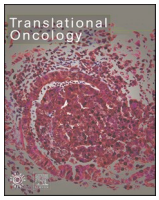
Translational Oncology 40 (2024) 101872

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon



Mesothelin methylation, soluble mesothelin related protein levels and inflammation profiling in workers chronically exposed to naturally occurring asbestos fibers

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ARTICLE INFO

Keywords:
Asbestiform fibers
Malignant pleural mesothelioma
Naturally occurring asbestos
Biancavilla
Fluoro-edenite
Epigenetic
Methylation
Mesothelin
Inflammation
Soluble mesothelin related protein

ABSTRACT

Exposure to asbestiform fibers, including chrysotile and amphibole, is carcinogenic, causing malignant pleural mesothelioma (MPM) when inhaled. Some populations globally face Naturally Occurring Asbestos (NOA) exposure, leading to MPM cases like in Biancavilla, Italy, from Fluoro-edenite (FE) contamination. Studies show NOA exposure causes epigenetic changes, focusing on mesothelin methylation, an MPM marker, and altered inflammation, emphasizing the health risks of FE and asbestos. This research, conducted from February 2022 to October 2022, studied 125 construction workers from Biancavilla and 125 controls from 40 km away without Biancavilla work history. With at least ten years in construction and no respiratory conditions, participants underwent medical assessments and gave blood samples for analysis, including inflammation markers, mesothelin methylation, and soluble mesothelin-related protein levels. The results showed similar demographics but differing inflammation and methylation levels in exposed workers, suggesting long-term cellular changes. Pearson correlation showed intricate biomarker relationships. Significant inflammatory differences were found between FE exposed and non-exposed workers, indicating potential health impacts from FE. This raises concerns for communities like Biancavilla, emphasizing the importance of extensive epigenetic research for public health.

Introduction

Exposure to all forms of asbestiform fibers (chrysotile, crocidolite, amosite, tremolite, actinolite and anthophyllite) is widely recognized as carcinogen [1]. Asbestos causes mesothelioma and cancer of the lung, larynx, and ovary [1]. The type of tumor most frequent following exposure is malignant pleural mesothelioma (MPM) triggered by the inhalation of asbestos fibers due to environmental or occupational exposure [2,3].

The silicate mineral physique variety may be fibrous or non-fibrous and, among the minerals which produce the airborne particulate, the highly harmful ones present a fibrous-asbestiform crystal frame [4]. The term Naturally Occurring Asbestos (NOA) indicates to asbestos minerals present in rocks and soils to differentiating them from those included in asbestos containing materials [5]. Though amphibole minerals are widespread, fibrous and asbestiform amphiboles are fewer and necessitate particular geologic activities that stimulate the evolution of fibers, in specific rock deformation throughout or successive to amphibole development [6].

Six fibrous silicate minerals fitting to the serpentine (i.e., chrysotile) and amphibole (i.e., tremolite, actinolite, anthophyllite, amosite, and crocidolite) mineral groups are classified as asbestos by law in Europe and in various states worldwide [4]. Nevertheless, several investigations establish that also these six types, others asbestiform fibers such as erionite, antigorite and Fluoro-edenite (FE) could also be hazardous if inhaled by humans, leading to several respiratory diseases [5,7,8].

Environmental exposure to NOA occurs as a result of a natural activity or social actions in regular lifetime situations, such as agriculture, transport, construction, leisure exposures and simply living close to the source of exposure [5,9–11].

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https://doi.org/10.1016/j.tranon.2023.101872
Received 30 September 2023; Received in revised form 10 December 2023; Accepted 15 December 2023
Available online 21 December 2023
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Several investigations have also demonstrated that there is an association between the beginning of MPM and environmental asbestos (natural and not natural) exposure [8,10,12–17]. Many populations worldwide are exposed to NOA, in Canada has been assessed the lifetime mortality risk of lung cancer and MPM related wide-ranging between 1.4 and 4.9 per 100.000 persons incessantly environmentally exposed to asbestos for 80 years in a mining city [18]. In Turkey, the incidence of MPM was higher in zones nearby NOA and associated with the prevalent wind direction [19]. Similarly, environmental research in Minnesota (US), discovered that environmental exposure to Libby fiber (vermiculite) increased the incidence and mortality of Asbestos Related Diseases (ARD), including MPM [20].

In Italy in the 1990s, a cluster of deaths from MPM was reported in a town in eastern of Sicily: Biancavilla [10,11]. An environmental assessment indicated that the stone quarries located southeast of the city could be a source of asbestos exposure due to FE [10,11]. The raw material mined from the quarries was utilized, for about 50 years, extensively in the local construction, so the buildings contain significant numbers of FE. The derived material had been used locally for construction purposes [10,11]. Subsequent some in vitro, in vivo and epidemiological studies the IARC (Lyon, France) classified FE as carcinogenic to humans, but only for MPM [21]. Consequently, there are prior surveys on health exposures due to environmental and occupational exposure to FE in people living around the source of exposure to FE. In fact, previous studies have shown an involvement of the inflammatory processes in exposed subjects [22–24].

Though the etiology of MPM is absolutely recognized, therapeutic advances have been inadequate. The effect of chemotherapy on the patients with MPM is even now poor, the median survival being around 8–12 months [25]. The elevated mortality rate connected with MPM is principally attributable to the lack of effective screening approach for early detection [25].

Previous in vitro tests implemented on normal pleural mesothelial cell line (MeT-5A) and MPM cell line (JU77) carried out in order to examine the carcinogenetic effects and epigenetic modulation stimulated by FE exposure showed that the expression levels of hsa-miR-323a-3p, hsa-miR-101–3p and hsa-miR-20b-5p were correlated with the exposure to FE [26], this results were confirmed *in silico* analysis carried out on MPM due to FE tissue [27].

Although genomic alterations are clearly correlated with oncogenesis, recent investigations suggests that modifications that are not instantly found in the DNA sequence as well perform an important role in carcinogenesis [28]. These “epigenetic” alterations involve sequential and spatial control of gene action necessary for homeostasis [29]. Moreover, epigenetics involves heritable and reversible modifications regulating a range of processes for instance RNA elongation, mitosis, DNA replication and repair and additionally plays a key role through carcinogenesis [28,29].

Furthermore, chronic exposure to NOA has been recently linked to distinct epigenetic shifts, with particular attention being given to the methylation patterns of mesothelin, a biomarker often elevated in malignant pleural mesothelioma cases [15,28,30]. Mesothelin methylation acts as a possible early indicator of carcinogenesis [30]. Moreover, these epigenetic changes are coupled with altered inflammation profiles, suggestive of an immune response to the inhaled fibers. Elevated levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, have been consistently identified in serum samples of individuals residing in areas with known FE contamination [22] . Such inflammatory markers, when analyzed in tandem with epigenetic modifications, provide a holistic understanding of the underlying molecular and immunological shifts prompted by FE. This dual-perspective is crucial, as it underscores the combined genetic and environmental factors that contribute to FE-related health risks. Therefore, the aim of this study is to investigate the epigenetic alterations, specifically mesothelin methylation, and the inflammation profiles of workers chronically exposed to FE fibers.

Materials and methods

Study design and population

In a reference to a previous study conducted in 2016 [22], the workers selected for the current investigation were part of a larger cohort. This cohort has been continuously and methodically monitored over the years by the research team, as documented in various publications [8,10,17,22,24,31,32]. This ongoing assessment provided a rich longitudinal data set, allowing for a more nuanced understanding of the health impacts over time.

For this nested case-control study, which involves selecting cases and controls from a pre-existing larger cohort, 125 construction professionals from Biancavilla (Sicily, Italy) were selected. Similarly, a control group comprising 125 construction workers, who had worked at least 40 km away from Biancavilla, was established. This study design is advantageous in leveraging existing data, allowing for a more efficient and in-depth analysis of the relationship between occupational exposure and health outcomes in a defined population.

For the sample selection, criteria for inclusion and exclusion of participants were applied. Participants were required to have at least ten years of experience in the construction sector. For those in the control group, any previous employment history within the Biancavilla area was a criterion for exclusion. Individuals previously diagnosed with respiratory disorders such as asthma, bronchopneumonia, and tuberculosis were not considered. Additionally, any historical asbestos exposure meant automatic disqualification for the control group members. Data gathering was facilitated by a structured questionnaire, covering areas like medical background, employment history, medication use, and lifestyle habits, encompassing smoking and alcohol consumption. Furthermore, each participant underwent a comprehensive medical evaluation, which included spirometry.

Blood collection, soluble mesothelin related protein levels, mesothelin methylation and inflammation profiling analysis

In the morning, after an overnight fast, 10 ml of venous blood was drawn to evaluate parameters including red and white blood cell counts, haematocrit, hemoglobin levels, erythrocyte sedimentation rate, C-reactive protein levels, and liver enzymes such as aspartate aminotransferase and alanine aminotransferase.

For the purpose of inflammation profiling and mesothelin levels assessment, blood was collected into vacuum tubes containing gel and a clot activator (Vacuette, Greiner Bio-One, Kremsmünster, Austria) in preparation for analysis. Once drawn, these samples were allowed to stand vertically at room temperature for a duration ranging from 30 to 60 min . Following this, they were centrifuged at 3500 rpm for a span of 10 min . The resultant serum was isolated and preserved at a temperature of −20 °C pending further analysis. Levels of serum interleukin-1β (IL-1β), IL-6, IL-8, and TNF-α were determined utilizing sensitive quantitative sandwich enzyme-linked immunosorbent assays (Quantikine ELISA Kit, R&D systems, USA) [22,33].

Soluble mesothelin related protein (SMRP) levels were quantified utilizing the MESOMARK™ ELISA kit (Fujirebio Diagnostics, Inc., Malvern, PA, USA) following the manufacturer’s guidelines. SMRP values at or exceeding the established threshold of 2.9 nM were interpreted as positive outcomes.

For mesothelin methylation (MSLN) analysis Genomic DNA was isolated from 200 µl of whole blood employing the QIAwave DNA Blood & Tissue Kit (QIAGEN, Hilden, Germania), adhering to the manufacturer’s guidelines. The NanoDrop 2000C (Thermo Scientific, USA) was utilized to ascertain the DNA’s quantity and purity. A sample of 500 ng of DNA underwent sodium bisulfite modification, after which it was purified using the EpiJET Bisulfite Conversion Kit (Thermo Scientific, USA). DNA was then eluted using twelve microliters of M-Elution Buffer and preserved at −80 °C for subsequent analysis.

Consistency in measurements was ensured by calibrating the instruments and conducting internal quality checks, using control and calibration materials from the same lot provided by the manufacturer throughout the research period.

Real-time PCR analysis of mesothelin methylation was conducted following the protocol delineated by Yu et al. [34]. In briefly, the specific primer sequences for MSLN methylation (M) and non-methylation (U) were: MSLN (M): (F) 5′-GGG GTA AAG TTT TTT ATT TAA TTG C-3′, (R) 5′-AAC ACC GTA AAT CCA CCG AT-3′, and the amplification length was 233 bp; MSLN (U): (F) 5′-GTT AGG GGT AAA GTT TTT TAT TTA ATT GT-3′, (R) 5′-AAA AAA CAC CAT AAA TCC ACC AAT-3′, and the amplification length was 241 bp.

The Fast PCR system (Applied Biosystems, USA) was employed with the following parameters: 38 cycles for MSLN (M) at 95 °C for 3 s and 64 °C for 30 s, and for MSLN (U), conditions included 95 °C for 3 s and 62.5 °C for 30 s. The methylation percentage (M %) was derived using the formula: M % = 100 × (amount of methylated DNA/total of methylated and unmethylated DNA). The combined quantities of methylated and unmethylated DNAs represented the total DNA amount of the target genes. DNA methylation levels were categorized based on M % values as follows: 0 for M % ⟨ 20.0; 1 for 20.0 < *M* % < 40.0; 2 for 40.0 < *M* % < 60.0; 3 for 60.0 < *M* % < 80.0; and 4 for M % ⟩ 80.0. Categories 0, 1–3, and 4 were designated as unmethylated (U), partially methylated (U/M), and fully methylated (M), in that order. As controls, both methylated and non-methylated human DNA sets (Zymo Research, USA) were incorporated.

Statistical analysis and ethical issues

Data were summarized as mean ± SD for continuous variables and frequencies for categorical variables. Normality was checked by the Kolmogorov-Smirnov test and homogeneity of variance by Levene's test. The T-test was used for analyzing continuous variables, and Fischer's test for categorical variables. Pearson correlation was employed to assess the linear relationship between two continuous variables. All statistical analyses were performed using jamovi.

The workers were recruited as part of the occupational health surveillance protocol mandated for all workers in Italy.

Ethics statement. The research protocol received the approval of the Ethics Committee of Catania University Hospital (Catania, Italy) and the written informed consent of all subjects was acquired including them in the study.

Results

In Table 1, a comparison between exposed and non-exposed workers provides insights into the potential effects of occupational exposure. In terms of demographic factors, both groups had comparable ages, with the exposed workers averaging 54.26 years and their non-exposed counterparts at 54.66 years, suggesting that age did not play a discriminating role between the two groups. However, this marked difference did not achieve statistical significance, suggesting that age might not be a determinant factor in this context. Similarly, the Body Mass Index (BMI) values for both groups were closely aligned, with exposed workers registering 21.28 and non-exposed workers at 20.82, with no significant variation between them. Smoking frequencies were identical across both groups, each at 6 %. The duration of employment in their respective fields, represented as working age, was also comparable between the two groups.

Turning our attention to the inflammatory markers and other relevant indicators, distinct patterns emerged. Levels of interleukin-1β (IL-1β) were significantly elevated in exposed workers compared to the non-exposed group, indicating potential inflammatory responses or other cellular activations specific to the exposed group. While the concentrations of IL-6 and IL-8 were fairly consistent between the two groups, a marked elevation in TNFα was observed in the exposed workers. This is notable as TNFα is often implicated in inflammatory processes and can be an indicator of heightened immune responses. Additionally, SMRP levels were significantly higher in the exposed group, which might be indicative of mesothelial cell activity or injury (Fig. 1).

Table 1
Comparative analysis of worker characteristics and health outcomes.

	Exposed workers (n.125)	Non exposed workers (n.125)	*p* Value
Age (years-mean ± SD)	54.26 ± 0.42	54.66 ± 2.40	n.s.
BMI (mean ± SD)	21.28 ± 1.39	20.82 ± 1.41	n.s.
Smoking (frequencies)	8 (6 %)	8 (6 %)	n.s.
Working age (years-mean ± SD)	24.54 ± 3.55	24.37 ± 3.34	n.s.
IL-1β	34.128 ± 5.977	22.175 ± 2.042	<0.001
IL-6	14.899 ± 2.666	14.630 ± 2.647	n.s.
IL-8	17.464 ± 1.703	17.771 ± 1.556	n.s.
TNFα	30.724 ± 3.426	10.083 ± 1.710	<0.001
SMRP	0.679 ± 0.406	0.372 ± 0.227	<0.001
MSLN			
U *	57 (46 %)	112 (90 %)	<0.001
*U/M**	13 (10 %)	8 (6 %)	<0.001
M *	55 (44 %)	5 (4 %)	<0.001

* U: unmethylated;U/M: partially methylated; M: fully methylated.

This table presents a detailed comparison of various characteristics and health markers between workers who were exposed and those who were not exposed to FE. The statistical significance of the differences is indicated by the p values.

Perhaps most compelling was the data on mesothelin methylation. A vast majority of non-exposed workers displayed an unmethylated profile, contrasting sharply with the exposed group where the distribution was almost evenly split between unmethylated and fully methylated profiles. The findings underscore the potential epigenetic modifications in exposed workers, potentially hinting at long-term cellular changes resulting from their occupational exposures.

From the Pearson correlation analysis (Fig. 2), it is evident that there exists a negative correlation between IL-8 and each of IL-1β, IL-6, and TNFα (*p* Value <0.001). Conversely, a positive correlation is observed between IL-1β and TNFα, and similarly between SMRP and both IL-1β and TNFα (*p* Value <0.001). These findings suggest distinct interrelationships among these biomarkers, underscoring the intricate biochemical interactions within the study population.

Discussion

The results of exposed and non-exposed workers revealed significant differences in specific inflammatory markers despite similar demographics. Notably, exposed workers exhibited increased IL-1β and TNFα levels, and pronounced alterations in SMRP values and mesothelin methylation profiles. These changes suggest potential inflammatory responses and cellular changes due to occupational exposure. The Pearson correlation further highlighted distinct inter-relationships among the biomarkers, indicating intricate biochemical dynamics within the study population. The significant disparities in inflammatory markers between exposed and non-exposed workers, despite analogous demographics, raise pivotal questions about the specific physiological repercussions of occupational exposures. In light of existing research, the elevated levels of IL-1β and TNFα in exposed workers are not unprecedented. Similar pro-inflammatory cytokine elevations have been observed in individuals exposed to agents like asbestos. This parallel suggests that FE, much like asbestos, might elicit an innate inflammatory response when inhaled. This inflammatory cascade is potentially the body's defense mechanism, attempting to combat the intrusion of FE particles into the pulmonary system. In fact, the presence of FE in the workplace environments of exposed individuals offers a plausible explanation for the observed biological variations. FE could be the driving factor behind the heightened IL-1β and TNFα levels seen in

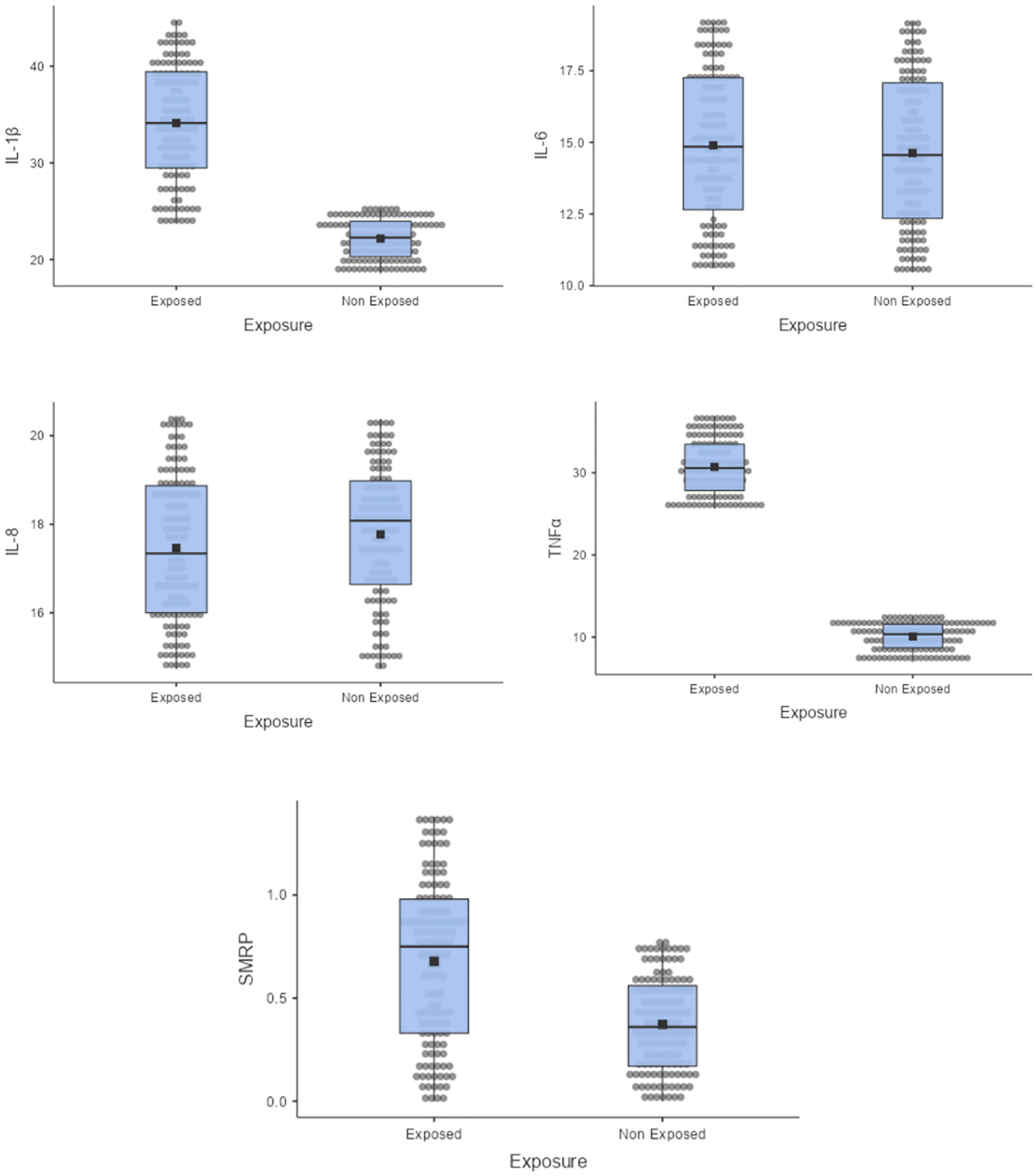

Fig. 1. Box and whisker plots illustrating the distribution of SMRP levels and inflammatory markers between exposed and non-exposed worker groups.

exposed workers. These specific elevations are consistent with the body's response to the asbestos like fiber [35]. It is important to note that TNF belongs to the family of pro-inflammatory mediators and has been previously correlated well with the degree of inflammation of the skin, the number of cells, and vascular changes in the inflamed area, as evidenced by prior studies [36]. Furthermore, elevated concentrations of TNF have been observed in other diseases, where it is associated with the formation of fibrous tissue in myelofibrosis and with carcinogenesis, adding to its significance in the context of FE exposure [37].

Interestingly, the uptick in SMRP values among the exposed group is a finding that bears significant clinical implications. Historically, heightened SMRP levels have been a hallmark in individuals diagnosed with mesothelioma, serving as an indirect indication of mesothelial cell distress or even potential malignancy [38]. Given this association, the elevated SMRP values among the exposed workers in our study raise concerns. Moreover, the conspicuous changes in SMRP values and mesothelin methylation profiles further imply that the cellular environment of exposed workers is responding to the external factor, the FE. The very nature of this mineral fiber, when inhaled, can instigate inflammatory cascades, tissue damage, and epigenetic alterations, phenomena that resonate with the observed changes in the aforementioned biomarkers [39]. The subsequent Pearson correlation analysis further emphasizes the complex interplay between these biomarkers, perhaps shedding light on the nuanced biochemical repercussions of FE exposure in the workplace.

The observed disparities in mesothelin methylation between the two

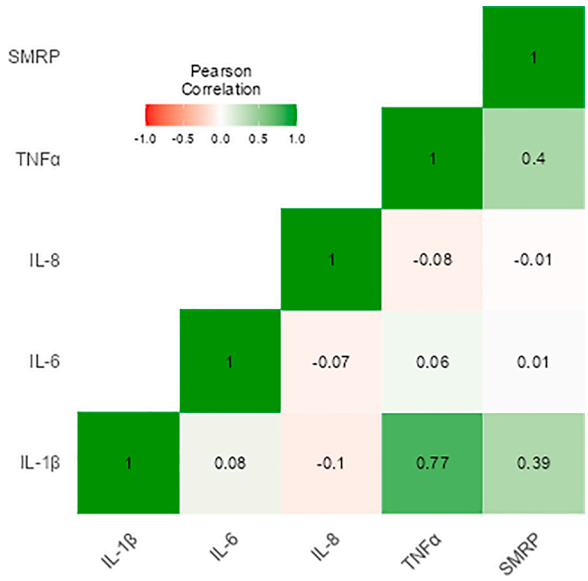

Fig. 2. Heatmap of Pearson correlation coefficients for SMRP and inflammatory biomarkers.

groups of workers are indeed striking and warrant closer attention. Methylation, as an epigenetic modification, often signals the regulation of gene expression without altering the underlying DNA sequence [28]. In the context of this study, the elevated methylation profiles in the exposed workers imply that their occupational environment might be triggering epigenetic alterations, specifically in the mesothelin gene.

The pronounced contrast, where the vast majority of non-exposed workers predominantly showcased an unmethylated profile as opposed to the nearly balanced distribution in the exposed group, suggests that the work environment of the latter has a profound influence on their genetic regulation. Mesothelin, being often implicated in various cellular pathways and notably in certain malignancies, highlights the importance of this epigenetic change [40].

The fact that methylation modifications can endure and even be passed down through cell divisions may be indicative of persistent and long-lasting cellular changes in exposed workers [41]. This can be especially concerning if we consider that such modifications might predispose individuals to health conditions or diseases in the long term. In essence, the stark differences in mesothelin methylation patterns reinforce the notion that environmental and occupational factors can leave lasting imprints at the cellular level, potentially impacting the health trajectory of exposed individuals.

The burgeoning awareness surrounding potential hazards associated with asbestos exposure is pivotal, primarily due to the extended latency period before the onset of asbestos-related diseases (ARDs) in particular to MPM [42]. Previous endeavors in enhancing the methodology for health surveillance of asbestos-exposed populations reflect a global cognizance of the issue at hand [43]. Our current research effort underscores three cardinal points, each contributing to the growing knowledge base on this matter.

The observation of the present investigation sheds light on the SMRP values within healthy individuals with a history of FE exposure. This highlights the significant differential effects between occupational and non-occupational exposure scenarios. Non-occupational exposure scenarios, such as household contamination and neighborhood exposure, present unique challenges due to the inherent difficulty in accurately quantifying exposure durations. Given that the exposure to FE is also environmental, this brings forth the vital realization that while SMRP might serve as an indicator, its utility as a discriminating biomarker between exposed and unexposed subjects remains contentious, given its independence from estimated exposure levels [44].

The results suggests that factors like age, BMI, and smoking function may not entirely account for the surge in SMRP values among exposed subjects. Smoking, a common confounder in respiratory studies, also did not significantly influence SMRP values. Such insights further bolster the potential application of SMRP as a diagnostic aid, especially when adjusted for these confounding variables [45].

Lastly, our inquiry into the methylation status of the MSLN promoter region offers nuanced insights into the epigenetic ramifications of asbestos exposure. The associations between aberrant methylation profiles and prolonged asbestos exposure, as highlighted by prior studies, underscore the multifaceted impact of this hazardous material [46]. Our attempt to draw correlations between methylation status and SMRP levels has indeed provided significant revelations. Even though the overarching methylation profiles remained relatively unchanged across groups, distinct variations in SMRP levels emerged. This dichotomy highlights the intricate relationships between genetic and epigenetic landscapes in the face of external exposures. Furthermore, the widespread applicability and ease of procuring peripheral blood as a sample source, as seen in this study and others [47–49], make it an attractive option for future research.

While our study contributes significantly to understanding the role of SMRP and methylation in the context of NOA exposure, it also emphasizes the complexity of the epigenetic landscape. The nuanced interactions between various CpG sites, methylation status, and external exposures [50], as evidenced in our findings, underscore the necessity for a more expansive, in-depth epigenetic analysis in the future. Unraveling these complex interrelationships will undoubtedly bolster our understanding of the pathogenesis of ARDs and MPM and potentially illuminate novel diagnostic and therapeutic avenues [51].

In the rapidly evolving field of environmental health research, there's an imperative need to understand not just the immediate effects of specific occupational exposures, but also the broader ramifications on populations living in close proximity to sources of these exposures. The town of Biancavilla presents a unique case where the endemic presence of FE extends the risk not only to workers directly handling this material but also to the general populace residing in the area.

FE, with its asbestos-like properties, poses a substantial health risk due to inhalation of airborne fibers, even at environmental levels. Past studies have predominantly focused on the acute effects in occupationally exposed individuals, highlighting inflammatory responses and other cellular alterations [23,23,26,32,32]. However, the health implications for the general population, subjected to chronic, albeit lower-level, environmental exposure remains under-explored. One of the crucial insights from previous research on occupational exposure is the revelation about mesothelin methylation profiles. Epigenetic modifications, especially DNA methylation, have emerged as sensitive indicators of environmental exposures and potential precursors to various health outcomes. Given that the mesothelin gene is often implicated in various cellular pathways and in certain malignancies, its methylation status could serve as an invaluable biomarker, even before the onset of clinical manifestations. It is, therefore, logical to hypothesize that the general population of Biancavilla, being continuously exposed to FE, might also exhibit similar epigenetic alterations. By extending the analysis of mesothelin methylation to the general populace, it could not only deepen our understanding of the broader health implications of FE exposure but also provide early indicators for potential adverse health outcomes. Furthermore, while the intensity of exposure might be attenuated for the general population compared to direct occupational exposure, the prolonged and consistent nature of their interaction with FE might culminate in significant epigenetic changes over time. Understanding these subtle changes can pave the way for early interventions, policy modifications, and public health advisories that ensure the well-being of the community at large.

The study, examining the health impacts of asbestos exposure due to FE in Biancavilla, Italy, has some strengths and weaknesses. Its major strengths include a comprehensive cohort selection of 125 construction

workers from Biancavilla and an equal number of controls from a distance, ensuring a robust comparative framework. The study's methodology is rigorous, involving participants with at least ten years in construction and no respiratory conditions, which helps minimize confounding factors. It significantly contributes to the field of epigenetic research by focusing on mesothelin methylation, a marker for MPM, and highlights the importance of such research for public health.

However, the study also has limitations. The geographical scope, limited to a specific region, might affect the generalizability of the findings to other populations. By focusing solely on construction workers, the study potentially overlooks the impacts of FE exposure in other occupations or the general population, which could have different risk profiles. Despite efforts to control for confounding factors, there may still be unaccounted variables such as lifestyle habits or genetic predispositions influencing the results.

In conclusion, there is a hope that authorities will consider the idea of using biomarkers for screening the general population of Biancavilla. This advancement not only deepens our understanding of the effects of FE but also highlights a comprehensive approach to public health. It recognizes that, within the interconnected ecosystems of the modern world, the line between direct and indirect exposure is ever more indistinct. Against this backdrop, the idea of expanding occupational health surveillance to all workers and the broader population of Biancavilla is being considered, which would facilitate an in-depth assessment of the long-term health impacts from environmental exposures. In light of this, the possibility of expanding occupational health surveillance to all workers and the general population of Biancavilla is being considered, which would allow for a comprehensive assessment of the long-term health impacts of environmental factors.

CRediT authorship contribution statement

Caterina Ledda: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Carla Loreto:** Conceptualization, Methodology, Project administration. **Claudia Lombardo:** Data curation, Formal analysis, Investigation. **Venera Cardile:** Conceptualization, Funding acquisition. **Venerando Rapisarda:** Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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