Update of *in vitro*, *in vivo* and *ex vivo* fluoro-edenite effects on malignant mesothelioma: A systematic review (Review)

VERONICA FILETTI[1], ERMANNO VITALE[2], GIUSEPPE BROGGI[3], MARIA P. HAGNÄS[4,5], SAVERIO CANDIDO[6,7], ANNA SPINA[8] and CLAUDIA LOMBARDO[3]

[1]Anatomy and Histology, Department of Biomedical and Biotechnological Sciences; [2]Occupational Medicine, Department of Clinical and Experimental Medicine; [3]Pathologic Anatomy, Department 'G.F. Ingrassia', University of Catania, I-95123 Catania, Italy; [4]Rovaniemi Health Centre, 96200 Rovaniemi; [5]Center for Life Course Health Research, University of Oulu, 90150 Oulu, Finland; [6]Oncologic, Clinic and General Pathology Section, Department of Biomedical and Biotechnological Sciences; [7]Research Center for Prevention, Diagnosis and Treatment of Cancer (PreDiCT), University of Catania, I-95123 Catania; [8]INPS Italian National Social Security Institution, I-95129 Catania, Italy

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Abstract. Fluoro-edenite (FE), asbestiform fiber found in Biancavilla (Sicily, Italy), presents various characteristics similar to the asbestos group, in particular two fibrous phases tremolite and actinolite. Indeed, epidemiological studies have shown that FE fibers have similar effects to those of asbestos fibers. Such studies have reported a high incidence of malignant mesothelioma (MM), an aggressive neoplasm of the serosal membranes lining the pleural cavity, in individuals residing there due to FE exposure in Biancavilla related to environmental contamination. Evidence has led to the classification of FE as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC). The aim of this systematic review is to compare the results achieved in *in vitro*, *in vivo* and *ex vivo* experimental studies involving FE in order to update the current knowledge on the pathogenesis and molecular mechanisms responsible for FE-mediated MM development as well as the availability of effective biomarkers for MM prevention and diagnosis. This review is focused on the pathophysiological mechanisms mediated by inflammation induced by FE fiber exposure and which are responsible for MM development. This review also discusses the discovery of new diagnostic and prognostic biomarkers for the management of this pathology. It is known that the risk of cancer development increases with chronic inflammation, arising from enhanced reactive oxygen species (ROS) and NO• production stimulated by the body to remove exogenous agents, causing DNA damage and enhanced signal transduction that may lead to activation of oncogenes. Studies concerning MM biomarker discovery indicate that several biomarkers have been proposed for MM, but mesothelin is the only Food and Drug Administration (FDA)-approved biomarker for MM, with limitations. In recent studies, *in silico* analysis to identify selected miRNAs highly deregulated in cancer samples when compared with normal control have been developed. This *in silico* approach could represent an effort in the field of biomarker discovery for MM.

Correspondence to: Dr Veronica Filetti, Anatomy and Histology, Department of Biomedical and Biotechnological Sciences, University of Catania, Via Santa Sofia 87, I-95123 Catania, Italy
E-mail: verofiletti@gmail.com

Abbreviations: FE, fluoro-edenite; MM, malignant mesothelioma; IARC, International Agency for Research on Cancer, ROS, reactive oxygen species; FDA, Food and Drug Administration; NOA, naturally occurring asbestos; AM, alveolar macrophage; PLC, phospholipase C; PKC, protein kinase C; PTK, protein tyrosine kinase; iNOS, inducible nitric oxide synthase; LDH, lactic dehydrogenase; VEGF, vascular endothelial growth factor; Rb, retinoblastoma; pRb, phospho-retinoblastoma; Fb-3, fibulin-3; Hsp70, heat shock protein 70; PGs, prostaglandins; AQP1, aquaporin-1; OS, overall survival; CK5, cytokeratin 5; HMGB-1, high mobility group box 1; miRNA, microRNA

Key words: fluoro-edenite, asbestos, fibers, malignant mesothelioma, *in vitro*, *in vivo*, *ex vivo*

Contents

1. Introduction

It is well known that the general population may be exposed to 'naturally occurring asbestos' (NOA) (1-4). The term NOA

refers to the mineral as a natural component of soils or rocks as opposed to asbestos in commercial products, mining or processing operations. NOA may be released as fibers into the air by human activities or natural weathering processes that represent a risk for human exposure (3).

A fiber is defined as a particle (length/diameter ratio 3:1) with certain characteristics that make it respirable, penetrating into the alveolar level and participating in gaseous exchanges (5). *In vitro*, *in vivo* and *ex vivo* studies have demonstrated that the dimension, surface property, shape, cristallinity, chemical composition, physical durability, exposure route, duration of exposure, dose (6) and genetic background of the host exposed (7) are all determinants for the biological activities of a certain fibrous element. Fiber dimensions are important because only fibers with diameter <0.4 μm and length <10 μm are respirable to the distal alveolar space. Dose is a crucial determinant for triggering inflammation. Indeed, high doses over short periods promote an acute neutrophil-predominant inflammation, whereas low doses over prolonged exposure periods promote alveolar macrophage (AM)-predominant chronic inflammation (7). In general, fibers greater than 20 μm in length are associated with asbestosis, and fibers greater than 10 μm in length are the most carcinogenic (5).

Carcinogenic mineral fibers are divided into asbestos and asbestiform fibers (8,9). The term 'asbestos' is used to identify silicate minerals belonging to two families: Amphiboles (amosite, anthophyllite, actinolite, crocidolite, tremolite, anthracite) and serpentine (crysotile) (6,10). Amphiboles are straight, rod-like fibers, whereas serpentines are curvilinear fibers. Asbestos has a significant industrial importance for its characteristics, its abundance and its low cost. Asbestos has sound-absorbing and sound-proofing properties, thermal stability at high temperatures, good mechanical resistance, good resistance to chemical and biological agents, and for this high versatility it has been widely used in different areas, especially in the building industry (11).

Exposure to asbestos fibers can cause several diseases such as asbestosis, lung and bronchus cancer, malignant mesothelioma (MM) of the pleura, peritoneum, pericardium and tunica vaginalis testis, neoplasms of the ovary, larynx and trachea carcinoma (11). There is evidence that the inhalation of asbestos fibers can provoke two types of inter-connected pathogenetic processes: Chronic inflammation and carcinogenesis, involving the lung after inhalation and deposition of asbestos fibers (12). Therefore, it has been established that cancer frequently arises in areas of chronic inflammation (13). Many lines of evidence have highlighted the ability of asbestos fibers to: Interfere with the mitotic apparatus; stimulate host cell proliferation; induce genetic and epigenetic alterations; induce cytotoxicity and fibrosis; produce oxidative stress by at least three sources including fiber surface reactivity, release from inflammatory cells especially AMs, and mitochondrial-derived ROS release from inflammatory and other target cells such as lung epithelial cells and mesothelial cells (7). The ROS production results in DNA damage, release of inflammatory cytokines and growth factors that collectively contribute to fiber pathogenicity (12,14) and H_2O_2 production in mediating asbestos pulmonary toxicity (7).

Several studies have reported a high incidence of MM due to asbestos exposure in: Finland (15), California, USA (16), China (17), New Caledonia (18), Corsica (19), Cyprus (20) and Greece (21). Yet, in many cases, it has been discovered that the cause of these MM cases has not been asbestos but asbestiform fibers.

The term 'asbestiform fiber' is commonly used to indicate erionite, winchite, magnesio-riebeckite, richterite, Libby asbestos, antigorite and fluoro-edenite (FE) fibers. Erionite is a mineral belonging to the zeolite family (22). Several studies have reported a high incidence of MM due to erionite exposure in rural regions of Turkey, Central Anatolia (23-27). Furthermore, individuals exposed to erionite may develop interstitial fibrosis and additional pulmonary pathology impacting lung function and patient survival (28).

Clark and Nye counties, in southern Nevada, USA, have shown a significantly high incidence of MM due to carcinogenic fibers including erionite, winchite, magnesio-riebeckite, and richterite (29). These are the same fibrous minerals present in Libby, Montana, USA, where they have been related to MM and other asbestos-related diseases (30).

Antigorite is a silicate mineral very similar in chemical composition to chrysotile and its asbestiform variant is present in serpentinite rocks associated with MM (31). Antigorite is found in the Western Alps (Piemonte, Italy) (32,33), in North America, Australia-Oceania, and Rowland Flat in South Australia (34,35).

Fluoro-edenite (FE), the amphibole of Biancavilla (Sicily, Italy), is a silicate mineral belonging to the amphibole family (36). This silicate mineral has been identified in the lavic products of Monte Calvario from stone quarries located in the southeast of Biancavilla (37), a small town of the Etnean volcanic complex, in Sicily. This silicate mineral presents some characteristics similar to the asbestos group (38,39); in particular it presents the same morphological and compositional aspect of the two fibrous phases tremolite and actinolite. The mineralization process led to the development of large prismatic crystals embedded in the matrix, small acicular crystals that line cavities or also fibrous and asbestiform (37). The salient feature, which nonetheless distinguishes the FE of Biancavilla not only from other fibrous minerals, but also from all the other known amphiboles, is the very anomalous composition characterized by high sodium, aluminum and fluorine contents, in comparison to other known oncogenic minerals (40).

Epidemiological studies have indeed confirmed that FE fibers have shown similar effects to those already reported after exposure to asbestos fibers (8,41-43) including cell necrosis with release of high mobility group protein B1 and activation of the Nalp3 inflammasone, leading to chronic inflammation, DNA damage and carcinogenesis (44).

Several studies have reported a high incidence of MM in Italy due to FE exposure in Biancavilla (45-48) concerning the time window 1980-2009. All of the data suggest that a mode of exposure to FE fibers is related to environmental contamination, rather than specific occupational activities (45). In fact, the stone material from the quarry of Monte Calvario has been used locally for about 50 years for building purposes (8,49,50) and none of the residents diagnosed with MM have been significantly exposed to asbestos during their professional lives (12).

Diseases related to erionite and FE fibers present with characteristics similar to asbestos-related pathologies. The underlying modes of action of asbestosis, lung cancer and MM

seem to be different in regards to the fiber type, lung clearance, and genetics. Several lines of evidence have led to the classification of asbestos, erionite, and FE as Group 1 human carcinogens (51; IARC, 1987).

Therefore, NOA represents an important environmental concern. Thus, asbestos and asbestiform fibers continue to cause a high health concern due to the long latency period of related diseases.

2. Literature search methodology

This systematic review was carried out in accordance with PICo criteria (52). The review/research question was defined, using PICo criteria, by identifying: Population, interest, and context of research. What are the *in vitro*, *in vivo* and *ex vivo* studies correlated with MM due to FE exposure in Biancavilla? (Fig. 1). The research was performed by using the following search term: 'Fluoro-edenite fibers'. A search of the research manuscripts suitable for inclusion in this systematic review, was carried out and the research papers of significance were collected and reviewed. The main topics and alternate terms from our PICo question that were used for the search were: Fluoro-edenite exposure, Fluoro-edenite, Fluoro-edenite fibers, Fluoro-edenite fibres, Biancavilla, Biancavilla's exposure, Malignant mesothelioma. The English language was used as a limit to our search. SCOPUS and Medline (using PubMed as the search engine) databases were used to search relevant research articles available from March 4 to August 4, 2020 (Fig. 1).

Inclusion and exclusion criteria. The following inclusion criterion was adopted: Experimental studies that assessed the effects of FE fiber exposure *in vitro*, *in vivo* and *ex vivo* models. The following exclusion criteria were applied: i) Scientific articles that were not published in the English language; ii) review or conference abstracts or letters to the editor; iii) experimental studies that did not concern *in vitro*, *in vivo* and *ex vivo* models. For duplicate studies, the article containing further detailed information was solely included.

Quality assessment and data extraction. Two reviewers (VF and CL) retrieved articles independently. The title, abstract and full text of each potentially pertinent study were reviewed. Any divergence on the eligibility of the studies was determined by debate. The following information was extracted from all qualified papers: Authors, year of publication, and study characteristics.

After a free search for scientific literature by reviewers, a total of 29 documents were collected. In conclusion, 5 studies were disqualified after review of the manuscript. A total of 24 studies satisfied the inclusion criteria and were included in this systematic review. A flow-chart depicting the choice of studies is documented in Fig. 2. The information concerning authors, year of publication, and characteristics of included studies have been included in Table I.

3. *In vitro* studies concerned with MM due to FE exposure

Various studies have been conducted to investigate the effects of FE fibers on several cell lines which are commonly used to evaluate the cytotoxicity of various silica dusts (53): A549 (human pulmonary epithelial cancer cells), MeT-5A (human pleural mesothelial cells), J774 (mouse alveolar monocyte-macrophage cells), JU77 (human MM cells), and human lung fibroblasts. Epithelial cells are involved in proinflammatory effects and they are the cells of origin of bronchogenic carcinoma. In addition, the transformation of mesothelial cells leads to mesothelioma, thus they are suitable to determine the direct effects of fibers. Alveolar macrophages are the first defense mechanism against particulates and fibers entering the lower respiratory tract (54); and fibroblasts represents a cell type of central regulatory potential in lung diseases (55).

Prismatic vs. fibrous FE. Several studies (12,56) have reported the cellular effects of prismatic and fibrous FE by using the A549 cell line. These cells, upon contact with prismatic FE, develop actin-rich protrusions from the plasma membrane, namely ruffles and filopodia, that allow the capture and internalization of material into the cytoplasm of epithelial cells. This phagocytic-like behavior of cells exposed to prismatic FE occurs only after the cells have reached the confluent state. The organization of the actin cytoskeleton, which represents one of the key target for a huge number of toxicants, remains well organized both in control and in treated A549 cells. In contrast with the results obtained with prismatic FE (12), the fibrous mineral provokes dramatic changes in the actin network of A549 cells (56). In particular, the actin stress fibers completely disappeared and actin-rich membrane ruffles arise from the cell surface following FE fiber exposure. In addition to these effects, the results obtained show that fibrous FE promote multinucleation, cell spreading and a dramatic increase in cell size, but without interfering with the passage of the resulting multinucleated cells through the cell cycle and without condemning cells to death.

Cytokines and growth factors derived from alveolar macrophages are implicated as mediators of asbestos-induced patho-physiological responses (57). Indeed, inflammation also characterizes the response of epithelial cells to external danger, which produce an array of mediators transmitting cellular signals (58). Interleukin (IL)-6 and IL-8 concentrations, that are respectively a multifunctional cytokine with immunoregulatory and proinflammatory effects (56) and a chemotactic cytokine involved in the recruitment of polymorphonuclear granulocytes to the site of injury (59), have been determined by Travaglione *et al* (12,56) after treatment of A549 cells with prismatic and fibrous FE. The results revealed that although prismatic FE interferred with cell physiology, by reducing the proliferation rate and increasing the release of the proinflammatory cytokine IL-6, this did not perturb the cell cycle and there was no evidence of any particular effects correlated to cellular transformation (12). On the contrary, in the case of fibrous FE exposure, an increase in both IL-6 and IL-8 secretion in the surnatant, in a time dependent-manner, was demonstrated (56). Cytokines, produced by epithelial cells in response to a cellular damage, activate neutrophils and macrophages which accumulate in the injured area. The recruited cells then produce ROS (60) and additional cytokines in an attempt to remove the unsafe agent. With chronic inflammation, tissue fibrosis can occur concomitantly with an enhanced risk of cancer development, arising from enhanced ROS production leading to DNA mutations and enhanced signal transduction that may lead to activation of oncogenes.

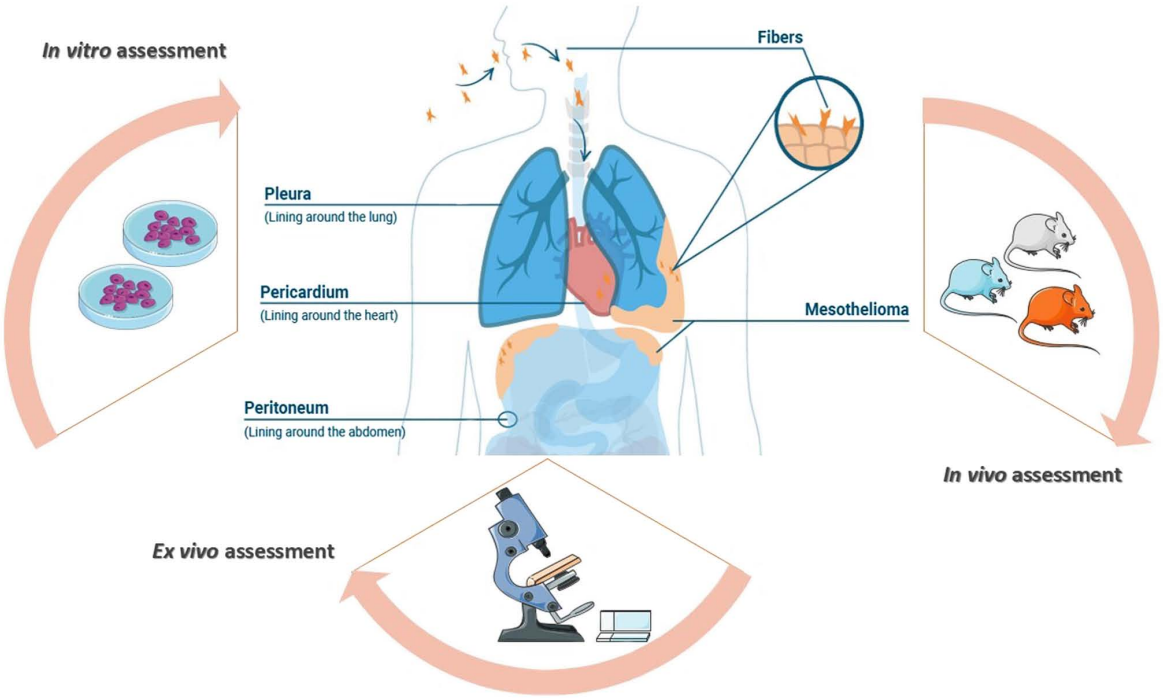

Figure 1. Graphical abstract.

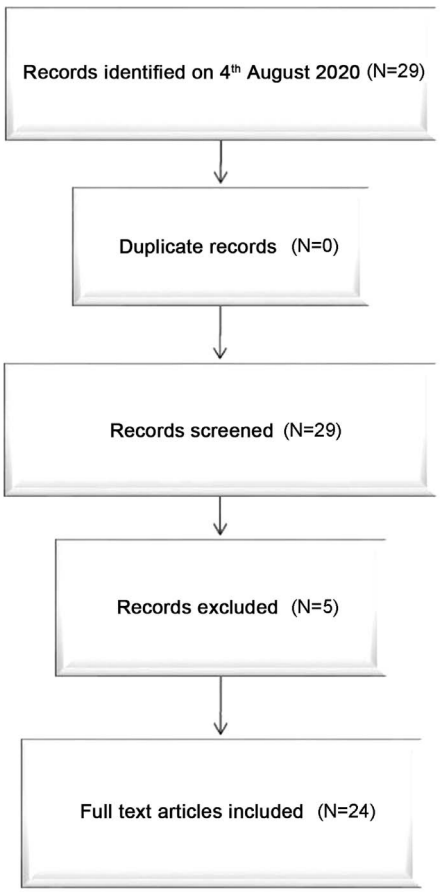

Figure 2. Flow chart outlining the included and excluded studies in this systematic review.

Therefore, these research studies, which demonstrated a pro-mesotheliomatogenic effect of fibrous but not prismatic FE in lung epithelial cells, highlight a differential cell response ensued by prismatic or fibrous FE.

Fibrous FE vs. crocidolite and tremolite. In lung epithelial cancer A549 cells, the behavior of FE fibers is similar to crocidolite (56), whose link with chronic inflammation and lung cancer is renowned (61,62). Fibrous FE causes actin reorganization and multinucleation accompanied by a significant increase in cell size. While these effects became evident after 48 h of FE exposure, in the case of crocidolite exposure the effects are observable at 24 h (56). Loreto *et al* (63) demonstrated that also FE 27 (70% of Fe^{3+}), FE 19 (50% Fe^{3+}) and tremolite fibers are able to promote the formation of multinucleated cells, but the phenomenom is greater in cells following crocidolite exposure (56,63).

Furthermore, the incubation with fibrous FE and crocidolite causes a comparable decrease in the number of viable A549 cells (56), J774 cells and human lung fibroblasts (64). Pääkkö *et al* (65) associated the observed reduction in A549 growth to the induction of the apoptotic process. The results reported that crocidolite is able to cause apoptosis in A549 cells, associating this phenomenom to the nuclear accumulation of the p53 protein, a transcription factor mainly involved in cell cycle arrest and apoptosis after DNA damage. In contrast, Travaglione *et al* (56) demonstrated that both fibrous FE and crocidolite were not able to influence the expression of p53 and of pro-Bax and anti-apoptotic Bcl-2 and Bcl-X_L proteins. Furthermore, neither fibrous FE nor crocidolite were able to induce alterations in the passage of A549 cells throughout the cell cycle; the percentages of cells in the G0/G1, S, G2/M phases did not vary between the control and cells exposed to the fibers.

In order to investigate the proinflammatory potential of FE 19 and FE 27, tremolite, and crocidolite fibers, the secretion of IL-8 (56), IL-6, IL-1β and TNF-α (63) were determined in A549 cells. All fibers increased the release of proinflammatory

Table I. Characteristics of the studies included in the systematic review.

	(Refs.)	Samples
In vitro N=11	Travaglione *et al* (12)	A549 cells
	Cardile *et al* (60)	J774 cells
	Cardile *et al* (64)	A549 cells
		J774 cells
		Human lung fibroblasts
	Travaglione *et al* (56)	A549 cells
	Cardile *et al* (32)	MeT-5A cells
		J774 cells
	Pugnaloni *et al* (54)	MeT-5A cells
		A549 cells
		J774 cells
	Loreto *et al* (63)	A549 cells
	Musumeci *et al* (69)	MeT-5A cells
		A549 cells
	Rapisarda *et al* (74)	MeT-5A cells
	Rapisarda *et al* (76)	Human lung fibroblasts
	Filetti *et al* (77)	MeT-5A cells JU77 cells
In vivo N=2	Soffritti *et al* (36)	Sprague-Dawley rats
	Belpoggi *et al* (88)	Sprague-Dawley rats
Ex vivo N=11	DeNardo *et al* (82)	Lung tissue of sheep
	Martinez *et al* (43)	Lung tissue of sheep
	Loreto *et al* (83)	Lung tissue of sheep
	Musumeci *et al* (84)	Lung tissue of sheep
	Musumeci *et al* (92)	Lung tissue of sheep
	Musumeci *et al* (85)	Lung tissue of sheep
	Loreto *et al* (86)	Lung and lymph nodes of sheep
	Rapisarda *et al* (93)	Tracheobronchial lymph nodes of sheep
	Ledda *et al* (87)	Tracheobronchial lymph nodes of sheep
	Angelico *et al* (94)	Human MM tissue
	Caltabiano *et al* (44)	Human MM tissue

MM, malignant mesothelioma. A549, human pulmonary epithelial cancer cells; MeT-5A, human pleural mesothelial cells; J774, mouse alveolar monocyte-macrophage cells; JU77, human MM cells.

cytokines in a time-dependent manner, but crocidolite was found to promote a more consistent secretion of the analyzed cytokines (59,63); on the contrary, tremolite induced a reduced release of the same (63). Among cytokines, it has been demonstrated that IL-1β and tumor necrosis factor (TNF)-α act through the phosphoinositide-specific phospholipase C (PLC) pathway to activate protein kinase C (PKC) (66), essential for many cellular functions such as the processes of secretion, differentiation, proliferation and cell growth (67). However, it is known that asbestos stimulates ROS generation by interaction with cellular membranes or through protein tyrosine kinase (PTK), PLC and PKC pathway activation in a dose-response manner (68). Loreto *et al* (63) aimed to ascertain whether exposure to FE 27 and FE 19 fibers may induce cytokine increase related to PLC activation in A549 cells comparing these effects with those of tremolite and crocidolite. The results revealed that all fibers induced PLC expression in lung epithelial cells following exposure to 50 μg/ml for 48 h but with a different level of expression induced by each fiber. Tremolite showed the highest level of expression of isoform PLC-β1 while FE 19 showed the highest level of expression of isoform PLC-γ1. FE 27 showed values of expression of both the comparable isoforms, while crocidolite fibers induced lower expression of PLC. The possible induction of oxidative stress was also examined by Cardile *et al* (64) by evaluating the intracellular ROS production, the amount of nitrite/nitrate and the expression of inducible nitric oxide synthase (iNOS). In this case, the cell lines exposed to FE and crocidolite included A549, J774 and human lung fibroblasts. The results indicated that the increase in ROS generation was directly proportional to fiber concentration and exposure time. Moreover, in all experimental cultures, $NO^{\bullet}$ synthesis and iNOS expression increased after crocidolite fiber but not after FE fiber exposure. Cell cytotoxicity was also evaluated assessing lactic dehydrogenase (LDH) release in A549, J774, human lung fibroblasts exposed to FE and crocidolite. The presence of LDH in culture medium is a marker of

membrane breakdown. The results revealed that LDH release was significantly increased when cell cultures, in particular A549 cells, were exposed to 50 μg/ml FE or crocidolite for at least 48 h, or the when the fiber concentration was increased to 100 μg/ml for 24 h (64).

The sensitivity to mineral fibers of A549 cells has also been compared with pleural mesothelial MeT-5A cells (54,69). The data demonstrated the critical role of epithelial and mesothelial cross-talk in FE fiber exposure and that these are able to induce functional modifications in a variety of parameters with crucial roles in cell cycle control, cancer development and progression. Pugnaloni *et al* (54) investigated the distribution of polymerized actin in A549 and MeT-5A cells exposed to FE fibers. Furthermore, the effects of FE exposure on the synthesis of vascular endothelial growth factor (VEGF), β-catenin (54) and retinoblastoma (Rb) (69), three critical steps of epithelial cell activation pathways, have been investigated. The results showed greater viability in A549 than in MeT-5A cells exposed to FE fibers (54,69) with consequent actin staining more irregular and granular than in the respective controls, suggesting that FE induces a dyregulated assembly of actin (54). The pathogenicity of FE fibers was demonstrated by the almost total arrest of cell movement at 48 h in both cell lines and by the flocculation of F-actin molecules. After a 48-h incubation, VEGF and β-catenin expression were evident in both cell lines (54), suggesting that FE fibers do not exert a primary toxic action inducing rapid cell death, but induce an abnormal cellular status with upregulated cell activities and a risk of cell transformation (54,70-72). In contrast to expectations, A549 and MeT-5A cells exposed to FE 19 fibers exhibited no change in Rb level, but overexpressed phospho-retinoblastoma (pRb) (69). The initial status of Rb changes with the cell cycle and it is regulated by the activity of cyclins D1, D2, D3 and CDK4 or CDK6 complexes in mid-G1 phase (73). Rb promotes cell cycle arrest and a return to the G1 phase; it has a central role in most instances of apoptosis, while pRb functions as a checkpoint in the G1 phase promoting cell growth. Since cyclin D1 is a sensor of cell division signals, it has been evaluated whether its expression is correlated with pRb expression; indeed, there was found a positive relationship in a dose-response manner in both cell lines (69). Furthermore, FE reduced the p27 expression both in A549 cells (69) and in MeT-5A cells (69,74), a tumor-suppressor gene due to its function as cell cycle regulator, that in cancer is often inactivated (75). The downregulation of p27 is associated with stathmin-1 upregulation in cancer, conferring an aggressive phenotype to cancer cells (74). In addition, fibulin-3 (Fb-3) overexpression may be responsible for the malignant transformation of MeT-5A cells after exposure to FE fibers (74). Fb-3 overexpression reflects a defensive response of the tissues after exogenous stimuli as FE exposure (76).

Functional *in vitro* experiments performed on MeT-5A and JU77 cells have been carried out in order to test the carcinogenetic effects and epigenetic modulation induced by FE exposure (77). The results showed that MeT-5A cells were more sensitive to FE fibers compared to JU77 tumor cells. The *in silico* analyses revealed a set of miRNAs strictly involved in MM and these have been used as *in vitro* experimental targets. The *in vitro* results showed that the expression levels of hsa-miR-323a-3p vared significantly in both supernatant- and cell-derived miRNAs derived from treated and untreated cells. Secreted and cellular hsa-miR-101-3p in MeT-5A cells exposed to FE fibers and JU77 cells showed different trends of expression. In regards to hsa-miR-20b-5p, there was no differential expression between secreted and cellular hsa-miR-20b-5p. This miRNA has shown a significant upregulation in JU77 cells vs. control and treated MeT-5A. Certainly, translational analyses will be performed on a subset of patients chronically exposed to FE fibers to further verify the clinical role of such miRNAs in high-risk individuals and their possible use as biomarkers of FE exposure or MM early onset (77).

The monocyte-macrophage J774 cells are more sensitive to FE than MeT-5A cells, suggesting that the primary site of the inflammatory response induced by mineral fibers could be the macrophage rather than the lung epithelium (78). The greatest sensitivity has been demonstrated in terms of heat shock protein 70 (Hsp70) induction, that was found to stimulate the formation of ROS and $NO^{\bullet}$. The form 19 was found to have a markedly strong effect on $NO^{\bullet}$ biosynthesis while the form 27 had a stimulatory effect on ROS generation in J774 cells, in contrast to MeT-5A cells. In contrast, detection of LDH release, which is a marker of cell necrosis, was found to be amplified in MeT-5A cells compared to J774 cell line (60,64,78). In general, tremolite is less effective than FE in producing more biological alterations, while it is inactive at 5 μg/ml. In opposition, at higher concentrations FE 19 is stronger than the other particulate (78).

Alveolar macrophages have an important role in the fibrotic process involved in silicosis and in other lung diseases (79). They are mediators in the interaction between inhaled particulates and different types of cells, by the release of a variety of inflammatory and growth-mediating factors (80). Cyclooxygenase-2 (COX-2) catalyses the conversion of arachinodic acid to prostaglandins (PGs) and it is mainly induced in response to proinflammatory stimuli, cytokines, growth factors and mitogens. It is known that PGs have an important role in cancer pathogenesis (81); therefore, Pugnaloni *et al* (54) demonstrated a time-dependent COX-2 overexpression and a PGE_2 increase in monocyte-macrophage J774 cells exposed to FE. PEG_2 derived from COX-2 was found to be involved in solid tumor pathogenesis through inhibition of apoptosis, facilitation of tumor cell invasiveness and promotion of angiogenesis (54).

4. *In vivo* studies concerned with MM due to FE exposure

It is important to underline that FE fibers were identified in the lungs of a housewife of Biancavilla, who died subsequently to a diagnosis of MM (45), in lung tissue (43,82-86) and lymph nodes (86,87) of sheep living in the Biancavilla area. Indeed, several studies have been performed to evaluate the relationship between FE and MM and to prove the biopersistence of these fibers in tissue.

Induced exposure of prismatic vs. fibrous FE. Different authors (36,88) have tested the same concentration of two mineral forms, prismatic and fibrous FE, with two administration methods, single intraperitoneal and intrapleural injection, on groups of Sprague-Dawley rats, to acquire more information on the potential relationship between exposure to FE and MM. The results are concordant and in line with previous preliminary data (64), and there is evident confirmation regarding a mesotheliomatogenic potential of FE fibers. In contrast, prismatic FE

failed to induce a mesotheliomatogenic response in the totality of the animals, in accordance to the controls (36,88).

The intraperitoneal injection of fibrous FE caused effects much stronger than those observable by intrapleural administration (36,88). In particular, 82.5% of the deceased rats treated by intraperitoneal injection died because of mesothelioma; only 14.3% of the deceased rats treated by intrapleural injection died due to mesothelioma induced by fibrous FE. The peritoneal tumors involved the abdominal cavity, with whitish and yellowish tissue on the surface of all the organs; furthermore a serosal effusion was present in almost all cases. Instead, the pleural tumors involved the visceral and/or parietal pleura and in 80% of the cases the diaphragm was largely involved with subsequent extension of the cancer into the peritoneal cavity (36).

Prismatic FE did not provoke mesothelioma in animal models. On the contrary, fibrous FE was found to cause a strong mesotheliomatogenic effect on the peritoneum and a milder extent on the pleura.

5. *Ex vivo st*udies concerned with MM due to FE exposure

Environmental exposure of fibrous FE. Sheep lung is comparable to human lung in architecture, volume, and respiratory parameters (89); therefore, it is a suitable model for toxicological studies concerning exposure to environmental pollutants (89), such as asbestos (20,90,91) or FE fibers (43,83-87,92).

One of the first experimental study (43) that analyzed sheep exposed to FE fibers present in the surrounding environment of Biancavilla, demonstrated that the first pathological event seems to involve the alveolar epithelium, resulting in classic honeycombing (43,83), and subsequently the interstitial matrix (43,83,84). Matrix metalloproteinase (MMP)-13 is mainly overexpressed in fibroblasts and epithelial cells, while immunopositivity of TNF-related apoptosis-inducing ligand (TRAIL) and its receptor death receptor 5 (DR5) are detected on alveolar surfaces and in the vascular stroma. The triggering event at the level of type I pneumocytes seems to damage the cytoplasmic membrane, resulting in loss of cell elements and exposure of underlying capillaries, and eventually in a series of reactions including macrophage activation, possible release of growth factors, metaplasic reconstruction of lung alveoli, and fibrosis (43). Loreto *et al* (83) demonstrated epithelial and interstitial Bax overexpression and negative Bcl-2 immunoexpression. pRb overexpression was also detected in FE-exposed sheep lung, in particular in alveolar epithelium and the interstitium, while Rb expression was absent (84). Immunopositivity for TRAIL and MMP-13 receptor (43), the changes in Bax and Bcl-2 (83), and the altered balance between Rb and pRb expression (84) can be considered a programmed response to protect the organism against uncontrolled cell proliferation, suggesting that apoptosis may be activated by FE fibers. A significant increase in the expression of CD68-positive macrophages, tryptase-positive mast cells, as well as a significant increase in microvascular density evaluated as CD31-positive areas in lung tissue of sheep exposed to FE fibers have been demonstrated. These data confirm the important role played by tumor microenvironment components in favor of angiogenesis in MM induced by FE exposure (85). Musumeci *et al* (92) investigated N-cadherin, ADAM-10 and aquaporin-1 (AQP1) expression in the lung tissue of sheep exposed to FE fibers, showing different patterns of immunolabeling. N-cadherin and ADAM-10 were more expressed in FE-exposed lung tissue, when compared with the control. On the contrary, AQP1 was more highly expressed in non-exposed lung tissue. These results suggest that N-cadherin, ADAM-10 and AQP1 are probably involved in different pathological processes induced by FE fiber exposure. The cellular and molecular toxicity mechanisms and the cellular response to FE fibers are still not well known, but these results highlight that molecules involved in carcinogenesis and in the inflammatory process participate in the network of events induced by exposure to FE fibers.

Loreto *et al* (86) demonstrated overexpression of MacroH2A.1, at the protein level, in lung epithelial cells and in lymph nodes of sheep exposed to FE fibers. The data suggest an involvement of MacroH2A.1 in the cellular response triggered by direct exposure to FE. The immunoreactions were detected in the areas where fibers were embedded and localized, in the lung, to the pulmonary and bronchial epithelium and not to the fibrotic interstitium. This significant research (86) seems to show a clear association between exposure to FE fibers and MacroH2A.1 expression view to identifying, in the future, a targeting epigenetics for cancer therapy.

Several studies have examined the lymph node draining pulmonary lobes of sheep grazing around Biancavilla (87,93). The results show a greater size of lymph nodes with signs of anthracosis. At the paracortical level, they show lymph-follicle hyperplasia with wide reactive corea and several macrophages containing grey-brownish particulate interspersed with elements with a fibril structure, forming nodules. Similar findings were detected in some peribronchiolar areas of the lung parenchyma. The FE fiber dimensions found in digested lymph nodes of sheep were similar to those found in the lung of a housewife from Biancavilla who died of MM as described by Paoletti *et al* (45). Indeed, sheep can be a biological indicator of environmental pollution by FE fibers, measuring these fibers in lymph node draining pulmonary lobes (87,93).

Several immunohistochemical investigations were conducted to demonstrate the implication of different compounds in MM due to FE fibers exposure and to investigate their potential role as diagnostic and prognostic markers. Angelico *et al* (94) demonstrated the prognostic role of AQP1 in FE-induced MM. In fact, the immunohistochemical overexpression of AQP1 was found to be associated with an increased median overall survival. Caltabiano *et al* (44) found high immunoexpression of Fb-3 in neoplastic cells with nuclear and cytoplasmic localization, demonstrating the implication of Fb-3 in MM due to FE exposure. Fb-3 could therefore have a potential role as a diagnostic and prognostic marker.

6. Discussion

The prevention of pathologies related to exposure to carcinogenic fibers such as asbestos and fluoro-edenite (FE), also includes the reduction of these fibers in the environment. Generally, this can be achieved in three ways: Reclamation, encapsulation and confinement. These interventions tend to eliminate airborne fibers to avoid exposure that can cause various diseases including cancer.

The observation of a significant incidence of MM, subsequently linked to the inhalation of FE fibers of Biancavilla, has

been reported by epidemiological studies (45-48). Previous cross-sectional studies conducted on subjects exposed to FE fibers confirm the *in vitro*, *in vivo* and *ex vivo* data. In fact, it has been demonstrated that exposure to FE fibers may induce autoimmunity (95,96), and the involvement of the inflammosome (41).

This malignant cancer is a highly aggressive neoplasm of the serosal membranes lining the pleural cavity (97). Only 5% of MM patients are diagnosed at an early stage (98) and the median survival is approximately 6-12 months (97,99). Moreover, current treatment for MM, which is based on surgery and standard chemotherapy, has a modest effect on the overall survival (OS), which remains dismal (4).

The diagnosis of MM is always challenging as MM may appear in patients up to 30-40 years after exposure to carcinogenic fibers; the clinical and imaging signs of MM are non-specific; and a definitive diagnosis, which relies on histology, can sometimes be very difficult to achieve, even with the use of immunohistochemistry. To date, no single marker or panel of soluble biomarkers is available for a clear diagnosis of MM (100).

Many biomarkers have been proposed for screening and diagnosis of MM in subjects exposed, such as calretinin, cytokeratin 5 (CK5), podoplanin, mesothelin, osteopontin, hyaluronic acid, Fb-3 (44), VEGF (101), AQP1 (94), high mobility group box 1 (HMGB-1) (102), and MacroH2A.1 (86). Mesothelin is the only Food and Drug Administration (FDA)-approved biomarker for MM (103-105), but with limitations (106). In fact, the poor sensitivity of mesothelin clearly limits the added value to the diagnosis of MM (100).

Some studies have been conducted to understand the link between common genetic variations in the molecular pathways and cancer risk with the final goal to develop novel therapeutic targets. Lim *et al* (107) reported mutations in SMO and SUFU and a novel multi-exonic deletion in PTCH1 in MM cell lines and tumors. These data suggest that aberrant activation of the Hedgehog (HH) signaling in MM is unlikely to be driven by mutations in the majority of tumors but instead activated through autocrine signaling (107,108). This pathway may represent a novel therapeutic target in MM for recently developed HH pathway inhibitors.

Several studies have demonstrated that microRNAs (miRNAs) may be used as valuable non-invasive diagnostic and prognostic biomarkers for various human diseases, including cancers (42,109,110). In the clinical setting, circulating cell-free miRNAs and fecal miRNAs are the main forms of RNA used as diagnostic biomarkers (111). In particular, a recent review of the literature by Ledda *et al* (112) indicates a list of miRNAs potentially involved in MM. Potential miRNA biomarkers for this malignant neoplasm include the following: miRNA-126-3p, miRNA-625-3p, miRNA-103a-3p, miRNA-16-5p, miRNA-143-3p, miRNA-145-5p, miRNA-192-5p, miRNA-193a-3p, miRNA-200b-3p, miRNA-203a-3p, and miRNA-652-3p. The scientific community has revealed that several miRNAs are involved in deregulation and in all molecular mechanisms associated with MM development (113,114) and constantly updates the miRNAs which can be associated with MM early diagnosis and prognosis.

In recent studies, several bioinformatics approaches to identify selected miRNAs highly deregulated in cancer samples when compared with normal control have been developed (77,115-118). The *in silico* study of the expression of certain miRNAs represents an effort in the field of biomarker discovery because in this way it is possible to analyze the data coming from multiple studies of miRNA profiling; in this way it is possible to have a large series of samples useful to obtain truthful expression data concerning miRNAs with a potential diagnostic and prognostic role in cancer. In addition, the development of new high-sensitivity technologies and the analysis of liquid biopsy samples and circulating tumor DNA are paving the way to new non-invasive validation studies aimed to discover new promising diagnostic and prognostic biomarkers for several pathologies, including MM (119-121).

Therefore, future studies will be conducted to understand the link between common genetic variations in the molecular pathways and cancer risk with the final goal to develop novel therapeutic targets. Research is needed in order to computationally select putative miRNAs involved in the development and progression of lung cancer or MM and to be validated in correspective *in vitro* tumor models and in a subset of patients chronically exposed to FE. It could also be particularly helpful to study and subsequently use a combination of several protein and molecular markers to improve diagnostic accuracy.

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Authors' contributions

Conceptualization of the review was accomplished by VF and CL. The research methodology was designed and conducted by VF, EV and GB. Validation of the research data was conducted by VF and CL. Formal analysis was carried out by VF. Investigation of the data was carried out by VF, EV and GB. Data curation was conducted by VF, MPH and SC. Writing-original draft preparation was carried out by VF and CL. Writing-review and editing was accomplished by VF, MPH, SC and AS. Supervision was conducted by CL. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors report that they have no competing interests.

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