



Absence of phagolysosomal activation and high clearance efficiency define the low pulmonary toxicity of short asbestos fibers

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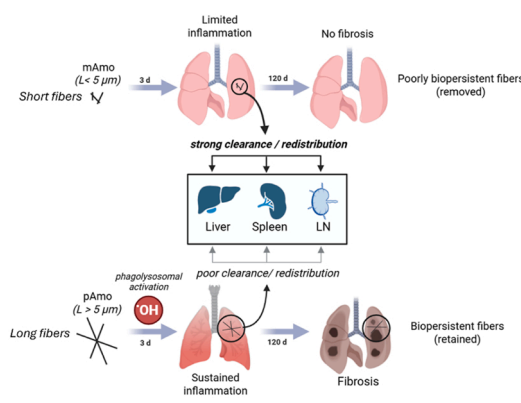
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HIGHLIGHTS

- Long amosite asbestos fibers (pAmo) induce stronger acute/chronic inflammation and fibrosis than short counterparts (mAmo).
- Phagolysosomal activation, immunogenic cytotoxicity, and sustained lung biopersistence drive long fiber toxicity.
- Short amosite fibers (mAmo) are not-immunogenic, efficiently cleared from the lungs, but show distribution to distal organs.
- ICP-MS provides a reliable and sensitive technique to quantify asbestos burden in tissues after exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

The physicochemical properties of fibers critically determine asbestos pathogenicity, driving inflammation, fibrosis, and lung cancer. The prevailing paradigm in fiber toxicology posits that long and biopersistent fibers pose a greater health risk than short fibers. However, this assumption is debated due to limited studies specifically assessing the pathogenicity of short fibers. In this study, we compared the lung proinflammatory and profibrogenic effects of two amosite fiber types, long pristine amosite (pAmo, L > 5 μm) and short milled amosite (mAmo, L < 5 μm). In instilled C57BL/6 mice, long pAmo were significantly more inflammogenic and fibrogenic than short mAmo, which only induced limited acute pulmonary toxicity. Radical production under acidic

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phagolysosomal conditions occurred with long pAmo but not mAmo, explaining the pronounced pulmonary responses to pAmo. ICP-MS analysis revealed that long pAmo persisted in lung tissue, whereas short mAmo was completely cleared within four months. Nevertheless, a substantial fraction of short mAmo was biodistributed to extrapulmonary organs, including liver, spleen, and mediastinal lymph nodes in comparison to long pAmo. In conclusion, these findings establish fiber length as a critical determinant of asbestos-induced pulmonary pathogenicity and demonstrate that the limited pulmonary toxicity of short amosite fibers results from their low reactivity and rapid lung clearance. Moreover, the significant extrapulmonary translocation of short amosite observed in our study underscores the importance of considering the potential systemic effects of small fibers.

1. Introduction

Asbestos is the commercial name of a group of naturally occurring fibrous silicate minerals having excellent properties (tensile strength, heat and chemical resistance) which result from peculiar physico-chemical features [1,2]. As evidenced in several epidemiological and toxicological studies, exposure to asbestos minerals, including fibrous amphibole minerals (e.g. crocidolite and amosite), and fibrous serpentine (i.e., chrysotile), is associated with severe chronic diseases, such as inflammation, asbestosis, mesothelioma and lung cancer [3–5]. For this reason, in 1989 the International Agency for Research on Cancer (IARC) included asbestos in Group 1 as carcinogen for humans [6], and more than 60 countries in the world have banned mining and using asbestos to date, with some exceptions [7]. Despite asbestos production and consumption has considerably declined worldwide [8], the exposure to other naturally occurring asbestos (NOA) or elongated mineral particles (EMPs, Aspect Ratio Length/Width ≥ 3 , following NIOSH 2011 definition) that exhibit asbestos-like structure and properties is of concern for human health [9,10]. The paradigm explaining inflammogenicity, fibrogenicity and carcinogenicity of asbestos and asbestos-like EMPs appears to lie on a complex interplay between fiber reactivity and dimension (length and width), ultimately impacting fiber pulmonary biopersistence [11–14]. Indeed, sufficiently long, thin, and poorly soluble fibers, such as crocidolite and amosite, are known to impair the ability of alveolar macrophage (AM) to dissolve or physically remove them from the lungs (frustrated phagocytosis) by the lymphatic system [15,16]. In addition to the direct carcinogenic effect, long and reactive asbestos fibers accumulated in the lungs generate radical species (ROS), trigger oxidative stress, and induce the release of damage-associated molecular pattern molecules (DAMPs) which activate immune responses synergistically operating to develop inflammatory pathologies [17,18].

The role of biopersistence and pulmonary clearance of long asbestos fibers, commonly associated with carcinogenic potential [3,19–22], has been extensively investigated in animal models [21]. Studies on asbestos fibers clearance carried out in rat showed a clearance half-time longer than 10 months for crocidolite, suggesting size-dependent biopersistence to be influenced by fiber dimension and solubility [23,24]. Conversely, a pulmonary clearance half-time of 11.4 days was observed for chrysotile fibers (which dissolve faster than crocidolite) longer than 20 μm [25]. To date, in contrast, there is no study specifically dedicated to address the biopersistence of short amphibole fibers ($L < 5 \mu\text{m}$), whose pulmonary toxicity is still debated [10,12,21,26–29]. Inhaled fibers accumulated in the pulmonary interstitium can be removed via lung lymph flow and then distribute to the whole body by the bloodstream [30,31]. Also, it is recognized that short fibers are physically cleared from the lungs following internalization by AM, if small enough to be completely internalized by cells without inducing frustrated phagocytosis [12]. Following translocation, inhaled short fibers may accumulate in the lymphatic system, inducing possible toxicity in draining lymph nodes following overloading [32,33]. In this context, extrapulmonary outcomes resulting from the potential biodistribution of asbestos fibers previously deposited in the lungs were suggested [34].

To detail the pulmonary pathogenicity of short amphibole fibers and elucidate the role of biopersistence in their in-vivo impact, we compared

the acute and long-term toxicity of two amosite fibers prepared ad hoc, pAmo (fiber length $> 5 \mu\text{m}$) and mAmo (fiber length $< 5 \mu\text{m}$), in a mouse model (C57BL/6). The amosite samples were characterized for their morphometric characteristics (length and diameter distribution), and surface reactivity in terms of free radical release and iron speciation was assessed. Taking advantage of a multi-technique platform based on ICP-MS and histology via polarized-light microscopy (PLM), we determined that the majority of administered pAmo persisted in the lungs, while mAmo fibers were removed. A fraction of the removed fibers accumulated into extrapulmonary organs (mediastinal lymph nodes, spleen, and liver). By confirming the limited pulmonary biopersistence of short amosite fibers, our findings highlight how fiber length and reactivity drive amosite-induced lung toxicity, and emphasize that fiber deposition, clearance, and redistribution are central processes in asbestos pathogenicity. From our study, we also propose ICP-MS and PLM as a convenient approach to quantify the burden of hazardous EMPs in biological samples.

2. Materials and methods

2.1. Amosite asbestos fibers

Pristine (pAmo) and milled (mAmo) amosite asbestos fibers were prepared in our laboratory from historical long (LFA) and short (SFA) fiber amosite samples, respectively. These two samples, originally prepared from a single batch of South African amosite by Johns-Manville Corporation (Littleton, CO, USA), were kindly provided by Dr. Lang Tran (Institute of Occupational Medicine, Edinburgh, UK). SFA were obtained by grinding LFA in a ceramic ball-mill system, followed by sedimentation in water [35]. Previous investigations revealed the characteristic crystalline structure of fibrous amosite in both samples, with no bulk structural alterations due to milling [36]. The detailed preparation of pAmo and mAmo samples used in this study is reported in a previous publication [37]. In brief, both LFA and SFA were suspended in phosphate-buffered saline (PBS) containing 0.14 % (w/v) bovine serum albumin (PBS-BSA; 3.2 mg/ml, total volume 3 ml) and subsequently subjected to ice-assisted ultrasonication (250 W/ml, 45 min; probe diameter 3 mm) using a VCX750 ultrasonic processor (Sonics, Newtown, CT, USA). This dispersion step enhanced fiber dispersion in biological media, and was optimized for delivery via oropharyngeal aspiration (o.p.a.; see Section 2.6). Resulting pAmo (obtained from LFA) and mAmo (obtained from SFA) samples were characterized for size, morphology, mineralogical composition, and surface reactivity. EPR-spin trapping spectroscopy, carried out to evaluate the generation of hydroxyl and carboxyl radicals after 1-hour incubation in a neutral pH solution, was consistent with previous findings, confirming minimal impact of sonication of surface properties with respect to the original fibers (Supplementary Materials, Figure S2a) [36].

2.2. Morphology and size distribution

Fiber morphology and dimensions (width, length, and aspect ratio = length/width) were assessed using a TESCAN VEGA 4 IMS Scanning Electron Microscope (SEM) equipped with an Ultim Max40 OXFORD EDS detector. pAmo and mAmo fibers were deposited onto graphite

tape, coated with a 10 nm gold layer, and imaged at 2500x, 5000x, and 10000x magnification (10–15 kV). Both samples included a substantial proportion of elongated particles (aspect ratio ≥ 3) as previously reported [37,38].

2.3. Chemical analysis

Mineral chemistry was assessed on petrographic sections with a Scanning Electron Microscope associated with Energy Dispersive X-ray spectroscopy (SEM-EDS) JEOL JSM IT 300 LV with Oxford INCA Energy 200 ED. Operating conditions: 15 kV, 5 nA, 10^5 counts per second (CPS), 30 s counting time; error: $< 1\%$ for major and $< 5\%$ for minor elements. Standards included pure elements and simple oxides/silicates. Five representative analyses for each fiber type are provided (Supplementary Materials, Table T1). Before the analysis, samples were coated with a carbon layer. Data were processed using OriginPro software.

2.4. Evaluation of free radical generation (In-chemico test)

Hydroxyl radical ($\bullet\text{OH}$) generation was evaluated over 72 h using EPR spectroscopy coupled with spin trapping, using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Cayman Chemical, Michigan, USA) as the spin trap, in two simulated biological fluids: Gamble's solution (GAS, pH 7.4) and Artificial Lysosomal Fluid (ALF, pH 4.5). For each experiment, 10 mg of pAmo or mAmo were suspended in 200 μL of either GAS or ALF [39]. After suspension, 100 μL of 0.17 M DMPO and 100 μL of 0.2 M H_2O_2 (Merck, Germany) were added. The mixtures were incubated in the dark at 37°C under gentle shaking (120 RPM, Horizontal Shaker model 701, ASAL S.r.l., Italy) for 10 min, followed by centrifugation (1 min at 11,000 RCF, Rotina 380, Hettich, Germany). A 50 μL aliquot of the supernatant was then collected for EPR analysis to quantify radical generation.

The remaining solid was re-incubated under identical conditions, replacing DMPO with 100 μL of Milli-Q water. Samples were maintained at 37°C under gentle shaking, and $\bullet\text{OH}$ generation was reassessed after 24 and 72 h of incubation using the same procedure. To ensure consistent oxidative conditions, the H_2O_2 -containing solution was renewed daily throughout the 3-day incubation period. To assess the potential contribution of leached ions to hydroxyl radical ($\bullet\text{OH}$) production, 10 mg of pAmo or mAmo were incubated in ALF with 100 μL of Milli-Q water and 100 μL of 0.17 M DMPO. The suspension was stirred at 37°C for 10 min in the dark. After incubation, samples were centrifuged (5 min, 11,000 RCF), and the supernatants were carefully collected. To each supernatant, 10 μL of 2 M H_2O_2 were added, resulting in final concentrations of 0.04 M DMPO and 0.05 M H_2O_2 . $\bullet\text{OH}$ radical production was then measured immediately and again after 10 min of incubation at 37°C .

1 l of GAS includes: 0.095 g of magnesium chloride, 6.019 g of sodium chloride, 0.298 g of potassium chloride, 0.126 g of sodium phosphate dibasic, 0.063 g of sodium sulfate, 0.368 g calcium chloride dihydrate, 0.574 g of sodium acetate, 2.604 g of sodium bicarbonate, and 0.097 g of sodium citrate dihydrate. ALF (1 l) contains: 0.05 g of magnesium chloride, 3.21 g of sodium chloride, 0.071 g of sodium phosphate dibasic, 0.039 g of sodium sulphate, 0.128 g calcium chloride dihydrate, 0.071 g of sodium citrate dihydrate, 6.0 g of sodium hydroxide, 20.8 g of citric acid, 0.059 g of glycine, 0.09 g of sodium tartrate dihydrate, 0.085 g of sodium lactate, and 0.086 g of sodium pyruvate. EPR spectra were recorded at room temperature using a Miniscope 100 X-band EPR spectrometer (Magnettech, Germany) with the following acquisition parameters: microwave power, 20 mW; center field, 3330 G; scan range, 120 G, and modulation amplitude, 1 G. Each measurement was repeated three times, and a blank control was included in every experimental set.

2.5. Surface iron characterization

pAmo and mAmo (0.5 mg/ml) were incubated with 1 mM ferrozine to evaluate the amount of removable Fe^{2+} , or 1 mM ferrozine + 1 mM ascorbic acid to evaluate the total amount of removable iron ($\text{Fe}_{\text{tot}} = \text{Fe}^{2+} + \text{Fe}^{3+}$). Fe^{3+} was calculated as Fe_{tot} minus Fe^{2+} . The amount of iron extracted after 1, 5, 24 and 48 h were measured in the supernatant using an Unikon 930 spectrophotometer (Kontron Instrument, Augsburg, Germany) by measuring the absorption of Fe^{2+} -ferrozine complex at 562 nm ($\epsilon_{\text{mM}} = 27.9 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.6. Animals and treatments

Female C57BL/6 mice (12 weeks old, weight ~ 20 g; $n = 80$) were obtained from Janvier SAS (St Berthevin, France). Animals were housed in polycarbonate cages with conventional sawdust bedding (Carfil, Oud-Turnhout, Belgium), provided with sterile rodent chow and acidified water, and housed in positive-pressure air-conditioned units (25°C , 50 % relative humidity) under a 12 h light/dark cycle. All experimental procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory animals and complied with Belgian and European regulations (EEC n° 86/609, LA1230312 and 2022/UCL/MD/019). After acclimatization, mice were randomly assigned to four main groups ($n = 20$ per group): unexposed control (PBS-BSA, Ctrl), pAmo-exposed, mAmo-exposed, and crocidolite-exposed (Crc; positive control). UICC crocidolite fibers have been widely used in in vivo toxicological studies [40], and their detailed physico-chemical characterization is reported in the literature [41,42]. Each group was further subdivided into two subgroups based on time points post-exposure: 3 days (3 d; Ctrl: $n = 10$, pAmo: $n = 10$, mAmo: $n = 10$, Crc: $n = 10$) and 120 days (120 d; Ctrl: $n = 10$, pAmo: $n = 10$, mAmo: $n = 10$, Crc: $n = 10$). Control received 50 μL of PBS-BSA via oropharyngeal aspiration (o.p.a.). Exposed groups (pAmo, mAmo and Crc) were administered a single dose of the respective fiber sample (0.1 mg/mouse, reference pathological dose), suspended in 50 μL of PBS-BSA, and delivered via o.p.a. to animals under general anesthesia. Anesthesia was induced by intraperitoneal injection of a mixture of Nimatek (1 mg/mouse; Eurovet, Bladel, The Netherlands) and Rompun (0.2 mg/mouse; Bayer, Leverkusen, Germany) as per Sironval et al. [43]. Fiber suspensions were prepared by ice-assisted ultrasonication in PBS-BSA as previously described (Section 2.1), and thoroughly vortexed immediately prior to administration to ensure homogeneous fiber dispersion. Oropharyngeal aspiration (o.p.a.) was used as a non-surgical alternative to intratracheal instillation. This method offers improved reproducibility of pulmonary outcomes, including inflammation and fibrosis, and is easier to perform consistently, as it does not require the technical expertise associated with surgical procedures [44,45]. Its efficacy for investigating pulmonary effects following asbestos exposure has been previously demonstrated in the literature [46]. To maximize pulmonary delivery and minimize accidental gastrointestinal exposure, the mice's tongues were gently extended with forceps to expose the oropharynx and obstruct the epiglottis. Their nostrils were occluded using forceps to promote oral inhalation and ensure direct fiber suspension aspiration into the lungs. Mice were continuously monitored during anesthesia and throughout recovery. The selected fiber dose (0.1 mg/mouse) aligns with established models used to assess asbestos-related pathogenicity [47–50], and corresponds to an estimated 6×10^6 pAmo fibers and 1×10^8 mAmo fibers per administration and mouse, based on calculations by Tomatis [36]. Animals were euthanized at either 3- or 120-days post-exposure by intraperitoneal injection of 200 μL sodium pentobarbital (60 mg/ml; Certa, Braine-l'Alleud, Belgium).

2.7. Bronchoalveolar lavage and lung homogenate preparation

Bronchoalveolar lavage (BAL) was performed on a subset of mice

($n = 5$ per group, per time point) 3 or 120 days after exposure to PBS-BSA, pAmo, mAmo, or crocidolite (Crc). Post-euthanasia, the lungs were perfused with 0.9 % NaCl, and the trachea was cannulated. BAL was conducted by flushing the lungs four times with 1 ml of 0.9 % NaCl. The recovered bronchoalveolar lavage fluid (BALF) was then centrifuged ($280 \times g$, 10 min, 4°C ; Centrifuge 5804 R, Eppendorf, Hamburg, Germany). The cell-free supernatant was collected and stored at -80°C for subsequent biochemical measurements. Pelleted BAL cells were stained using the Diff-Quick method and analyzed microscopically. A differential cell count was performed by light microscopy, with at least 200 cells counted per sample. After BAL collection, whole lungs were surgically excised via a midline sternotomy. Lungs were promptly homogenized in 2 ml ice-cold PBS using an Ultra-Turrax T25 homogenizer (Janke and Kunkel, Brussels, Belgium), and stored at -80°C for further analysis.

2.8. Evaluation of acute and long-term responses in the BALF of mice

Lactate dehydrogenase (LDH) activity was measured in 3-day BALF using Arras et al. protocol [51]. Cell-free DNA (cfDNA), indicative of necrotic cell death and used a biomarker of pulmonary toxicity [52,53], was quantified using the Quant-iT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Pro-inflammatory cytokines were quantified in 3-day BALF samples using DuoSet ELISA kits (R&D Systems, Minneapolis, USA): IL-1 α (Cat. # DY400), IL-1 β (Cat. # DY401), IL-6 (Cat. # DY406), TNF- α (Cat. # DY410), CXCL1 (Cat. # DY453). Osteopontin (OPN), a matricellular cytokine implicated in inflammation and fibrosis [54,55], was quantified in 120-day BALF using the DuoSet ELISA kit (Cat. # DY441, R&D Systems) [56,57].

2.9. Lung collagen quantification

Soluble collagen in 120-day lung homogenates was quantified via Sircol assay after hydrolysis (6 N HCl at 108°C for 24 h) as described in [58].

2.10. Tissue collection for fiber qualitative/quantitative analysis via histology/imaging or ICP-MS

A separate cohort of animals, comprising both unexposed controls (Ctrl) treated with PBS-BSA (50 μl) or exposed for 3 or 120 days to one of the three fiber types (pAmo, mAmo, or Crc: 0.1 mg/mouse; $n = 5$ per group, per time point) via o.p.a. as already reported (Section 2.6), was allocated for histological studies and ICP-MS analysis. Mice were sacrificed as described in Section 2.6, and bronchoalveolar lavage (BAL) was omitted. To minimize cross-contamination, selected organs were excised in a predefined order: spleen, liver, mediastinal lymph nodes, then lungs. This order was designed to ensure that extrapulmonary tissues were removed before the thoracic cavity was opened, thus reducing the risk of fiber redistribution from lung tissues during necropsy. Throughout dissections, dedicated instruments were used for each organ, or were thoroughly rinsed with sterile saline between uses. Each organ was immediately rinsed with sterile saline after removal to eliminate loosely attached material and then placed in sterile containers for further processing. These procedures are consistent with standard practices for particle biodistribution studies [59–61], where tissue collection is typically performed after euthanasia. Post-excision, the spleen and liver were bisected. The left half of the spleen and the left lateral lobe of the liver were fixed in 3.65 % paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS for subsequent histological investigation. The right half of the spleen and the remaining liver lobes were weighed, homogenized, and stored at -80°C for later ICP-MS analysis. For the lungs, the left lung (upper, middle, and lower regions) was fixed for histological processing, while the right lung lobes (superior, middle, inferior, and post-caval) were homogenized and

stored at -80°C for ICP-MS. Mediastinal lymph nodes were processed entirely due to their small size.

2.11. Histology

Paraffin-embedded left lung sections (5 μm) from mice exposed for either 3 days (Ctrl: $n = 5$, pAmo: $n = 5$, mAmo: $n = 5$, Crc: $n = 5$) or 120 days (Ctrl: $n = 5$, pAmo: $n = 5$, mAmo: $n = 5$, Crc: $n = 5$), and not subjected to BAL, were stained with hematoxylin and eosin (H&E, nucleus and cytoplasm staining). Images were acquired using both bright field (BF) and polarized light microscopy (PLM) with a Zeiss Axioscan slide scanner (Carl Zeiss, Oberkochen, Germany) and processed using Zeiss Zen 3.8 software.

2.12. Inductively coupled plasma-mass spectrometry (ICP-MS)

Fiber burden was assessed in homogenized tissues from animals not subjected to bronchoalveolar lavage (BAL). Samples were obtained at two time points (see Section 2.10): 3 days post-exposure (right lung lobes: superior, middle, inferior, and post-caval), and 120 days post exposure (right lung lobes, right liver lobes, right halves of the spleen, and mediastinal lymph nodes) following exposure to pAmo and mAmo fibers. Quantification of silicon (Si) and/or manganese (Mn), used as tracers of fiber exposure, was performed using inductively coupled plasma mass spectrometry (ICP-MS) with an Agilent 7500ce Octopole plasma mass spectrometer (Agilent Technologies, Santa Clara, USA). These elements are characteristic of South African amosite asbestos, where Si as the primary structural component and Mn presented as a trace element. [62,63]. ICP-MS, due to its high sensitivity, is widely applied for trace element analysis in biological samples [64]. The analytical approach was adapted from Leinardi et al. [65]. Briefly, 500 μl of weighed, homogenized tissue from control and fiber-exposed animals, was transferred into PTFE digestion vessels and mineralized at 200°C for 2 h in an alkaline environment obtained by adding 200 mg NaOH pellets (final NaOH concentration: 10 M, pH ~ 15). Following cooling, residues were dissolved in 4.6 ml of deionized water (dH_2O) and neutralized with 400 μl concentrated HNO_3 . A 500 μl aliquot of the neutralized mixture was transferred into polypropylene tubes and diluted 1:10 with ICP-MS diluent (a 0.5 % HCl, 1 % HNO_3 solution containing Sc, Ir, Ge, Rh as internal standards). To prevent silicon contamination, all procedures were conducted in a cleanroom environment using only non-glass material and under laminar flow conditions. Calibration curves for Si and Mn were generated using TraceCERT silicon standard (10,000 mg/l Si in nitric acid) and Multi-Element ICP-MS Standard Solutions (Sigma-Aldrich, St. Louis, MO, USA). Laboratory duplicates were included in all analyses to ensure reproducibility. To minimize carry-over effect between samples, the instrument was rinsed with 2 % HNO_3 for 60 s followed by 1 % HNO_3 for 30 s. Instrumental limit of quantifications (LOQs) considered for each element were determined with the blank method [66].

3. Results

3.1. Pristine (pAmo) and milled (mAmo) amosite fibers show distinct and well-defined size distributions

A primary objective of this study was to elucidate the differential toxic mechanisms of long versus short asbestos fibers by comparing samples with distinct size distributions (long pAmo and short mAmo, specifically). A quantitative fiber size distribution (FSD) analysis of pAmo and mAmo was conducted using scanning electron microscopy (SEM), measuring the length and width of approximately 300 fibers per specimen. The resulting FSD scatter plot revealed two clearly distinct clusters (Fig. 1). The pAmo sample was composed predominantly of long, thin fibers, with 80 % of fibers meeting the World Health Organization (WHO) criteria for respirable fibers for humans (aspect ratio

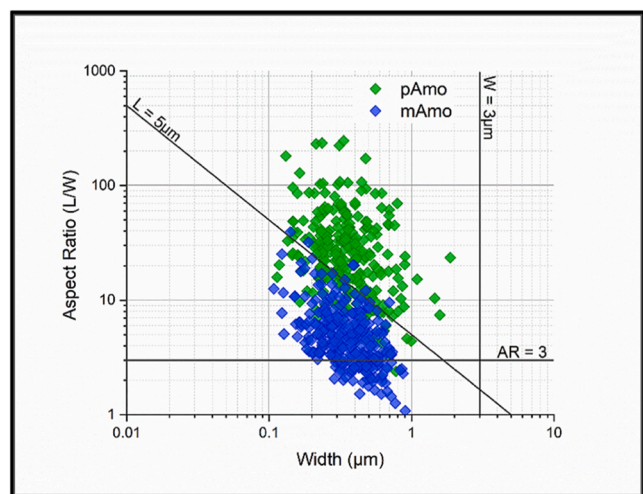


Fig. 1. Size distribution of pAmo and mAmo amosite fibers illustrates two length-distinct fiber samples. A scatter plot of fiber aspect ratio (AR, Length/Width) versus fiber width shows the distribution patterns of pAmo (green diamonds) and mAmo (blue diamonds). The pAmo sample comprises predominantly long ($L > 5 \mu\text{m}$) and respirable fibers, while the mAmo sample consists mainly of shorter fibers ($L < 5 \mu\text{m}$), in accordance with WHO respirability criteria [67], mAmo (blue diamond) consists of a population of shorter fibers ($L < 5 \mu\text{m}$). Despite significant differences in length, both fiber types display a comparable width distribution ($0.1 < W < 1 \mu\text{m}$). Detailed width and length frequency distributions, and representative SEM images of the two fiber samples, are provided in [Supplementary Materials Fig. S1](#).

[AR] ≥ 3 , length [L] $> 5 \mu\text{m}$, and width [W] $< 3 \mu\text{m}$ [67]. In contrast, the mAmo sample was distinctly shorter, with 98 % of fibers having lengths below $5 \mu\text{m}$ and widths $< 1 \mu\text{m}$, despite maintaining AR ≥ 3 . Notably, 99 % of pAmo fibers and 73 % of mAmo fibers exhibited aspect ratios ≥ 3 , with pAmo showing a much higher mean aspect ratio (33) compared to mAmo (mean AR of 5). While both fiber types had comparable width distributions, ranging from 0.1 to $1 \mu\text{m}$, the average length of pAmo fibers far exceeded that of mAmo, highlighting length as the primary distinguishing feature between the two samples (Fig. 1; [Supporting Materials S1a, b](#)).

Mineral composition was analyzed by energy-dispersive X-ray spectroscopy (EDS) (Table 1; [Supplementary Table T1](#)). Both fiber types were primarily composed of silicon dioxide (SiO_2) and iron oxide (FeO), together accounting for approximately 90 % of the total oxide content. Magnesium oxide (MgO), manganese oxide (MnO), potassium oxide (K_2O), and titanium dioxide (TiO_2) were present in comparable amounts in both samples. Minor differences were observed in the levels of aluminum oxide (Al_2O_3) and calcium oxide (CaO), which were slightly more represented in mAmo. The structural water content (H_2O , 2.3 %) was consistent across both fiber types. Overall, the two materials

Table 1

Mineral composition of pAmo and mAmo. Representative chemical analysis (in weight percent of oxides) carried out via EDS spectroscopy. For each specimen, 30 different punctual spectrum have been collected.

Oxide	pAmo	mAmo
NaO	0.03	0.00
MgO ₂	6.06	6.32
Al ₂ O ₃	0.17	1.24
SiO ₂	49.62	49.20
K ₂ O	0.06	0.17
CaO	0.00	0.22
TiO ₂	0.00	0.04
MnO	3.45	3.32
FeO	38.20	37.20
H ₂ O	2.30	2.30
TOTAL	100.00	100.00

displayed similar chemical profiles, while differing markedly in terms of fiber length.

3.2. Exposure to long pAmo induces severe acute pulmonary responses with respect to short mAmo fibers

The acute pulmonary response induced by oropharyngeal administration of long pAmo and short mAmo fibers was assessed in the BALF and in pulmonary tissues collected from instilled mice 3 days (3 d) post exposure (0.1 mg fiber/mouse; pAmo, ca. 6×10^6 fibers; mAmo, ca. 1×10^8 fibers; 50 μl) (Fig. 2A). Control mice (Ctrl) were treated with the same volume of a PBS-BSA solution. An additional set of mice was exposed to the same mass of reference crocidolite fibers (Crc) (Fig. 2A). We evaluated pAmo and mAmo cytotoxicity by quantifying BALF LDH and cell-free DNA (cfDNA) content. Proinflammatory cytokine (IL-1 α , IL-1 β , IL-6, TNF- α and CXCL1) levels and absolute neutrophil counts were used to assess alveolar inflammation. Compared to Ctrl mice, both pAmo and Crc treatments significantly increased BALF LDH and cfDNA (Fig. 2B and C), suggesting lung acute cytotoxicity characterized by lytic cell necrosis. Accordingly, mice exposed to pAmo and Crc showed significantly increased the levels of proinflammatory cytokines and chemokines (Fig. 2D-H), accompanied by the recruitment of inflammatory neutrophils (Fig. 2I). Notably, a less marked impact characterized by limited increase in BALF content of LDH, cfDNA, and proinflammatory cytokines, and minor influx of neutrophils, was observed for mAmo (Fig. 2B-I). These data underscored fiber length as the key parameter in triggering detrimental short-term responses in the lungs of exposed animals.

3.3. Contrasting acute responses among pAmo and mAmo are not related to the potential clearance of short fibers from the lungs

To clarify whether the distinct acute responses triggered by long pAmo and short mAmo might be ascribed to different pulmonary bio-persistence between the two fibers, we monitored their effects and presence in histology lung tissue sections stained with hematoxylin and eosin (H&E) by applying bright-field (BF) and polarized light microscopy (PLM). In accordance with previous observations, we noticed that 3 d post exposure the lung architecture of animals treated with long pAmo was strongly compromised with respect to control animals (Figs. 3A and B, and 3D and E). Indeed, pAmo-exposed lungs showed granulomatous lesions and inflammatory cell infiltrations in BF. In contrast, short mAmo exposure poorly impacted the tissue, and few inflammatory infiltrations were detected (Fig. 3G and H). Notably, PLM showed the widespread occurrence of both mAmo and pAmo in the lungs of exposed animals. pAmo were often organized in clusters co-localized with inflammatory lesions (Fig. 3F), while mAmo were dispersed through the pulmonary tissue (Fig. 3I). This suggested that the distinct acute effects triggered by the two fibers did not depend on a different pulmonary burden. To support this aspect, we monitored the pulmonary loading of pAmo and mAmo by measuring lung silicon (Si) and manganese (Mn) content via ICP-MS (Fig. 3J). Comparable pulmonary levels of Si content were detected in animals exposed to pAmo or mAmo, with respect to untreated mice (Fig. 3J). The same effect was observed by measuring Mn as trace element (Fig. 3J). This demonstrates that a comparable mass of fibers is present among pAmo- and mAmo-exposed animals and indicates that the difference in short-term pulmonary effects between the two fibers does not depend on early clearance mechanisms.

3.4. Long pAmo surface reactivity is enhanced in phagolysosomal-like acidic conditions

Free radicals released after asbestos exposure are known to be determinant in acute inflammation induced by fibers [68]. To better understand the role of surface reactivity in driving the contrasting acute

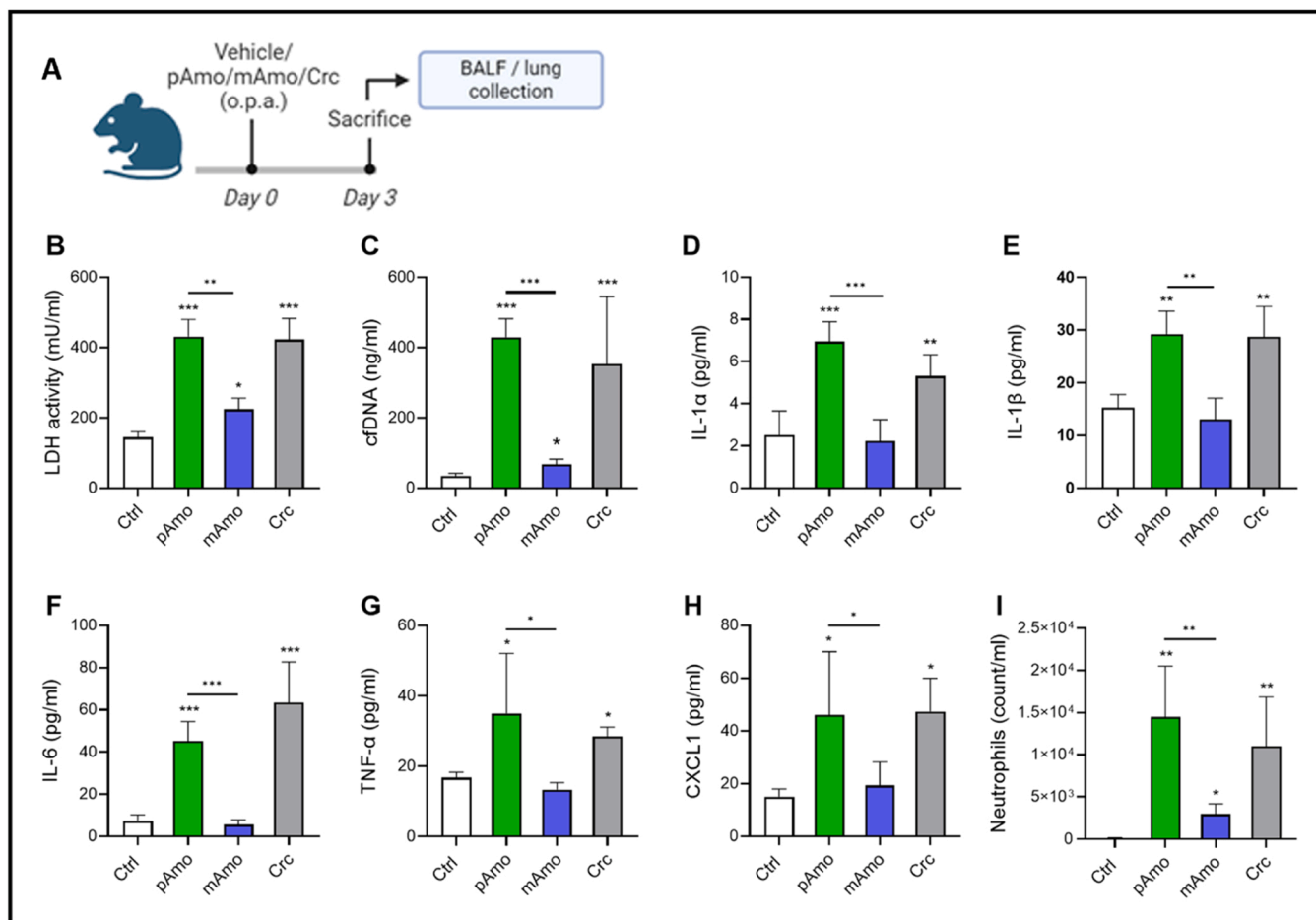


Fig. 2. Pulmonary inflammatory response magnitude is strongly dependent on fiber length. pAmo elicits a greater acute proinflammatory response than mAmo in the lungs of C57BL/6 mice. (A) Experimental setup: mice were treated via oropharyngeal aspiration (o.p.a.; 50 μ l/mouse) with PBS-BSA (Ctrl), pAmo (0.1 mg) or mAmo (0.1 mg). Crocidolite fiber (Crc, 0.1 mg) was used as a reference. Three days post-treatment, lung inflammation was assessed in bronchoalveolar lavage fluid (BALF) by measuring: (B) LDH activity and (C) cell-free DNA (cfDNA) as indicators of cytotoxicity; and (D) IL-1 α , (E) IL-1 β , (F) IL-6, (G) TNF- α , (H) CXCL1, and (I) neutrophil number as markers of proinflammatory activity. Cytokines and chemokine levels were quantified by ELISA. * P < 0.033, ** P < 0.002, and *** P < 0.001 relative to controls, or between pAmo- and mAmo- treated groups. ANOVA with Tukey's multiple comparisons test. Data are presented as mean $\pm$ SD (n = 5).

responses observed three days post exposure, we assessed the ability of long and short fibers to generate hydroxyl radicals ($\bullet$ OH) in simulated lung environments. Gamble's solution (GAS, pH 7.4) was used to mimic the interstitial lung fluid, whereas artificial lysosomal fluid (ALF, pH 4.5) simulated the acidic environment of macrophage phagolysosomes. This approach allowed us to assess the influence of pH on the ability of fibers to generate free radicals. Furthermore, considering that surface properties, and therefore fiber reactivity, may evolve over time, $\bullet$ OH production was monitored over a 72-hour incubation in these simulated fluids (Fig. 4). In GAS, both fiber types exhibited comparable and limited $\bullet$ OH generation, particularly within the first 10 min (Fig. 4A and B), with a shared time-dependent decline in reactivity. In contrast, a pronounced difference was observed in ALF: pAmo showed a markedly enhanced $\bullet$ OH generation under acidic conditions, while mAmo exhibited consistently low reactivity, unaffected by the change in pH (Fig. 4C and D). Both fibers exhibited a progressive reduction in reactivity over time (Fig. 4C and D). Similar trends were confirmed in phosphate buffer solutions at both neutral and acidic pH (Supplementary Materials, Fig. S2b). These results suggest that the limited lung inflammation elicited by short mAmo is attributable to its poor surface reactivity under phagolysosomal conditions, likely due to reduced ability to generate $\bullet$ OH radicals following phagocytosis by lung macrophages.

To further elucidate the influence of the biological environment in

driving pulmonary responses, we evaluated the generation of hydroxyl radicals ($\bullet$ OH) by the two fibers in the lysosomal fluid (ALF) supernatant (Fig. 5). EPR spectroscopy revealed that incubation of pAmo resulted in a strong $\bullet$ OH signal in the ALF supernatant after 10 min and 72 h (Fig. 5A and C, black lines), indicating persistent reactivity. Consistent with previous observations (see Fig. 4), the direct surface reactivity of pAmo fibers was high at 10 min, but declined by 72 h, although remaining well detectable (Fig. 5A and C, red lines). In contrast, mAmo induced only modest $\bullet$ OH generation in the ALF supernatant at 10 min, and this signal was almost completely abolished after 72 h (Fig. 5B and D, black lines). As expected, the direct surface reactivity of mAmo remained minimal and unchanged over time (Fig. 5B and D, red lines). Altogether, these results indicate that long pAmo fibers maintain a higher oxidative reactivity in the phagolysosomal-like environment, compared to short mAmo. This differential behavior, likely driven by distinct surface chemical properties under acidic conditions, contributes to the pulmonary toxicity observed upon pAmo exposure.

3.5. Distinct iron speciation contributes to the differential phagolysosomal reactivity observed between the two fiber types

To investigate the role of surface chemistry in the differential reactivity of the two fiber types under phagolysosomal conditions, we assessed the availability of bioaccessible iron from pAmo and mAmo.

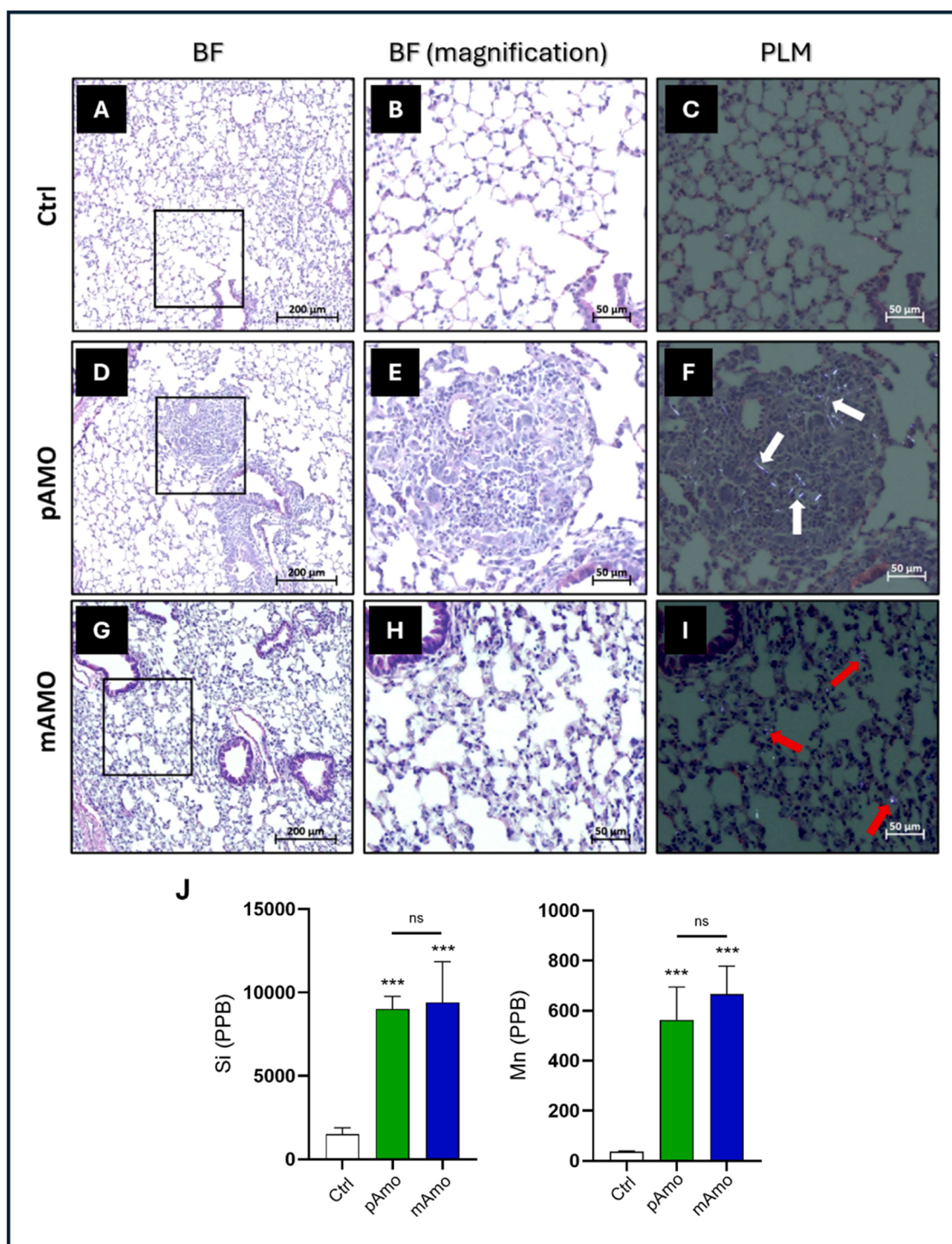


Fig. 3. Distinct acute pulmonary responses to pAmo and mAmo are not due to differences in early clearance. pAmo and mAmo fibers accumulate to comparable levels in the lungs of exposed mice 3 days post administration. C57BL/6 mice were treated via oropharyngeal aspiration (o.p.a.; 50 μ l/mouse) with PBS-BSA (Ctrl), pAmo, or mAmo fibers (0.1 mg/mouse). Lung tissue was examined using bright-field (BF) and polarized light microscopy (PLM) to assess fiber deposition 3 days after exposure. Representative images are shown for the control group (A: BF, full size; B: BF, magnification; C: PLM of B), pAmo-exposed group (D: BF, full size; E: BF, magnification; F: PLM of E), and mAmo-exposed group (G: BF, full size; H: BF, magnification; I: PLM of H). Fibers are indicated by white arrows (pAmo) or red arrows (mAmo) in PLM images. (J) Quantification of pAmo and mAmo burden in the lungs was performed by measuring silicon (Si) and manganese (Mn) content, respectively, via ICP-MS analysis of 500 μ l lung homogenate collected 3 d post administration. Scale bars: 200 μ m (full-size images) and 50 μ m (magnified views). *** P < 0.001 relative to controls, or between pAmo- and mAmo-treated groups; ns: not significant. ANOVA with Tukey's multiple comparisons test. Data are presented as mean $\pm$ SD (n = 5).

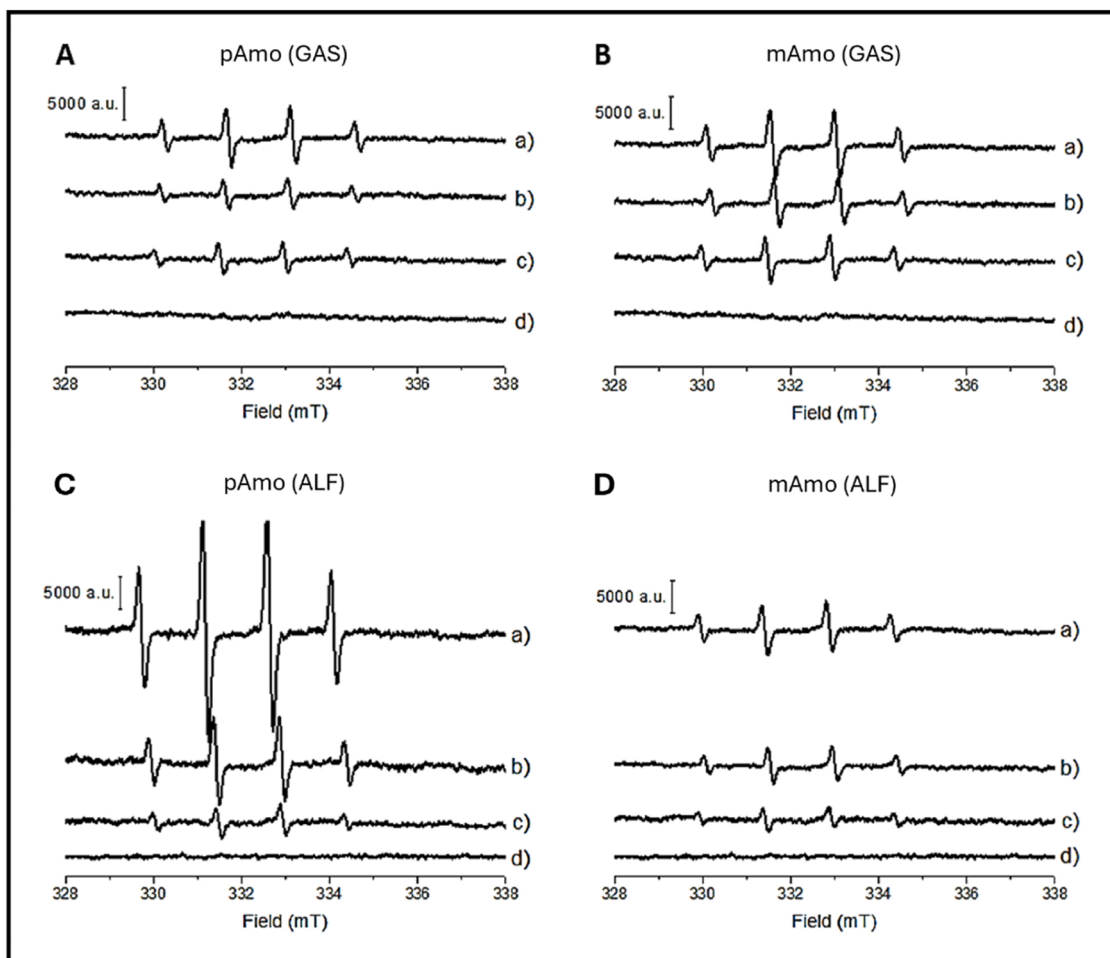


Fig. 4. Long pAmo reactivity is enhanced in an acidic environment, whereas short mAmo remains poorly reactive. Representative EPR spectra of [DMPO-OH]• adducts were recorded for pAmo and mAmo fibers incubated in Gamble's solution (GAS, pH 7.4) and Artificial Lysosomal Fluid (ALF, pH 4.5). The [DMPO-OH]• adduct signal exhibits a characteristic four-peak pattern with an intensity ratio of 1:2:2:1. Spectra were collected at 10 min (a), 24 h (b) and 72 h (c) of incubation. (d) represents the blank control. Panels correspond to (A) pAmo in GAS, (B) mAmo in GAS, (C) pAmo in ALF, (D) mAmo in ALF.

Bioaccessible iron can catalyze redox reactions and contribute to oxidative stress. Iron release was quantified at 1, 5, 24 and 48 h using two approaches: ferrozine in the presence of ascorbic acid to measure total leachable iron (Fe_{tot}), and ferrozine alone to detect ferrous iron (Fe^{2+}). Ferric iron (Fe^{3+}) levels were calculated by subtracting Fe^{2+} from Fe_{tot} ($\text{Fe}^{3+} = \text{Fe}_{\text{tot}} - \text{Fe}^{2+}$). As shown in Fig. 6A, both fibers exhibited similar kinetic profiles for Fe^{2+} release, reaching a plateau by 24–48 h. However, pAmo released significantly higher amounts of Fe^{2+} compared to mAmo at all time points (Fig. 6A). In contrast, mAmo released substantially more Fe^{3+} than pAmo, indicating a higher degree of surface oxidation (Fig. 6B). These results suggest that the outermost iron-containing layers of both fibers, likely composed of oxidized Fe^{3+} species, were rapidly removed within the first hour of incubation with chelators. The remaining surface of pAmo, richer in Fe^{2+} , was more reactive in the lysosomal environment and potentially more capable of driving hydroxyl radical generation via Fenton-like mechanisms. Conversely, the more oxidized (Fe^{3+} -rich) surface of mAmo appears less reactive in this regard. These findings account for the increased phagolysosomal activity observed with long pAmo fibers relative to short mAmo, and are also consistent with the acute inflammatory response elicited by the long – but not short – amosite asbestos fibers.

3.6. Long amosite pAmo fibers induce long-term profibrotic responses, while short mAmo are inert for the lungs

We then evaluated long-term responses after treatment with long and short fibers. C57BL/6 mice were exposed via o.p.a. to a single dose (0.1 mg/mouse) of pAmo or mAmo fibers and sacrificed 120 days post exposure (Fig. 7A). Animals were also treated with crocidolite fibers as a reference group. The BALF levels of osteopontin (OPN), a key regulator of fibrosis, were monitored, and lung collagen accumulation was assessed by measuring soluble collagen (Sircol assay) and histology via Red Sirius staining (Supplementary Materials, Fig. S3). In line with short-term results, long pAmo exposure induced robust lung fibrosis characterized by augmented levels of OPN, and a significant collagen deposition (Fig. 7B and C). Increased pulmonary accumulation of lymphocytes, a cell population linked to lung injury and fibrosis, was also observed in pAmo-exposed mice (Fig. 7D). Similar outcomes were induced by Crc fibers (Fig. 7). In contrast, absence of profibrotic responses and lymphocyte accumulation was noticed in mice exposed to mAmo (Fig. 7). Our data confirm that only long pAmo but not short mAmo induced chronic inflammation and fibrosis.

3.7. The negligible long-term pulmonary impact of short mAmo is associated with pulmonary clearance

To clarify the potential role of biopersistence in influencing long-

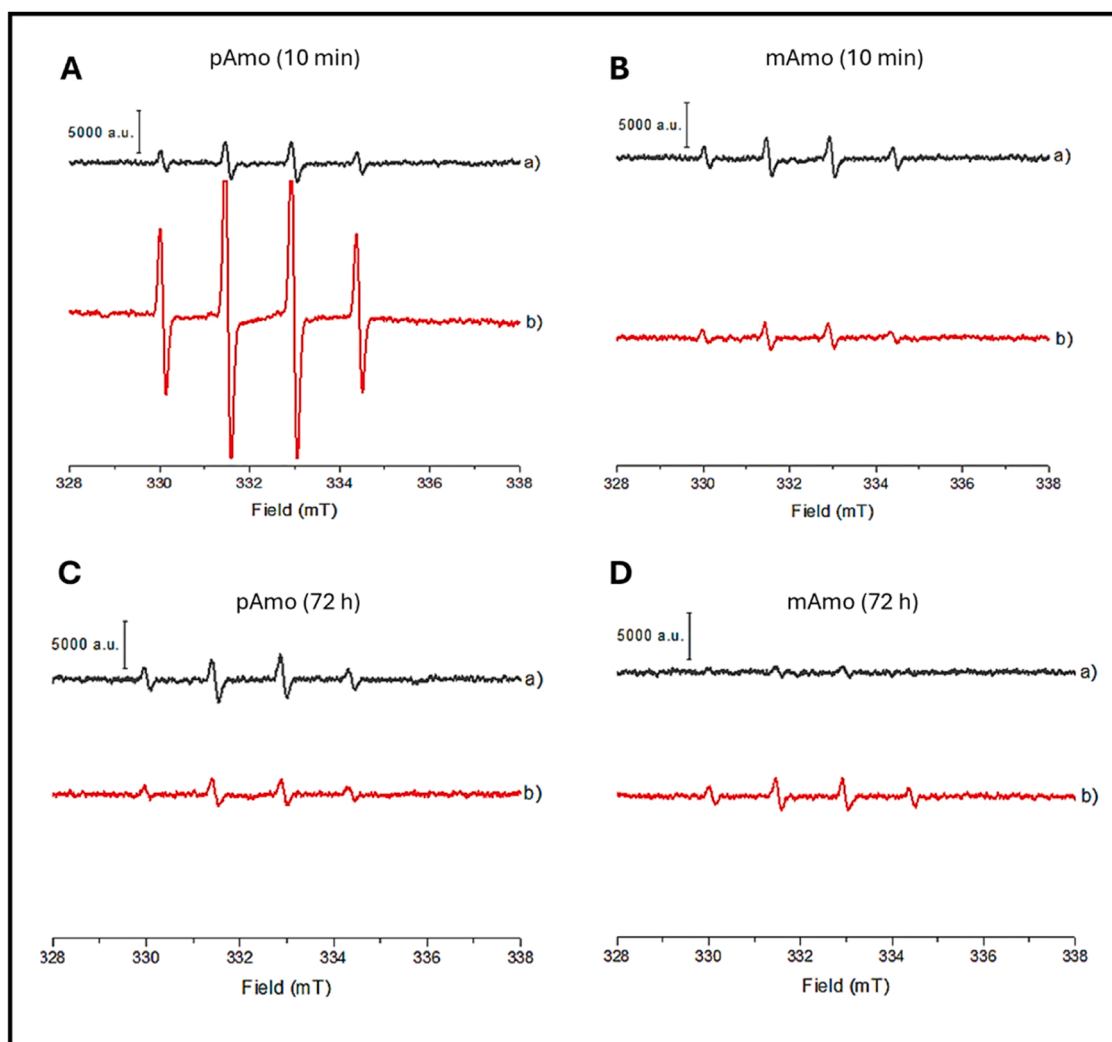


Fig. 5. Phagolysosomal activation enhances $\cdot\text{OH}$ release from pAmo and contribute to acute pulmonary responses. Representative EPR spectra of $[\text{DMPO-OH}]\cdot$ adducts were recorded for supernatants collected after fiber incubation in ALF (a) - black lines, or purified fibers (b) - red lines, at two time points (10 min and 72 h). Panel show (A) pAmo at 10 min; (B) mAmo at 10 min; (C) pAmo at 72 h; (D) mAmo at 72 h. No EPR signals were detected in blank controls (data not shown for clarity).

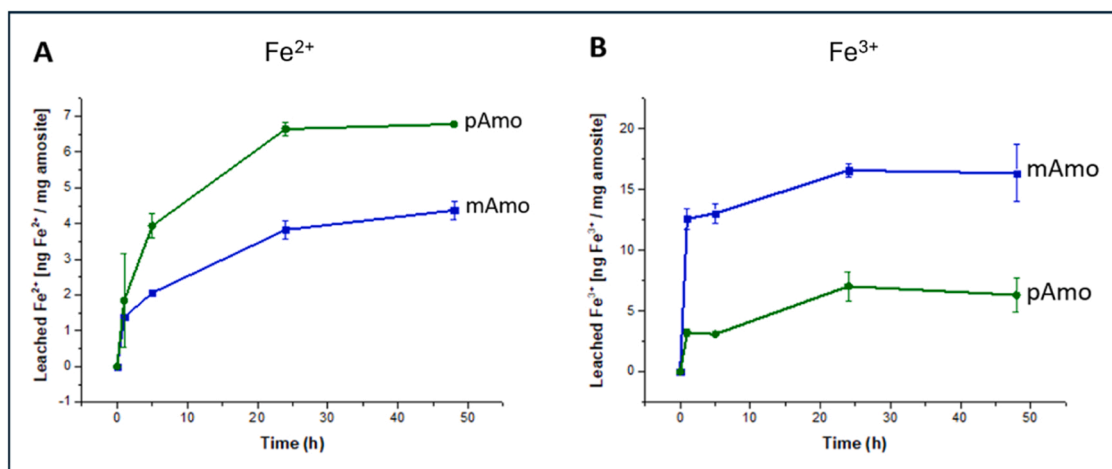


Fig. 6. pAmo fibers show less oxidized surface iron with respect to mAmo. Assessment of iron speciation in pAmo and mAmo fibers through bioaccessible iron release. Kinetics of (A) Fe^{2+} and (B) Fe^{3+} release from pAmo and mAmo at pH 4.5, measured using the ferrozine assay in the presence of ascorbic acid after 1, 5, 24, and 48 h of incubation.

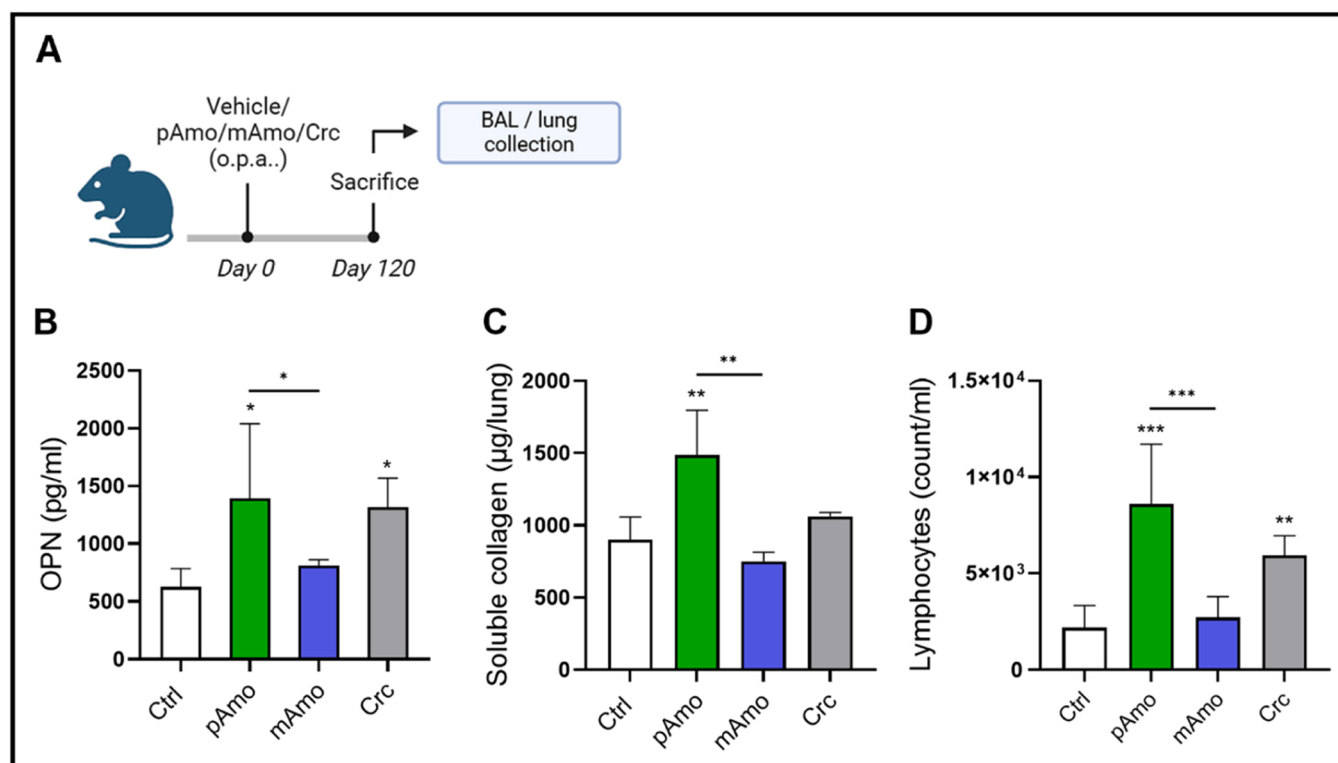


Fig. 7. pAmo exposure induces profibrotic responses in mouse lungs, while mAmo remains inert. (A) Experimental design: C57BL/6 mice were administered PBS-BSA (control, Ctrl), pAmo, or mAmo fibers (0.1 mg/mouse) via oropharyngeal aspiration (o.p.a.; 50 μl/mouse). Crocidolite fibers (Crc, 0.1 mg) served as reference control. 120 days post-exposure, profibrotic responses were assessed by measuring (B) osteopontin (OPN) in bronchoalveolar lavage fluid (BALF) and soluble collagen (C) levels in lung homogenates. Lymphocyte accumulation in BALF was also quantified (D). * $P < 0.033$, ** $P < 0.002$, and *** $P < 0.001$ relative to controls, or between pAmo and mAmo-treated group. ANOVA with Tukeys' multiple comparison test. Data are presented as mean $\pm$ SD ($n = 5$).

term pulmonary responses associated with pAmo or mAmo exposure, we monitored their presence in the fiber-treated lungs at day 120 by PLM and ICP-MS. With respect to vehicle-treated animals (Fig. 8A, B and C), major lung tissue disorganization, dense granulomatous nodules, and diffuse inflammation were observed upon long-term pAmo treatment (Fig. 8D and E). Polarized microscopy highlighted the presence of long fibers embedded with granulomatous lesions (Fig. 8F), indicating persistent pAmo fiber retention. Conversely, mAmo-treated lungs exhibited only minor, localized lesions with limited inflammatory infiltrates (Fig. 8G and H). Notably, no mAmo fibers were clearly distinguished in the pulmonary tissue at 120 d (Fig. 8I). These histological findings were corroborated by ICP-MS analysis (Fig. 8J). Indeed, pulmonary levels of the trace elements Si and Mn were elevated in the lungs of pAmo-treated animals in comparison to unexposed mice. In contrast, in mAmo-exposed mice, Si and Mn pulmonary levels were indistinguishable from controls (Fig. 8J). Together, these results provide strong evidence that the chronic pulmonary effects observed with long pAmo fibers are linked to their prolonged retention in lung tissue. In contrast, the absence of long-term responses following short mAmo exposure is associated with effective fiber clearance.

3.8. Short mAmo and long pAmo fibers deposited in the lungs accumulate in distal organs following extrapulmonary translocation

To explore the possibility that fibers cleared from the lungs may reach distal organs via extrapulmonary redistribution, we monitored the presence of pAmo and mAmo fibers in the liver, spleen and mediastinal lymph nodes (MLN) at 120 days post-exposure using polarized imaging and ICP-MS analysis. PLM revealed birefringent structures morphologically consistent with both pAmo (white arrows) or mAmo (red arrows) fibers in sections from the liver (Fig. 9A) and spleen (Fig. 9B). Despite these observations, no significant histopathological alterations—such as

granuloma formation or marked infiltration of inflammatory cells—were detected in the examined tissues.

Elemental analysis by ICP-MS analysis supported the microscopy findings, showing increased Si content in the liver (Fig. 9C), spleen (Fig. 9D), and MLN (Fig. 9E) of mice exposed to either fiber type compared to controls. Interestingly, animals exposed to short mAmo fibers exhibited significantly higher Si levels in the liver and spleen than those exposed to pAmo, potentially reflecting their higher pulmonary clearance efficiency and extrapulmonary translocation. Taken together, these findings are consistent with the hypothesis that a portion of both long pAmo and short mAmo fibers initially deposited in the lungs may reach extrapulmonary sites following lung clearance. The higher extrapulmonary distribution observed for mAmo fibers is likely to contribute to their complete lung clearance and the limited chronic pulmonary responses observed in exposed animals.

4. Discussion

It is accepted that fiber dimension (length and width), surface reactivity (i.e., iron ions) and biopersistence (long-term accumulation despite clearance mechanisms), are key actors in the asbestos health hazard [14,15,69,70]. In line with comparable outcomes observed in animals treated with long crocidolite and amosite fibers, our findings support the key role of fiber length and surface reactivity in inducing detrimental pulmonary responses [48,71–74]. We observed that pAmo fibers, characterized by greater length, exhibited significant oxidative reactivity in a phagolysosomal-like acidic environment. The potential impact of environmental pH in modulating surface activity is already well described in the literature [75,76]. pAmo reactivity was primarily driven by their surface chemistry, including a higher proportion of bioavailable Fe²⁺ ions, in line with previous studies [36]. This is critical, because Fe²⁺ can efficiently catalyze hydroxyl radical ($\bullet$ OH) generation

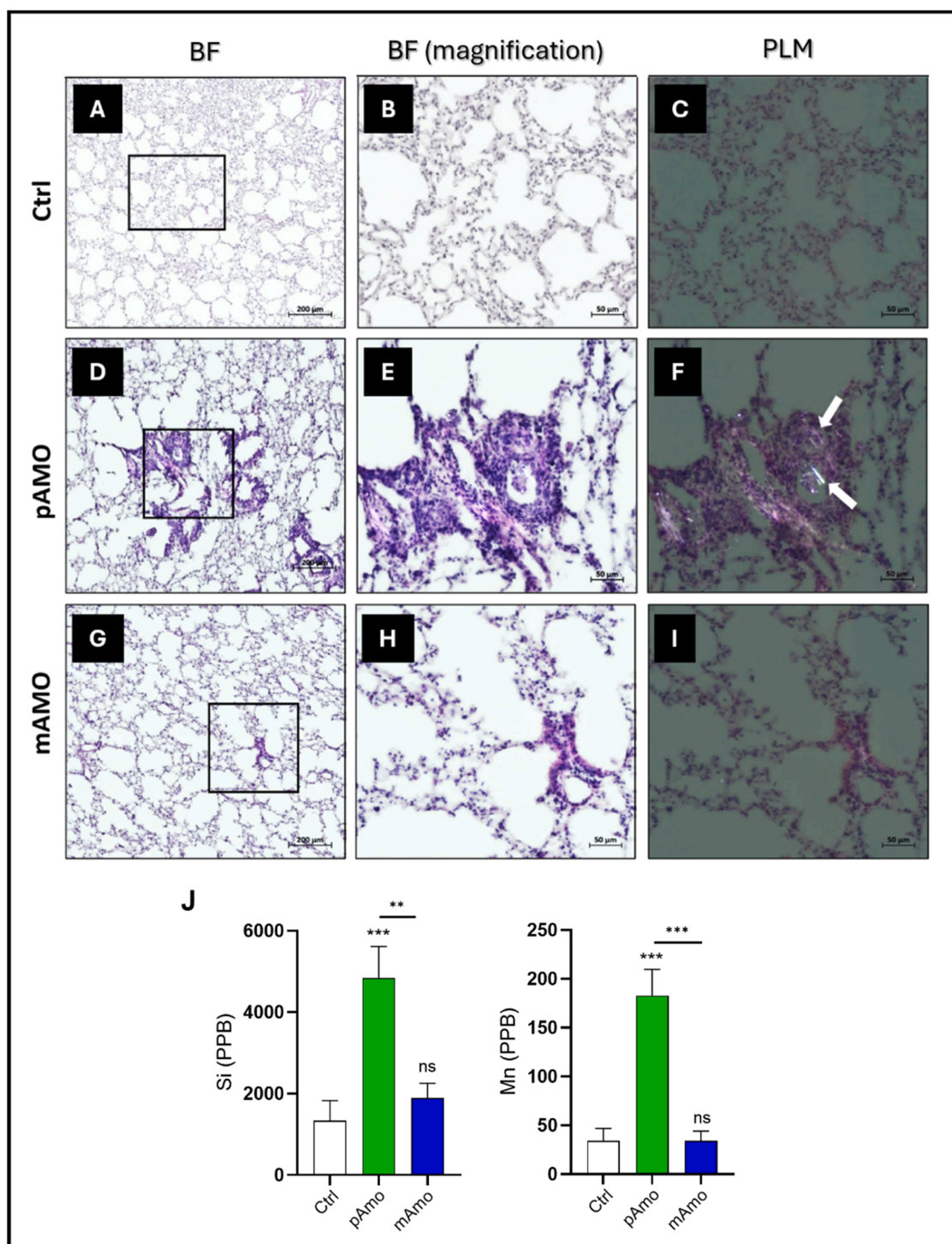
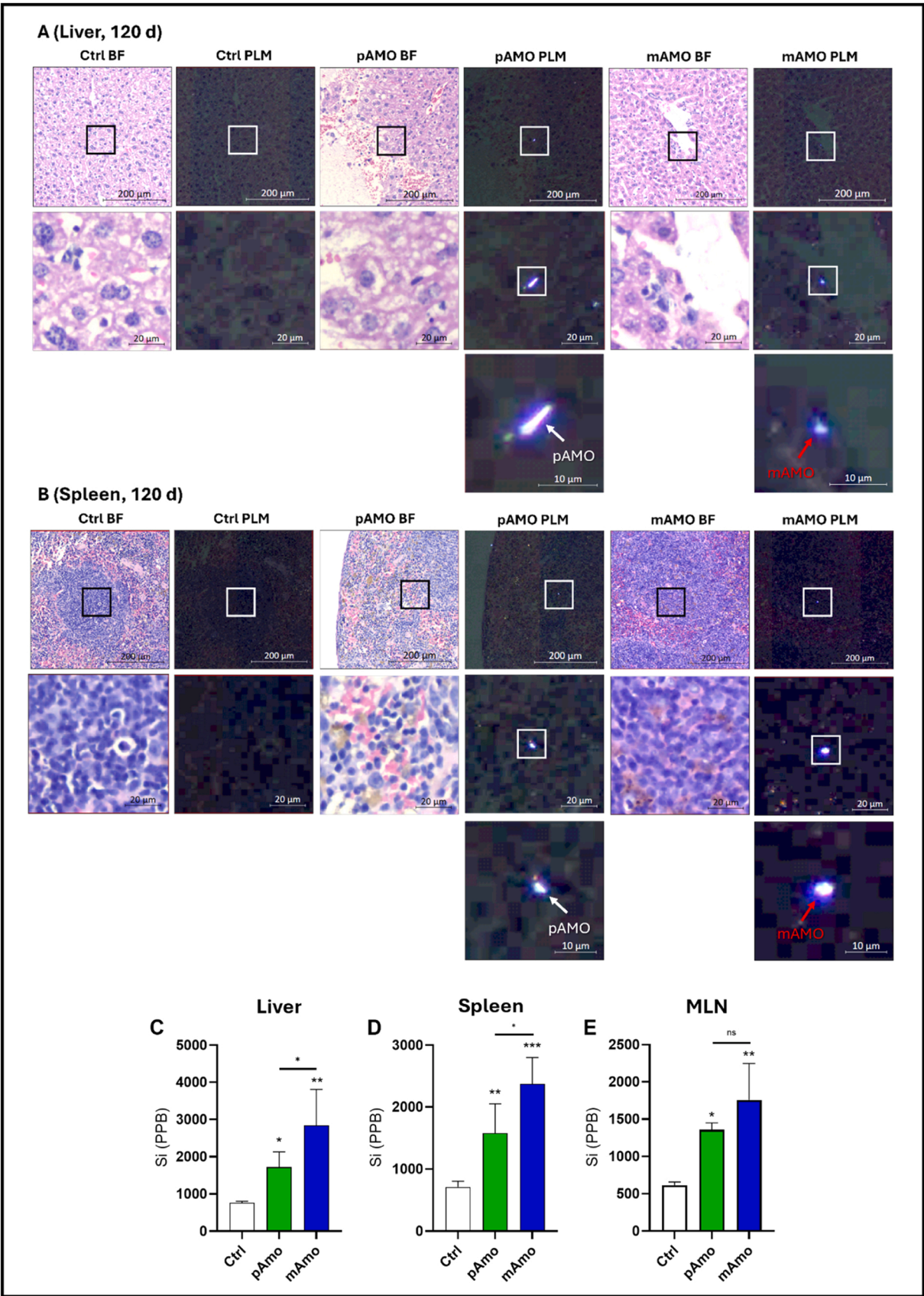


Fig. 8. Absence of mAmo fibers in the lungs of treated mice 120 d post exposure. C57BL/6 mice ($n = 5$ per group) were treated via oropharyngeal aspiration (o.p.a.; 50 μ l) with PBS-BSA (control, Ctrl), pAmo or mAmo (0.1 mg/mouse). Lung tissue was analyzed 120 days post-treatment using bright-field (BF) and polarized-light microscopy (PLM) to assess pulmonary fiber burden. Representative images from the control group (A) BF, full size, (B) BF, magnification, (C) PLM of B; pAmo-exposed group: (D) BF, full size, (E) BF, magnification, (F) PLM of E; mAmo-exposed group: (G) BF, full size, (H) BF, magnification, (I) PLM of H. pAmo fibers are indicated by white arrows in PLM images. Panel (J) shows quantification of pAmo and mAmo in lung homogenates (500 μ l) by measuring silicon (Si) and manganese (Mn) content via ICP-MS as exposure tracers. Scale bars: 200 μ m (full-size images) and 50 μ m (magnified views). ** $P < 0.002$, and *** $P < 0.001$ relative to controls, or between pAmo- and mAmo-treated groups; ns: not significant. ANOVA with Tukey's multiple comparison test. Data are presented as mean $\pm$ SD ($n = 5$).

via the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$). This sustained oxidative burst led to immunogenic cell death (ICD) of phagocytes attempting to engulf the long fibers, in a process known as frustrated phagocytosis [77,78]. As a consequence, damage-associated molecular patterns (DAMPs) including cell-free DNA and IL-1 α , and

nuclear proteins were released, contributing to early neutrophilic inflammation [79]. In addition, increased levels of proinflammatory cytokines such as IL-1 β , indicative of NLRP3 inflammasome activation, and IL-6, which has been implicated in asbestos-induced immune responses, further shaped the inflammatory environment [80,81].



(caption on next page)

Fig. 9. Long-term bioaccumulation of pAmo and mAmo fibers was detected in liver, spleen, and lymph nodes. Extrapulmonary bioaccumulation of pAmo or mAmo was observed 120 d post exposure. Representative bright-field (BF) and polarized light microscopy (PLM) images of H&E-stained tissue (Section 5 μ m) from vehicle-treated mice (Ctrl) and mice exposed to 0.1 mg pAmo or mAmo fibers 120 days post administration via oropharyngeal aspiration (o.p.a.; 50 μ l, 0.1 mg/mouse, $n = 5$ per group). (A) liver and (B) spleen; fibers are indicated by white arrows (pAmo) or red arrows (mAmo). Quantification of extrapulmonary fiber burden was performed by measuring silicon (Si) content using ICP-MS in the (C) liver, (D) spleen, and (E) mediastinal lymph nodes (MLN). Fibers (bright white) deposited in the tissues are pointed by white or red arrows (pAmo and mAmo, respectively). pAmo or mAmo burden in extrapulmonary organs was measured quantifying Si content via ICP-MS analysis. * $P < 0.033$, ** $P < 0.002$, and *** $P < 0.001$ relative to controls, or pAmo- vs. mAmo-treated groups; ns: not significant. ANOVA with Tukey's multiple comparison test. Data are presented as mean $\pm$ SD ($n = 5$).

Notably, we also observed elevated pulmonary TNF- α levels in animals exposed to pAmo. This response is likely mediated by Toll-like receptor 4 (TLR4) signaling, an intracellular pathway previously linked to asbestos-induced inflammation [82]. Together, these findings underscore the pivotal role of inflammatory cytokines in orchestrating the pulmonary immune response following long pAmo exposure. Moreover, they highlight such cytokines as potentially useful biomarkers for assessing the pulmonary hazard of elongated mineral particles in predictive toxicology studies.

Over time, this sustained inflammatory environment favored the accumulation of immune cells - likely T lymphocytes that contribute to pathological responses following mineral particle exposure [83] - and promoted profibrotic remodeling. These processes resulted in collagen deposition and structural tissue changes, indicating ongoing fibrosis. Such effects were also promoted by the inability of macrophages to efficiently clear apoptotic cells, a cell death subtype recognized for amosite asbestos [37], in a process named impaired efferocytosis [17, 84].

In contrast, short mAmo fibers exhibited markedly lower surface reactivity and poor hydroxyl radical production under acidic conditions. Their surfaces were more oxidized than pAmo, containing a higher quantity of Fe³⁺ ions, known to react more slowly with H₂O₂ and contributing less to radical generation, when compared to Fe²⁺. Indeed, the reaction rate of Fe³⁺ with H₂O₂ is about two orders of magnitude lower than that of Fe²⁺, since it must first be reduced to release $\cdot$ OH ($\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{HO}_2 + \text{H}^+$) [85]. mAmo fibers, therefore, though exhibiting partial activity, were efficiently internalized and cleared by lung macrophages, without triggering significant inflammatory and fibrotic responses. This contrasts with findings from a recent *in vitro* study by our group, which demonstrated greater cytotoxicity of mAmo fibers compared to pAmo in macrophage cell line [37]. We attribute this apparent discrepancy to the complexity in simulating, in a 2D *in vitro* model, key *in vivo* mechanisms such as the continuous recruitment of new macrophages, and the overall frustrated phagocytosis process.

Using a combined histological and ICP-MS approach, we tracked the clearance of amosite fibers from the lungs. This platform overcomes the common limitations of polarized light microscopy, which is unable to properly detect small objects and fragments commonly affecting short fiber preparations [37,86]. pAmo exhibited substantial retention in the lungs up to four months post-exposure, underscoring their high pulmonary biopersistence, responsible for marked chronic effects. This was consistent with findings from animals exposed to long crocidolite (Crc) fibers (Supplementary Materials, Fig. S4). In contrast, mAmo fibers were almost completely cleared from the lungs after 4 months, consistent with their smaller size and greater potential for cellular internalization. Silicon and manganese were effective elemental tracers, providing robust quantification of fiber burden over time despite the possible incongruent dissolution of the fibers, which could result in a preferential release of Mn cations with respect to Si [87]. Notably, at four months post-exposure, both fiber types were detected in extrapulmonary organs, including the liver, spleen, and mediastinal lymph nodes, with mAmo fibers showing higher bioaccumulation than pAmo. These findings are consistent with previous clinical and experimental reports suggesting that fiber deposition beyond the lungs, including in regional lymph nodes and systemic organs, may occur following inhalation exposure [34,88,89]. Several mechanisms could potentially contribute to the extrapulmonary translocation of fibers [30], including lymphatic

drainage (primary translocation) and systemic distribution via the bloodstream (secondary translocation). Inflammation, especially in response to highly proinflammatory pAmo fibers, may raise pulmonary interstitial pressure and facilitate fiber translocation across the alveolar-capillary barrier into the circulation [30]. This hypothesis is supported by our prior work demonstrating bloodstream-mediated biodistribution of other inflammatory particle types, such as crystalline silica [65]. In contrast, the smaller size and lower surface reactivity under phagolysosomal conditions of mAmo fibers may favor their internalization by phagocytic immune cells, including macrophages and dendritic cells, which are capable of migrating across epithelial and endothelial barriers. These immune cells may facilitate the transport of short fibers along lymphatic pathways, potentially explaining the presence of mAmo in immune-related sites such as the draining mediastinal lymph nodes [90]. While certain studies suggest that splenic accumulation of inhaled particles can occur through lymphatic drainage, the potential involvement of bloodstream transport in mediating the distribution of mAmo in the spleen, at least in part, cannot be excluded [91–93]. Although our study does not allow definitive identification of the exact translocation routes, the observed patterns suggest that fiber characteristics such as size, reactivity, and inflammation potential may influence the mechanisms by which asbestos fibers reach extrapulmonary compartments [34]. The detection of amosite fibers in immune-related organs also raises question regarding possible immunotoxic systemic effects, including immune modulation. Indeed, prior work from our group has reported increased levels of anti-dsDNA autoantibodies in asbestos-exposed mice, suggesting a potential link between fiber distribution and autoimmune outcomes [94].

Our study aimed to isolate the role of fiber length in asbestos-induced lung toxicity by employing two amosite samples of identical mineralogical origin but distinct morphologies, along with a reference long fiber (UICC crocidolite) with well-established pathogenicity at the investigated dose (see Section 2.6). This approach enabled us to attribute differences in biopersistence and biological activity primarily to fiber dimensions, and secondarily to surface reactivity, while minimizing confounding effects related to compositional variability. Also, the approach presented in this study could be extended to other environmental and naturally-occurring respirable EMPs such as fibrous antigorite and zeolites, to more accurately characterize the potential pathological hazards associated with exposure to these materials [95, 96]. Certain cytotoxic fibrous antigorites have recently been suggested to exhibit high biopersistence in simulant fluids, indicating the potential for long-term detrimental pulmonary effects upon exposure [97].

We acknowledge, however, that the present study has certain limitations. First, it is important to highlight that we administered the two fibers to animals in equal masses, but not equal numbers (fiber number of mAmo > pAmo). However, our results are not influenced by this scenario, as the pathological impact of mAmo, despite being administered in higher numbers than pAmo, was markedly lower. This indicates that the intrinsic and fiber-specific inflammatory and fibrotic potency of mAmo is minimal compared to that of pAmo. Future investigations could aim to expose animals to equivalent fiber numbers of mAmo and pAmo to better assess their relative inflammatory and fibrogenic potency under controlled numerical conditions.

Another notable limitation of this study lies in the species-specific anatomical and physiological differences between rodents and humans, which can profoundly influence the deposition, retention, and

clearance of airborne fibers within the respiratory tract [91]. This issue is especially relevant because the World Health Organization's definition of a respirable mineral fiber is based on human respiratory physiology and may not directly apply to murine models. Emerging computational approaches hold promises for bridging the gap between rodent data and human risk assessment. Such models can enhance the accuracy of predictions regarding fiber behavior, including clearance and translocation, within the human respiratory system [98,99].

The bolus administration method employed in this study (namely oropharyngeal aspiration or o.p.a.) offers practical advantages such as precise dose control and technical feasibility [44,45]. In addition, toxicological studies employing oropharyngeal aspiration have shown that this administration route produces pulmonary lesion severity and particle effects comparable to those observed with established methods such as inhalation or intratracheal instillation. These findings support the validity and reliability of o.p.a. as an accepted model for investigating pathological mechanisms at pulmonary level [44,45,100–102]. Nonetheless, this exposure route does not fully replicate the continuous, dynamic nature of real-world inhalation exposure. Our findings therefore reflect the effects and biodistribution of asbestos fibers following a single, acute exposure, which may not capture the responses that occur under chronic or repeated exposure scenarios. In addition, the fiber suspensions used here were prepared in phosphate-buffered saline (PBS) supplemented with bovine serum albumin (BSA) and optimized through ultrasonication and vortexing to ensure homogenous and stable dispersions for consistent pulmonary delivery. While this preparation enhances reproducibility, it may not fully reflect the physicochemical state of fibers in environmental aerosols. Furthermore, this preparation likely results in biocorona compositions distinct from those formed during inhalation, where fibers are delivered as aerosolized dry particles. Fiber aggregation and deposition patterns are also expected to differ between aspiration and inhalation routes, potentially influencing regional distribution within the lungs and cellular interactions [101]. Future studies employing reference inhalation exposure protocols are warranted to better mimic environmentally and occupationally relevant conditions, and to validate the observed effects under physiologically realistic exposure regimens.

A final consideration relates to the physiology of the pulmonary lymphatic system. The lung's lymphatic network is characterized by a series of one-way valves that maintain directional fluid flow from the alveolar interstitium toward draining lymph nodes under physiological conditions [103]. Upon death, the cessation of the one-way lymphatic flow, which under physiological conditions is maintained by valves and respiratory-driven forces [104], raises the theoretical possibility that fibers still present in the lymphatic vessels could redistribute into extrapulmonary tissues during necropsy. While this represents a conceivable contamination route, our study design minimizes the risk. Specifically, we confirmed lung clearance at the 120-day time point - when ICP-MS and PLM confirmed that short fibers were no longer detectable in the lungs - and collected extrapulmonary organs prior to lung removal under sterile conditions and saline rinsing, consistent with protocols described in published studies within the particle biodistribution and inhalation toxicology literature [59,60,105]. Although deep freezing of whole animals prior to necropsy has been suggested to prevent lymphatic backflow, this approach may introduce practical limitations such as disruption of tissue integrity due to the formation of ice crystals, which could interfere with downstream histological or analytical procedures [106–108]. Nonetheless, we acknowledge this theoretical risk and recommend further studies to explore complementary strategies for confirming extrapulmonary fiber localization.

5. Conclusions

Our findings highlight the central role of phagolysosomal activation and pulmonary retention in participating in the mechanisms underlying asbestos-induced lung pathology. These processes appear to be essential

for the toxic potential of fibers: when fibers do not possess characteristics such as a length that prevents complete internalization by phagocytic cells and sustained surface reactivity under phagolysosomal conditions, they are markedly less pathogenic. Although short fibers induced minimal lung pathology in our study, their potential to reach extrapulmonary sites should not be disregarded. The presence of fiber-associated signals in secondary organs suggest that systemic distribution may occur under certain conditions, warranting further investigation into potential long-term or immune-related effects. We suggest ICP-MS analysis as a reliable, sensitive, and easy-to-perform option for quantifying fiber burden in lung tissue and extrapulmonary target sites, thereby complementing existing methodologies for evaluating fiber clearance and redistribution following exposure.

Ethics approval and consent to participate

Animal studies were approved by the Institutional Animal Care and Use Committee (EEC n°86/609, LA1230312 and 2022/UCL/MD/019), respectively. A proof/certificate of approval is available upon request.

Environmental implication

Persistent long asbestos fibers (length > 5 µm, aspect ratio ≥ 3:1) are airborne carcinogens associated with lung inflammation and fibrosis. In contrast, the health risks posed by short asbestos fibers (length < 5 µm, aspect ratio ≥ 3:1) remain debated. Our comparative study demonstrates that short amosite fibers cause minimal lung toxicity due to their low reactivity and efficient pulmonary clearance. Although they present limited pulmonary hazard, their systemic distribution to secondary target organs underscores the need to consider potential non-pulmonary health effects in future risk assessments and regulatory decisions.

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Jasmine Rita Petriglieri: Writing – original draft, Methodology, Investigation. **Riccardo Leinardi:** Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **François Huaux:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Valérie Lecqueur:** Writing – review & editing, Funding acquisition, Conceptualization. **Saloua Ibouaadaten:** Investigation. **Yousof Yakoub:** Resources, Investigation. **Amandine Pochet:** Investigation. **Francine Uwambayinema:** Investigation. **Francesco Turci:** Writing – review & editing, Conceptualization. **Alain Lescoat:** Methodology. **Maura Tomatis:** Methodology, Investigation.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.139648.

Data availability

Data will be made available on request.

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