalable and most carcinogenic fibres; it has the highest Si/Al ratio (3.38) and highest values of BET-specific surface area (8.14 m^2/g). These data become much more helpful when integrated and compared with analysing selected spin probes' electronic paramagnetic resonance (EPR) spectra (Mattioli et al., 2016a; Cangiotti et al., 2017, 2018; Gualtieri et al., 2018). EPR data reveal that GF2 has a homogeneous site distribution intercalating more and less hydrophilic sites. Consequently, the GF2 surface interacts well with all spin probes, representing the chemical moieties in biological environments. The GF3a mesolite sample consists of tiny fibres and fibrils in the range defined as the most carcinogenic fibres. Compared to the GF2 sample, GF3a shows a lower Si/Al ratio (1.56) and a smaller BET-specific surface (1.55 m^2/ g). The surface of GF3a is less homogeneous and close polar sites were identified using EPR analyses. Massive crystals with very few thin fibres form the GF3b thomsonite sample, which shows the lowest Si/Al ratio (1.23) and the lowest BET-specific surface area (0.38 m^2/g). Like the mesolite sample, the surface of GF3a is less homogeneous than the GF2. These results suggest that the potential toxicity of a fibrous mineral is most likely related to the interacting capability of the fibres and their interaction sites. This interaction capability increases by increasing the surface area, the availability of the interaction sites, and different well-distributed polar sites. Therefore, we expect potential toxicity to decrease in the series order erionite ≫ mesolite > thomsonite. This indication was also tested by an *in vitro* study (Betti et al., 2022), which aimed to determine if and how these fibrous zeolites induce toxic effects on two different *in vitro* cellular models, the adherent murine hippocampal (HT22) and human immortalized T lymphocyte (Jurkat) cell lines. Results showed a cytotoxic effect of erionite in both cellular models and revealed different toxic behaviour of the mesolite and thomsonite fibres, suggesting other potential mechanisms of action. The outcome of this study would be a first step for further research on fine biochemical interactions of zeolite fibres with cells and future *in vivo* investigations.

Other contributions to the first subject come from Giordani et al. (2022b) and Mattioli et al. (2022). In these two papers, morphological, mineralogical, and physico-chemical features of suspected toxic mordenite and ferrierite fibres were studied. In Giordani et al. (2022b), three mordenite samples from Northern Italy were investigated. All the analysed samples show similar structural and chemical characters: they are Na-rich (Na >Ca>K), and the Al content decrease reflects the unit cell volumes in the series. Despite the size differences, all the studied mordenite samples are characterized by "respirable" fibres that could reach the lungs' deeper parts. These results indicate that fibrous mordenite may represent a potential health hazard and should be tested for toxicity and carcinogenicity, as it could be a non-safe zeolite.

The other paper (Mattioli et al., 2022) investigated the morphology, crystal structure, chemistry, and surface activity of a variety of ferrierite recently found in Northern Italy (Mattioli et al., 2016b), which shows particularly fibrous-asbestiform habit and chemical-physical properties very similar to those of ferrierite described by Gualtieri et al. (2018). Results from Mattioli et al. (2022) demonstrate that a notable amount of ferrierite fibres are breathable (average length ~22 µm, average diameter 0.9 µm, diameter-length ratio ≫1:3) and able to reach the alveolar space (average D_{ae} value 2.5 µm). The prevailing extra-framework cations are in the Mg> (Ca≈K) relationship, R is from 0.81 to 0.83, and the Si/Al ratio is high (4.2-4.8). The <T-O> bond distances suggest the occurrence of some degree of Si,Al ordering, with Al showing a site-specific occupation preference T1>T2> T3>T4. Ferrierite fibres show high amounts of adsorbed EPR probes, suggesting an increased ability to adsorb and interact with related chemicals. These outcomes, in agreement with Gualtieri et al. (2018), indicate that fibrous ferrierite should be considered a potential health hazard.

A comparison of the interaction capability (as adsorbed percentage of probes) of the investigated samples with that of different fibrous zeolites (data from Mattioli et al., 2016a, 2018, 2022; Cangiotti et al., 2017; Giordani et al., 2016, 2017; Gualtieri et al., 2018) is presented in Figure 5. The percentage of adsorbed probes is very high for the carcinogenic erionite samples, and is comparable with that measured for ferrierite and offretite. These zeolites exceed 95% and can be considered highly toxic to carcinogenic. Mesolite and thomsonite show lower interaction capability (60-70%) and can be considered harmful, while scolecite shows no interaction and can be assumed to be a non-toxic zeolite. As a more relevant result, it emerges that the interaction capability (taken as an adsorbed percentage of probes) seems to be a very useful parameter in assessing the toxicity of a mineral, which could also be considered in the assessment of potential health hazard calculation of the fibre potential toxicity index (FPTI) (Gualtieri, 2018).

Another contribution to the characterization of fibrous minerals is Giordani et al. (2022c), where study on the

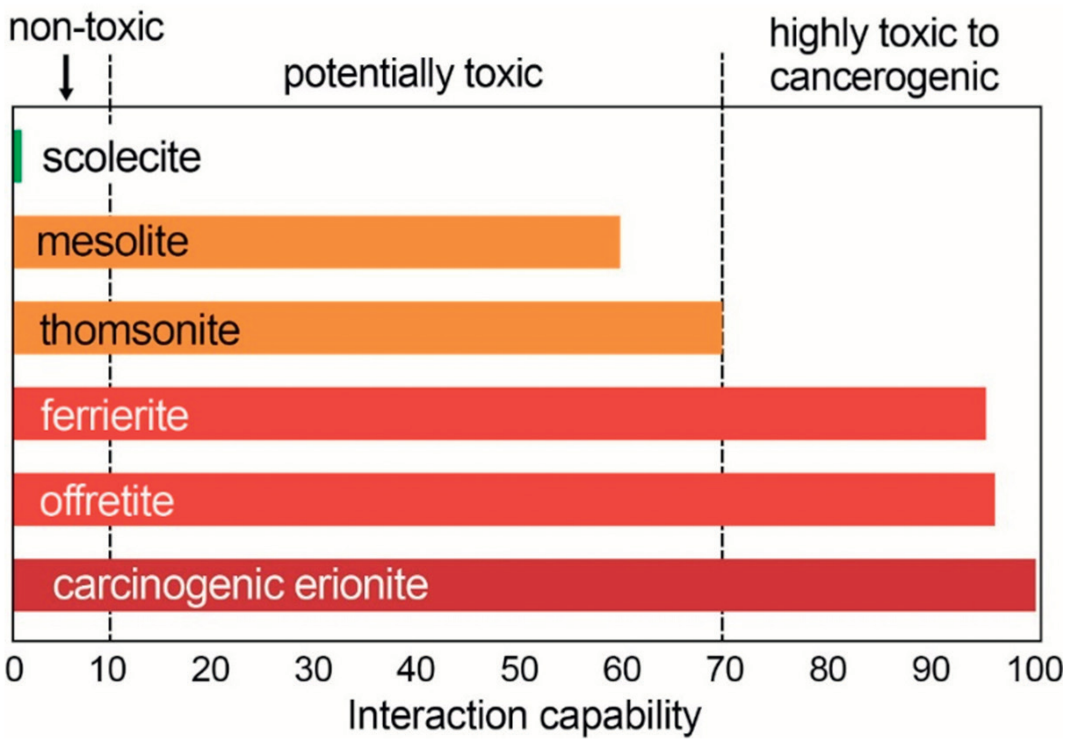

Figure 5. Comparison of the interaction capability of the investigated fibrous zeolites (modified from Giordani et al., 2022a).

water-soluble natural epsomite fibres from Perticara Mine (Italy) was conducted using SEM-EDS, XRPD, ICP-AES and alpha spectrometry measurements. The morphological and morphometric results showed that most fibres are inhalable (D_{ae}=5.09 μm) and can be potentially adsorbed from all parts of the respiratory tract. Chemical analysis reveals significant amounts of toxic elements (As, Co, Fe, Mn, Ni, Sr, Ti, Zn) and surprisingly high contents of radioactive isotopes (^{210}Po and ^{228}Th) in epsomite crystals, making the inhalation of these fibres potentially hazardous to human health. This study opens a new window on soluble minerals, such as epsomite, which can be present in both natural and anthropic environments and have never been considered from the point of view of their potential hazard.

Regarding the second topic, the research stems from the results of previous papers (e.g., Yao et al., 2017) showing how the interaction of living cells during *in vitro* tests is extremely dependent on the textural features of the used depositions. For example, Yao et al. (2017) observed that the distribution of fibres in their depositions were extremely different for increasing amounts of particles in the used suspension, with the paradoxical result that higher amounts of asbestos in the system were correlated with a lower toxicity. The result of the deposition depends on the physical aspects of an evaporating suspension and on the interaction between the solvent and the substrate (e.g., Macis et al., 2018). Assuming a negligible drop-surface interaction, as it is the case of water-based liquids on hydrophobic substrates, during the evaporation the contact angle of the edge of the drop remains constant, the shape of the drop remains spherical but the contact area between liquid and surface continuously decreases (Della Ventura et al., 2023). In such a case, because of the "stain effect" (see Macis et al., 2018) the result is the deposition of fibres forming bundles and aggregates within a small area, particularly at higher concentrations; this feature may strongly affect the results of fibre-cell toxicity experiments. Thus, we tested a novel procedure using an MD-K-130 instrument of Microdrop Technologies (GmbH Norderstedt, Germany) that generates droplets with a volume of 180 pl. This device allows to release droplets at well-defined and repeatable positions. In such a way, the suspension is efficiently distributed spatially on a substrate, and the amount of fibres can be easily controlled by successive depositions. The samples obtained from the same suspensions by employing both the classical pipette method and the microdrop technique were studied by using image processing of optical and SEM images. The results showed clearly that the depositions obtained with the microdrop method are extremely more homogeneous and, in particular, the number of single fibres deposited via the micro-dispenser is far higher than that resulting from the micropipette technique (Della Ventura et al., 2023).

CONCLUSIONS

A report of the major results accomplished within the multidisciplinary project PRIN 2017 *FIBRES* has been illustrated.

A thorough systematic characterization of regulated and non-regulated mineral fibres has been accomplished, with special attention to metal and metal-containing mineral impurities. This preparatory research line regarded chrysotile and UICC amosite standard from Penge mine (South Africa), potentially toxic natural fibrous zeolites and water-soluble natural epsomite fibres from Perticara Mine (Italy) which revealed significant amounts of toxic elements and radioactive isotopes (^{210}Po and ^{228}Th). The crystal-chemical characterization also regarded amphibole asbestos (crocidolite and tremolite) and erionite during leaching in a mimicked Gamble's solution at acidic pH up to 1 month. Cation exchanged forms of erionite have also been investigated for predictive purposes whenever in contact with biological fluids, with special attention to the role or iron. Moreover, the products of thermal transformation of amphibole asbestos fibres have been characterized with the aim to contribute to the understanding of the potential toxicity.

Portable micro-Raman spectroscopy coupled with portable microscopy (p-μR) and portable X-ray fluorescence (p-XRF) have been used as complementary techniques for the identification and characterization of asbestos and applied with other experimental techniques to the characterization of Naturally Occurring Asbestos (NOA) and Naturally Occurring Non-Asbestos (NONA) from different Italian and Spain localities.

The *in vitro* toxicity of chrysotile and crocidolite and fibrous erionite has been systematically investigated in detail. To this aim, representative batches of short (length ≤5 mm) and long (length >5 mm) chrysotile fibres were especially prepared by cryogenic milling and tested *in vitro*.

Novel ground-breaking approaches regarded: (i) the study of *in vitro* cellular acute toxicity (0 to 8h) on macrophage phenotypes of chrysotile, crocidolite and fibrous erionite observed by time-lapse video microscopy; (ii) the study of mechanisms of toxicity (apoptosis, membrane disruption, oxidative stress, DNA damage, onset of inflammatory processes) on macrophage phenotypes of chrysotile, crocidolite and fibrous erionite; (iii) the study of chrysotile toxicity on the two barriers fundamental for survival during foetal and adult life i.e., maternal-foetal barrier and respiratory lung barrier; (iv) the study of mechanisms of acute (24-48 h) and chronic toxicity (7 d) of chrysotile on human macrophages and endothelial cells and of the cross-talk among the different lung cell types for the onset of chronic inflammatory/carcinogenic processes; (v) the study of the release of metals from the fibres and the biological interaction between cells and chrysotile, amphibole asbestos and fibrous erionite fibred by synchrotron based techniques such as XANES-XRF spectroscopy and microtomography (SR-μCT); (vi) the design of a novel method to prepare fibres depositions to be used for toxicological experiments.

The results of this project have shed light on the role of active surface iron, biodurability, the 'Trojan horse effect', and ion exchange of the erionite zeolite fibres and will support the scientists working in the field of medicine, toxicology, and epidemiology to better understanding the mechanisms by which mineral fibres prompt adverse effects *in vivo* leading to carcinogenicity.

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