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BONSALL, Sam, ISSITT, Theo, PEAKE, Nick, HERIGSTAD, Mari, WEBBER, Jason and HAYWOOD-SMALL, Sarah <http://orcid.org/0000-0002-8374-9783>

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Asbestos mineral fibre exposure significantly affects volatile organic compound profiles of mesothelial cell lines in vitro

Sam Bonsall[1], Theo Issitt[1], Nick Peake[1], Mari Herigstad[1], Jason Webber[2] & Sarah Haywood-Small[1]✉

Malignant mesothelioma (MM) is a rare cancer caused by exposure to asbestos, this condition continues to represent a significant diagnostic and therapeutic challenge. Due to the long disease latency, non-invasive diagnostic modalities such as breath analysis may enhance MM early detection by identifying the disease specific pattern of volatile organic compounds (VOCs) in exhaled breath. In this study, solid phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS) was used to extract VOCs from the headspace of two mesothelioma cell lines: MSTO-211 H (biphasic mesothelioma) and NCI-H28 (epithelioid mesothelioma) in addition to MET-5a (non-malignant mesothelial cell line), following exposure to asbestos mineral fibers (actinolite, amosite, crocidolite and chrysotile) and a non-asbestos fiber control (wollastonite). Multivariate statistical analysis was applied to identify VOC-based biomarkers associated with asbestos exposure within the cell lines. Data confirms that exposure to mineral fibers (including the non-asbestos fiber control) induces significant alterations in the levels of as many as 24 VOCs in each cell line. This is the first study to investigate the impact of asbestos mineral fiber exposure on the VOC profile of MM cell lines. This may be relevant to studies involving mesothelioma patients, as these VOCs could also serve as indicators of asbestos exposure in individuals who have been exposed to asbestos.

Keywords Malignant mesothelioma, Volatile organic compounds, Asbestos

Malignant mesothelioma (MM) is a rare, incurable cancer affecting the lining of the lung, primarily associated with occupational asbestos fibre exposure. While asbestos usage is banned in the UK, the mining, importation, and usage of asbestos continues in many countries, despite recent studies directly linking a decreased incidence of MM to asbestos bans[1].

MM represents an ongoing diagnostic challenge. An overwhelming majority of MM cases are diagnosed at an advanced stage of disease progression, precluding patients from receiving curative treatment[2]. The largely non-specific symptoms associated with MM, coupled with a median latency period of approximately 40 years between initial asbestos exposure and development of malignancy both contribute to the difficulty of detecting MM at an earlier stage. Symptoms of MM, such as pleural effusion and shortness of breath arise at a much later stage of disease progression[3]. MM can only be clinically diagnosed via invasive pleural biopsy and thoracentesis at the onset of late-stage symptoms[4]. This highlights the need for a more effective diagnostic method for MM that can detect the disease at an earlier and potentially more treatable stage. Currently, MM has a median survival time between 8- and 14-months without treatment[5]. Early detection of MM could lead to better patient outcomes and enable further research into potential curative treatments to be conducted on patients with earlier stage disease.

An emerging diagnostic modality for cancer is breath analysis, whereby the levels of endogenous volatile organic compounds (VOCs) are measured in an individual's breath, usually via gas chromatography/ mass spectrometry (GC/MS). VOCs are low vapor pressure compounds which are released from cells as part of regular metabolic processes. Internal metabolic processes associated with disease can be disturbed, altering the VOC metabolites released and consumed by cells[6,7]. Therefore, individual VOCs or a VOC profile can serve as biomarkers of disease or progression towards a diseased state. In the context of cancer, this is a particularly

[1]Biomolecular Sciences Research Centre, Sheffield Hallam University, Sheffield, UK. [2]Institute of Life Science, Faculty of Medicine, Health and Life Science, Swansea University, Swansea , UK. ✉email: s.haywood-small@shu.ac.uk

attractive diagnostic option, as the accumulation of genetic mutations on top of rapid, uncontrolled cellular division may result in distinct VOC profiles related to these characteristics.

While high levels of accuracy and sensitivity have been achieved in studies investigating the validity of prospective diagnostic breath tests for pleural malignancies including MM[8,9], wide scale clinical validation is still required for breath tests to be adopted for use in the diagnosis of cancer. The precise relationship between malignancies and the various VOCs that have been identified as potential biomarkers is not yet fully understood. In general, cellular stress is thought to result in increased levels of lipid peroxidation, which in turn leads to altered production of VOCs[10,11] which can then be detected via breath test. MM is commonly referred to as an inflammatory cancer, and is characterised by a prolonged period of inflammation arising from frustrated phagocytosis of asbestos fibres and iron-mediated ROS/RNS production, which can lead to altered VOC profiles[12]. Additionally, recent research has shown that BAP1 mutations lead to significant alterations in VOC profile in mesothelial cells in vitro[13]. Furthermore, recent studies have successfully differentiated malignant mesothelial cell lines from non-malignant mesothelial and lung cancer cell lines based on VOC profile alone, indicating that MM induces a VOC profile distinct from both lung cancer and non-malignant mesothelium, which could potentially be detected in an individual's breath[14,15].

For the first time, this research describes the effect of asbestos mineral fiber (AMF) exposure upon cellular VOC profiles within a controlled, laboratory-based setting. We hypothesize that AMF exposure (actinolite, amosite, crocidolite and chrysotile) and a non-AMF control (wollastonite) will result in significant, detectable changes in VOC profile for mesothelial cell lines (MSTO-211 H, biphasic and NCI-H28, epithelioid) and non-malignant mesothelial cell line, MET5a. While the SV40-transformed MET-5 A cell line employed in this study may not perfectly recapitulate the characteristics of normal mesothelial cells due to its altered p53 status, this non-malignant model nonetheless provided a valuable in vitro platform to investigate the early molecular changes associated with mineral fiber exposure. Primary mesothelial cell cultures would have offered a more physiologically relevant system to study the progression to mesothelioma; however, access to such cell lines was not feasible within the timeframe of this research. Despite this limitation, the comprehensive analysis of fiber-induced alterations in the volatile metabolome, as reported here, represents an innovative approach that could enhance non-invasive early detection of disease through breath analysis. Overall, these findings advance mechanistic understanding of mineral fiber toxicity and lay the groundwork for developing improved strategies for mesothelioma prevention and intervention.

Materials and methods

Cell culture

MET-5a, MSTO-211 H and NCI-H28 cells were purchased from ATCC. MSTO-211 H and NCI-H28 cells were cultured in RPMI-1640 medium with L-glutamine (Thermo Fisher, Loughborough, UK) supplemented with 10% v/v FBS and 1% v/v Penicillin/Streptomycin (Thermo Fisher). MET-5a cells were cultured in Medium 199 (Merck, London, UK) supplemented with 10% v/v FBS, 1% v/v Penicillin/Streptomycin, 400nM HEPES, 440μM hydrocortisone, 870μM zinc free bovine insulin and 3.3nM human epidermal growth factor as previously described[16]. cell lines were maintained at 37 °C in a 5% C2 atmosphere. Culture medium was renewed every 3 days. Cells were subcultured at 70–80% confluency by detachment with 0.4% trypsin-EDTA solution. All cell lines were routinely screened throughout the study and found to be negative for mycoplasma with the MycoAlert detection kit (Lonza).

Headspace gas analysis of mesothelial cell lines

1.8×10^6 cells were seeded into standard T75 culture flasks and incubated for 24 h. Culture medium was then discarded and replaced with 15 mL culture medium containing 5 μg/mL AMFs. Cell culture medium was still removed and replaced for untreated cells and contained 50 μL PBS as a vehicle control. Cells were then incubated in the presence of a single AMF, a non-AMF control, or without additional treatment for a further 48 h. A 50/30μM DVB/CAR/PDMS SPME fiber was then used to extract VOCs from culture flask headspace by piercing the cap filter and exposing the SPME fiber to the flask headspace for 15 min, then retracting the fiber into the fiber holder assembly. The assembly and filter cap were sealed with parafilm for the duration of sampling. The SPME fiber was cleaned by insertion into a GC-MS inlet for 10 min at 250 °C prior to VOC extraction. VOC extraction was performed inside a cell culture incubator under standard culture conditions. New SPME fibers were conditioned in a GC-MS inlet at 270 °C for 30 min in accordance with manufacturer instructions. "Blank" flasks containing only cell culture medium with corresponding AMFs were also analyzed identically. While the order of sample analysis was not randomized, and blinding was not employed during data acquisition, no evidence of batch effects was observed, as demonstrated by the distinct clustering of asbestos-treated and untreated samples. To account for background signals, the study design included six biological replicates per group along with blank samples and media controls. Due to the inherent limitations of the SPME approach, internal standards were not utilized, and the analysis focused on relative abundances of detected compounds rather than absolute quantification. Despite these methodological considerations, the comprehensive workflow employed in this investigation provides robust, reproducible data to elucidate the volatile metabolomic alterations induced by asbestos exposure.

Asbestos mineral fibres

Asbestos mineral fibers were managed as described previously[17]. The Union for International Cancer Control-accredited fibers (Health and Safety Laboratories, UK, as described in Table 1) were prepared in specially designed laminar flow hoods (Santia Asbestos Management Ltd. laboratories) in a phosphate-buffered saline (PBS) solution to a final stock concentration of 2 mg/ml. AMFs pose a minimal risk of inhalation in an aqueous

solution and were stored in PBS and regularly checked to ensure they remained in solution. Solutions were sterilized at 121 °C in an autoclave and stored at room temperature, protected from light.

GC-MS analysis of VOCs

GC-MS analysis of VOCs was conducted as previously described (Little et al., 2020). An Agilent 7890 A with an Rtx-VMS capillary column (30 m x 0.25 mm x 1.4 µm; Restek, Saunderton, UK) coupled to a MS-5975 C triple axis detector was used for VOC analysis. The GC-MS inlet temperature was set to 250 °C for initial injection of the SPME fiber. The oven program was set as follows: Initial temperature of 35 °C held for 5 min, ramped to 140 °C at a rate of 4 °C min^{-1} and held for 5 min, then finally ramped to 240°C at a rate of 20 °C min^{-1} and held for 4 min. This gave a total run time of 45.25 min for one sample. The MS transfer line was set to 260 °C and VOC analysis was performed in full scan mode with a range of 35–300 a.m.u. VOCs were analyzed via direct injection of the SPME fiber into the GC-MS inlet. The SPME fiber was exposed for 10 min after insertion of the fiber assembly needle into the GC-MS inlet to simultaneously release VOCs for analysis and clean the SPME fiber for subsequent VOC extractions.

Cell viability

All cells were assayed for viability via trypan blue exclusion test following VOC extraction to confirm that AMF treatments did not negatively impact cell viability. T75 flasks containing cells were briefly washed with PBS, then trypsinized with 0.4% trypsin-EDTA to allow full detachment of cells. Cell viability and count were then assessed using 0.05% trypan blue in a 1:1 ratio with cell suspensions. Cell counts were performed with a countess II automated cell counter (Thermo Fisher).

Data processing and analysis

Mass spectra were exported from masshunter as CDF files and deconvolved and analyzed using the *eRah* R package[18,19]. Spectra were deconvolved with the parameters: min.peak.width = 1,1; avoid.processing.mz = c(35:45), and the resulting deconvolved spectra were then aligned with the parameters: min.spectra.cor = 0.8; max.time.dist = 4; mz.range = 35:300. Metabolites were then identified via spectral match to the MoNA GC/MS spectra database[20]. Prior to analysis, data was normalized with Metaboanalyst, using the "normalise by sum" function, resulting in a dataset which adhered to the assumptions of parametric testing. All subsequent data analysis was performed on a normalized data matrix. Significantly different compounds between culture medium and their corresponding cell line/treatment combination were retained, and insignificant compounds were omitted from subsequent analysis. Compounds with less than 80% spectral match were also omitted from subsequent analysis. The resulting aligned data matrix containing tentative compound identities was then analyzed using MetaboanalystR 4.0[21,22]. Principal component analysis (PCA) and sparse-partial least squares discriminant analysis (s-PLSDA) plots were produced with the default command parameters, and significant compounds associated with each AMF treatment were determined using independent t-Tests for each individual compound, comparing untreated cells to each AMF exposure group individually. Additionally, to address the issue of multiple comparisons, the Benjamini-Hochberg procedure was used to adjust p-values, reducing the risk of false positive findings due to the large number of compounds analyzed.

Results

Effect of asbestos fibre stimulation on cell viability

Trypan blue exclusion tests were conducted following VOC analysis to ensure asbestos fiber exposure did not affect cell viability. Asbestos fiber stimulation did not significantly affect cell viability or cell number for any cell line or fiber treatment (Fig. 1.)

PCA and SPLS-DA analysis

Background VOCs and siloxane compounds originating from cell culture medium, flasks and artefacts from the sampling process were removed prior to data analysis as described previously[12]. Each cell line displayed separation between untreated and AMF treated cells on both PCA and SPLS-DA plots. All cell lines showed some degree of separation between AMF treatments following PCA (Fig. 2).

The MET-5a cell line treatments clustered into two distinct groups, an untreated group and a second, tightly clustered group consisting of every AMF treatment, including the non-asbestos control fiber, wollastonite. The NCI-H28 cell line displayed three main groups. The first consisted of untreated cells, and the second of chrysotile

Fibre Name	Fibre Morphology
Actinolite	Amphibole
Amosite	Amphibole
Crocidolite	Amphibole
Chrysotile	Serpentine
Wollastonite	Non-Asbestos Fibre Control

Table 1. AMFs and control fibre used in this study, and their associated morphology.

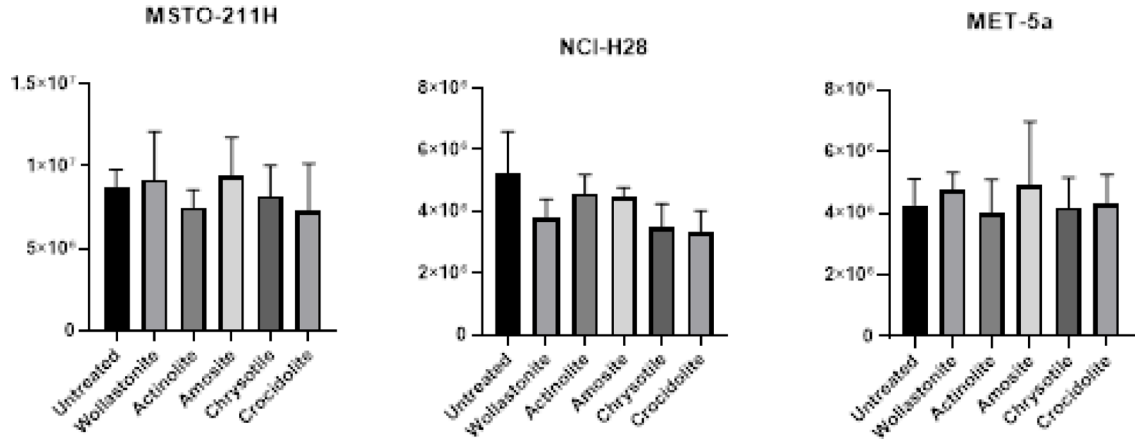

Fig. 1. Trypan blue exclusion test total live cell count results for all cell lines stimulated with 5 µg/mL AMF for 48 hours. One way ANOVA analysis did not reveal significant differences in cell number or viability following AMF stimulation (P>0.05).

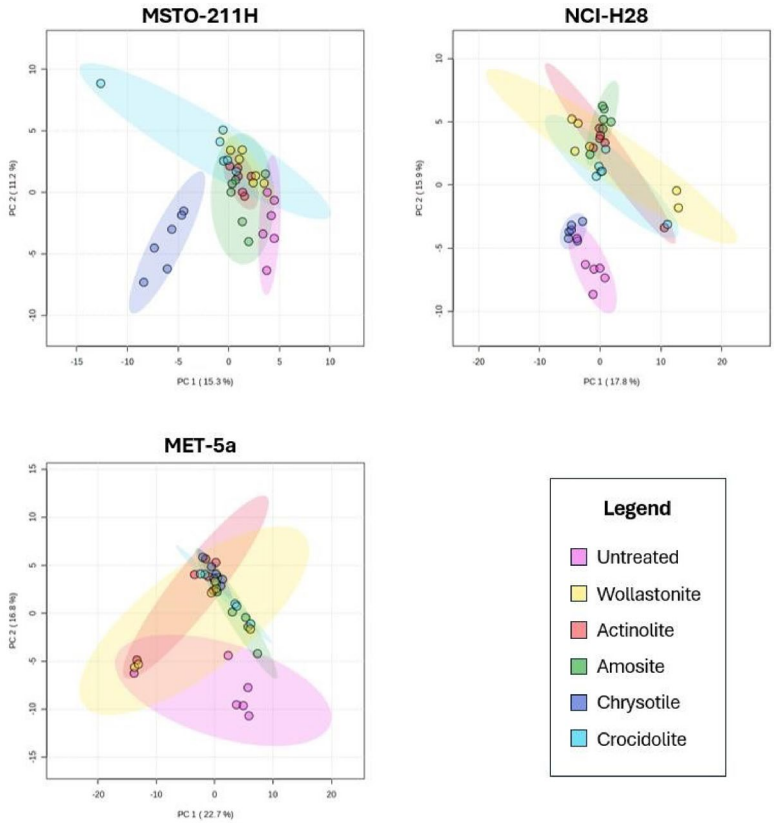

Fig. 2. PCA plots of mesothelial cell lines treated with AMFs. Each individual point represents a single experimental repeat ($n = 6$ per treatment).

treated cells, with minor overlap of a single experimental repeat from the untreated cells between these two groups. The third group consisted of relatively tightly clustered points from all remaining AMF treatments (Fig. 3). The MSTO-211 H cell line separated into two groups, one containing only chrysotile treated cells, and the second group consisting of all other AMF treated cells and untreated cells. All treatments in the second group were closely clustered.

Applying sPLS-DA resulted in much greater separation between the groups and a clearer differentiation between untreated and AMF treated VOC profiles across all cell lines (Fig. 3). sPLS-DA resulted in similar clustering and numbers of treatment clusters to PCA. The largest observed difference was the separation of the

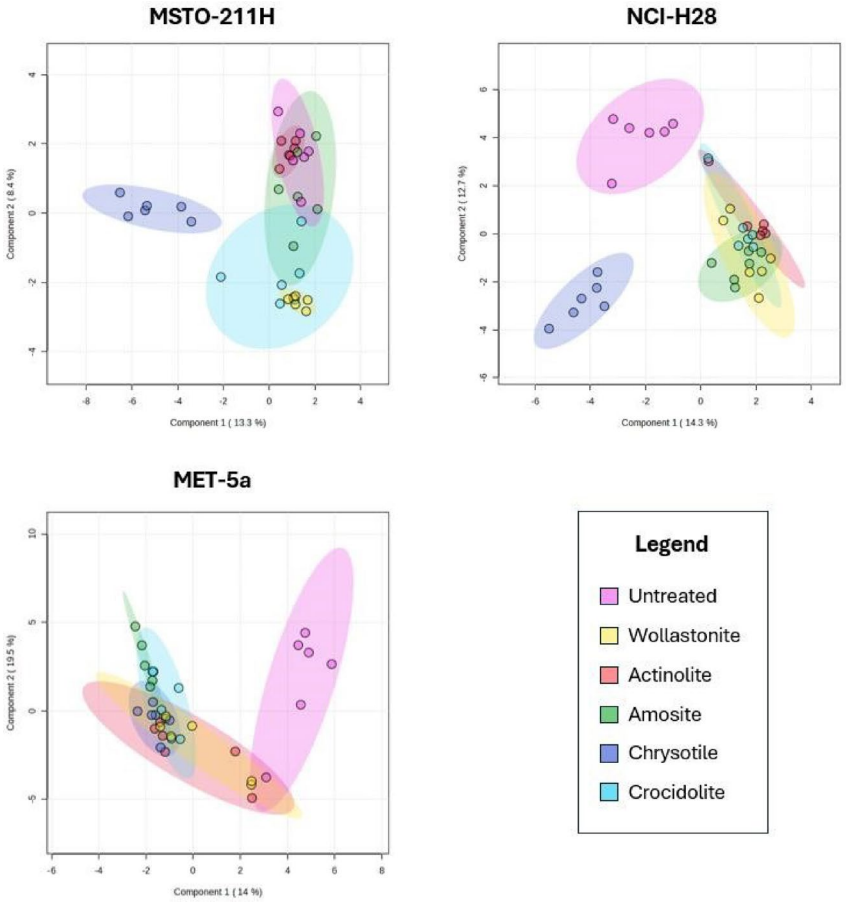

Fig. 3. SPLS-DA plots of mesothelial cell lines treated with AMFs. Each individual point represents a single experimental repeat ($n = 6$ per treatment).

NCI-H28 AMF treatments into three distinct groups rather than two, with untreated and chrysotile treated cells clearly separating, rather than clustering together as they did following PCA.

VOCs associated with AMF exposure

Following filtering and normalization of GC-MS results, statistical analysis revealed forty-seven VOCs which were significantly increased or decreased for at least two mineral fiber treatment for the MET-5a cell line. Subsequent filtering of compounds with low quality matches to the MoNA database produced a final panel of 29 VOCs which were significantly altered ($P < 0.05$ vs. untreated) in the MET-5a cell line following AMF stimulation (Table 2).

Discussion

Enhancing the clinical diagnostic options for malignant mesothelioma (MM) remains an urgent priority to improve patient outcomes. The identification of clinically relevant early detection biomarkers for MM would eliminate the need for current invasive procedures such as thoracentesis and pleural biopsy. Here, we have successfully demonstrated VOC metabolites specific to asbestos fiber exposure in a controlled environment, generating potential target VOC based biomarkers of asbestos exposure in MM. MM is classified as an inflammatory cancer, and its extended latency period suggests that patients undergo a prolonged period of oxidative stress in the pulmonary region, which may be reflected in an individual's breath which could be tested for the VOCs presented in this research.

Previous studies[14,15] investigated the VOC profile of MM cell lines in vitro. Little et al. showed that mesothelial cell subtype can be differentiated based on VOC profile, with different mesothelial subtypes, biphasic and epithelioid, having distinct VOC profiles. While the differentiation of mesothelial subtype based on VOC profile represents a step forward in the pursuit of a diagnostic breath test for MM, it is important to recognise the pathogenesis of MM involves a lengthy latency period following initial exposure to asbestos[23]. An ideal breath test for MM would be capable of detecting MM at an earlier, more treatable stage than current diagnostic methods. Previous studies have focused on the differentiation of healthy volunteers from MM patients[8,9,24], however the prolonged period of inflammation arising from asbestos exposure has not been explored in relation to its effect on patient breath VOC profile. Additionally, the effect of AMF exposure on the VOC profile of mammalian cell lines has not been explored either.

MET-5a		
Average RT	**Tentative ID**	**Functional group**
2.89	Isopropyl Alcohol(2-D)	Alcohol
2.99	Isoprene	Alkene
3.73	Butane	Alkane
7.12	Isopropenyl Methyl Ether	Ether
8.35	Benzene	Aromatic
13.81	Isopentyl Phenylacetate	Aromatic
14.44	Thymol	Aromatic
16.18	2-Hexanone	Ketone
16.97	Ethylbenzene	Aromatic
17.00	Benzyl Isovalerate	Aromatic
19.49	3-Methyl-1-Pentanol	Alcohol
19.67	Isopropylbenzene	Aromatic
20.12	5-Nonanone	Alkane
20.69	Undecane	Alkane
20.98	Propylbenzene	Aromatic
21.34	Decane	Alkane
21.45	Hexamethylethane	Alkane
23.11	2-Methylheptane	Alkane
23.45	Tridecane	Alkane
23.88	Nonacosane	Alkane
24.03	3-Phenylindole	Aromatic
24.18	Octadecane	Alkane
24.25	Benzaldehyde	Aldehyde
25.06	Methyl Hexyl Ketone	Ketone
27.85	4-Hydroxymandelic Acid	Aromatic
28.08	Eicosane	Alkane
28.34	Hexadecane	Alkane
31.79	Naphthalene	Aromatic

Table 2. Significantly altered VOCs ($P < 0.05$ vs. untreated) in the MET-5a cell line following AMF exposure as determined by independent t-tests applied to relative abundance values for each cell line. These compounds were detected in at least two AMF treatments. The same procedure was followed for the MSTO-211 H and NCI-H28 cell lines, where thirteen and twenty-nine significant VOCs were identified respectively (Tables 3 & 4). Individual identities of VOCs varied between each cell line, however significantly altered levels of alkanes, methylated alkanes, alcohols, and aromatic compounds were observed in every cell line following AMF exposure.

Asbestos is biopersistent, and therefore cells which have undergone malignant transformation in response to asbestos will still be exposed to asbestos in patients – We have observed evidence of this in mesothelioma tissue samples using LA-ICP-TOFMS imaging in a previous publication[25]. Therefore, asbestos exposure of both malignant and non-malignant cells (MSTO-211 H and MET5A respectively) models both the interaction of the untransformed and transformed cells with asbestos fibres.

Additionally, we aimed to identify whether there were cell-type specific responses to asbestos exposure, as well as responses that were observed across all cell types, both malignant and non-malignant. This approach allows us to identify alterations in VOC profile which are specific to individual cell types, as well as alterations which may be universal across cell types when exposed to asbestos. The comparison between AMF and untreated cells aims to model the impact of asbestos exposure versus no exposure, while the comparison between AMF and Wollastonite-treated cells helps to distinguish asbestos-specific responses from general responses to foreign particles. A detailed summary of all comparisons and the corresponding results is provided in Supplementary Table X."By building a VOC profile which can be attributed to AMF exposure in vitro, it may be possible to detect MM in asbestos exposed individuals during the latency period utilising the same VOCs as targets. Following asbestos exposure, it is likely that that there is a transitional period as mesothelial cells progress from a healthy state to a malignant state as genetic mutations accumulate[26], which may be reflected in exogenous VOCs arising from an altered metabolic state because of in situ AMFs.

This study investigated the impact of four AMFs and one non-AMF on the VOC profiles of mesothelial cell lines. Every mineral fibre treatment was found to induce alterations in VOC profile in every cell line. Initial PCA and sPLS-DA of cellular VOC profiles revealed clear separation of untreated cell VOC profiles from both AMF and wollastonite treated cell VOC profiles (Fig. 2/3). This initial exploratory data analysis confirmed the hypothesis that AMF stimulation induced a detectable, differential VOC profile in mesothelial cells, prompting

further exploration of the specific VOC identities which were affected following asbestos exposure. It was interesting to note that wollastonite, a non-asbestos mineral fibre, still induced significant alterations in VOC profile, which were unable to be differentiated from asbestos treated VOC profiles via PCA or sPLSDA. It is plausible that the physical presence of mineral fibres within and in proximity to cells is responsible for the metabolic response which induces an altered VOC profile, rather than a specific molecular or structural property of asbestos itself. While wollastonite is not categorized as an asbestiform fibre, it induces similar changes in cellular VOC profile to AMFs as shown by PCA and SPLS-DA plots (Figs. 2 and 3).

There were differences observed between each AMF treatment in terms of the specific VOCs which were altered. For example, of the twelve total VOCs which were altered in the MSTO-211 H cell line, only four VOCs were altered following all AMF treatments (Table 5), suggesting that these VOCs could function as generalised markers of AMF exposure regardless of the identity of the AMF. Conversely, actinolite and chrysotile AMF exposure induced changes in VOCs which were unique to that AMF treatment. Amosite and chrysotile AMF exposure induced changes in a total of 11 VOCs, whereas the wollastonite fibre treatment induced changes in seventeen VOCs, the most of any AMF treatment.

AMF exposure did not significantly affect live cell counts in these experiments (Fig. 2). Variations in the number of viable cells would change the levels and identities of VOCs which are detected[27], the lack of any significant alteration in cellular viability or overall cell count suggests that any alterations in VOC profile following asbestos fibre stimulation are a direct result of asbestos fibre stimulation and not reduced cellular viability.

The initial statistical analysis of significantly altered compounds revealed a panel of forty one unique VOCs associated with AMF exposure across the three cell lines. Most of the identified VOCs were long chain alkanes, aromatics and alcohols, classes of compounds which have been previously linked to lipid peroxidation and oxidative stress[28].

Previous small-scale studies have successfully differentiated healthy volunteers from asbestos exposed (Aex) individuals and MM patients based on breath VOC profile[29–32], which supports the notion that in situ AMFs within the mesothelium may induce, or contribute to, a detectable change in breath VOC profile long before malignancy develops.

Four VOCs were found to be significantly altered in every cell line following AMF stimulation (Table 5), indicating that these specific compounds could potentially serve as general markers for asbestos exposure.

Of the compounds which were identified as generalised markers of asbestos exposure, some have previously been identified as discriminatory VOCs in human breath for the diagnosis and detection of lung cancer and mesothelioma. Benzaldehyde has been previously identified differentiating VOC between healthy volunteers and mesothelioma patients[33] and has also been shown to be a discriminatory VOC in lung cancer patients[34]. Decane has also been identified in multiple studies [35–37] as a discriminatory VOC for lung cancer, but to our knowledge has not been specifically reported in mesothelioma previously. Isopentyl phenylacetate has not been previously reported as a VOC based marker of cancer. Additionally, 5-nonane has not been identified as a VOC based marker of cancer previously, but isomers, such as 2-nonanone have been identified as cancer biomarkers in both breast cancer cell lines [38] and patients [39].Classification of the identified VOCs into their functional groups aids the understanding of how AMFs influence cellular VOC profile. Many studies cite the identities of individual VOCs as individual biomarkers for mesothelioma; however, it is unlikely that a single VOC can function as a lone biomarker for disease. Rather, an overall profile attributed to specific classes of VOCs may be more clinically relevant[28]. Human metabolism is complex, and metabolic pathways involving lipid peroxidation arising from oxidative stress can result in a multitude of VOC products which can be linked back to the initial oxidative stress inducing factor. We have identified alkanes, methylated alkanes, alcohols, ketones and aromatics as five classes of exogenous VOCs which are significantly affected by AMF exposure (Tables 2, 3 and 4). Future studies may include the deeper investigation of the exact mechanisms responsible for their alteration via asbestos exposure.

MSTO-211 H		
Average RT	**Tentative ID**	**Functional group**
3.59	2-Methyllactic acid	Carboxylic acid
13.81	Ethylbenzene	Aromatic
16.97	5-Nonanone	Ketone
20.12	Benzaldehyde	Aromatic
21.46	Cyclohexanone	Ketone
21.58	Oxalic Acid Dibutyl Ester	Ester
22.77	(E)−3-Phenyl-2-Propanal	Aldehyde
23.44	N-Dodecane	Alkane
24.26	Isopentyl Phenylacetate	Aromatic
24.62	Decane	Alkane
27.85	Nonadecane	Alkane

Table 3. Significantly altered VOCs ($P < 0.05$ vs. untreated) in the MSTO-211 H cell line following AMF exposure as determined by independent T-Tests applied to relative abundance values for each cell line. These compounds were detected in at least two AMF treatments.

NCI-H28		
Average RT	**Tentative ID**	**Functional group**
1.35	Dimethylamine	Amine
4.02	Hexane	Alkane
8.26	Benzene	Aromatic
13.81	Isopentyl Phenylacetate	Aromatic
15.76	Undecane	Alkane
16.19	2-Hexanone	Ketone
17.46	Meta Xylene	Aromatic
18.66	Butyl Lactate	Ester
19.47	1-Hexanol	Alcohol
19.77	Isopropylbenzene	aromatic
20.11	5-Nonanone	Alkane
20.88	2-Methylheptane	Alkane
20.88	Hexamethylethane	Alkane
21.10	Propylbenzene	Aromatic
21.46	Decane	Alkane
21.58	Oxalic Acid Dibutyl Ester	Ester
23.44	Tridecane	Alkane
23.44	N-Octadecane	Alkane
24.26	Benzaldehyde	Aldehyde
25.05	2-Allyloxyethanol	Alcohol
25.06	Methyl Hexyl Ketone	Ketone
25.43	Isobutylbenzene	Aromatic
25.56	Hexadecane	Alkane
27.91	Nonadecane	Alkane
27.99	Phenol	Aromatic
28.51	Docosane	Alkane
28.97	Isobutyl Phenyl Ketone	Ketone
30.80	1,2,4-Trichlorobenzene	Aromatic
38.94	Hexacosane	Alkane

Table 4. Significantly altered VOCs ($P < 0.05$ vs. untreated) in the NCI-H28 cell line following AMF exposure as determined by independent t-Tests applied to relative abundance values for each cell line. These compounds were detected in at least two AMF treatments. In summary, a total of four VOCs were identified which were consistently significantly altered following AMF exposure in every cell line (Table 5), however, it should be noted that no VOCs were found which were significantly altered by every AMF treatment in every cell line.

All cell lines	
Average RT	**Tentative ID**
13.81	Isopentyl Phenylacetate
20.12	5-Nonanone
21.34	Decane
24.25	Benzaldehyde

Table 5. Significantly altered VOCs ($P < 0.05$ vs. untreated) which were detected in every cell line for at least two AMF treatments *as determined by independent t-Tests applied to relative abundance values.*

The trends seen in volatile levels in mesothelial cell lines following AMF exposure point towards the alteration of endogenous levels of specific classes of VOCs, rather than an increase or decrease in a single VOC. Every AMF treatment induced changes in at least one VOC belonging to these classes of compounds in every cell line. While the specific identity of VOCs varied between each cell line, the impact on VOC classes was similar for all cell lines. For example, it is unlikely that there is a major difference between the release of pentadecane and tetradecane metabolically, as they are both long chain alkanes differing by the incorporation of a single carbon. It is more likely that the presence of long chain alkanes in general is indicative of oxidative stress[28] because of acute AMF exposure.

While this study represents an important first step in understanding the relationship between AMF exposure and cellular VOC profiles, it is important to acknowledge some limitations. While the MET-5a cell line used in

this study is classified as non-malignant, this cell line is rendered immortal via SV40 transformation, resulting in total deletion of p53. Such alterations to a fundamental tumour suppressor gene should be considered when classifying this cell line as a model of normal mesothelial cells (ATCC, CRL-9444).

Comparison of the effects of AMF stimulation on non-malignant and malignant cell lines is important as it shows how aberrant genetic states may impact the VOCs released because of oxidative stress. AMFs are biopersistent and remain in situ in the mesothelium [40], therefore it is likely that MM patient breath VOC profiles will continue to be affected by AMF stimulation following transformation to a malignant state. While chrysotile has been shown to not have the same biopersistence as amphibole AMFs [41], exposure to AMFs in general may still result in an altered VOC profile. The MET-5a cell line is a non-malignant, immortalized cell line which can be used as a model for normal mesothelial cells in vitro. The effects of AMF stimulation on MET-5a cell lines may be analogous to the effect of AMF fibres on healthy human mesothelial tissue.

Conclusions

This study exposed cell lines to AMFs for a period of 48 h. Compared to the latency period of MM, this is a short period of time, however this was sufficient to induce detectable changes to cellular VOC profile. Additionally, previous studies investigating the effects of AMF and AMF-like fibres on mesothelial cell lines showed that an exposure time of 48 h was sufficient to induce detectable transcriptomic changes [42,43]. Longer term AMF exposure to mesothelial cell lines would be a logical next step and would reveal how cellular VOC profile may change in response to longer term exposure.

Biomarkers associated with asbestos exposure presented in this research present the first step in generating targeted approaches for upcoming breath studies. Translation from in vivo studies to human breath is a complex process[28] and the VOCs presented require further validation to assess their potential as MPM biomarkers.

In conclusion, we have shown that AMF stimulation significantly affects the VOC profile of both malignant and non-malignant mesothelial cell lines. Data identifies and supports a panel of VOCs which correspond to general AMF exposure. Our results agree with a recent systematic review which identified classes of compounds that are significantly altered in a state of oxidative stress are primarily long chain alkanes, aromatic compounds, and alcohols[28]. The results presented herein may be applicable to human volunteer studies, as these VOCs may also be indicators of asbestos exposure in asbestos exposed individuals and could potentially act as candidate target markers for investigation in asbestos exposed populations and mesothelioma patients.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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References

1. Zhu, W., Liu, J., Li, Y., Shi, Z. & Wei, S. Global, regional, and National trends in mesothelioma burden from 1990 to 2019 and the predictions for the next two decades. *SSM Popul. Health*. **23**, 101441. https://doi.org/10.1016/j.ssmph.2023.101441 (2023).
2. Zhang, W., Wu, X., Wu, L., Zhang, W. & Zhao, X. Advances in the diagnosis, treatment and prognosis of malignant pleural mesothelioma. *Annals Translational Med.* **3**, 182. https://doi.org/10.3978/j.issn.2305-5839.2015.07.03 (2015).
3. Guzmán-Casta, J. et al. Prognostic factors for progression-free and overall survival in malignant pleural mesothelioma. *Thorac. Cancer*. **12**, 1014–1022. https://doi.org/10.1111/1759-7714.13814 (2021).
4. Brims, F. Epidemiology and clinical aspects of malignant pleural mesothelioma. *Cancers (Basel)*. **13**, 4194. https://doi.org/10.3390/cancers13164194 (2021).
5. Zhang, Y. et al. Predicting survival for patients with mesothelioma: development of the PLACE prognostic model. *BMC Cancer*. **23**, 698. https://doi.org/10.1186/s12885-023-11180-y (2023).
6. Pham, Y. L. & Beauchamp, J. Breath biomarkers in diagnostic applications. *Molecules* **26**, 5514. https://doi.org/10.3390/molecules26185514 (2021).
7. Pereira, J. et al. Breath analysis as a potential and Non-Invasive frontier in disease diagnosis: an overview. *Metabolites* **5**, 3–55. https://doi.org/10.3390/metabo5010003 (2015).
8. Lamote, K. et al. Exhaled breath to screen for malignant pleural mesothelioma: a validation study. *Eur. Respir J.* https://doi.org/10.1183/13993003.00919-2017 (2017).
9. Lamote, K. et al. Detection of malignant pleural mesothelioma in exhaled breath by multicapillary column/ion mobility spectrometry (MCC/IMS). *J. Breath. Res.* **10**, 046001. https://doi.org/10.1088/1752-7155/10/4/046001 (2016).
10. Sutaria, S. R. et al. Lipid peroxidation produces a diverse mixture of saturated and unsaturated aldehydes in exhaled breath that can serve as biomarkers of lung cancer—A review. *Metabolites* **12**, 561. https://doi.org/10.3390/metabo12060561 (2022).
11. Zanella, D. et al. Comparison of the effect of chemically and biologically induced inflammation on the volatile metabolite production of lung epithelial cells by GC×GC-TOFMS. *Analyst* **145**, 5148–5157. https://doi.org/10.1039/D0AN00720J (2020).
12. Little, L. D., Carolan, V. A., Allen, K. E., Cole, L. M. & Haywood-Small, S. L. Headspace analysis of mesothelioma cell lines differentiates biphasic and epithelioid sub-types. *J. Breath. Res.* **14**, 046011. https://doi.org/10.1088/1752-7163/abaaff (2020).
13. Janssens, E. et al. Headspace volatile organic compound profiling of pleural mesothelioma and lung cancer cell lines as translational Bridge for breath research. *Front. Oncol.* **12**, 851785. https://doi.org/10.3389/fonc.2022.851785 (2022).
14. Ju, L. et al. Different cellular response of human mesothelial cell MeT-5A to Short-Term and Long-Term multiwalled carbon nanotubes exposure. *Biomed. Res. Int.* **2017**, 2747215. https://doi.org/10.1155/2017/2747215 (2017).
15. Voloaca, O. M. et al. Laser ablation inductively coupled plasma mass spectrometry as a novel clinical imaging tool to detect asbestos fibres in malignant mesothelioma. *Rapid Commun. Mass Spectrom.* **34**, e8906. https://doi.org/10.1002/rcm.8906 (2020).
16. Hayton, C. et al. Changes in lung epithelial cell volatile metabolite profile induced by pro-fibrotic stimulation with TGF-β1. *J. Breath. Res.* https://doi.org/10.1088/1752-7163/acf391 (2023).
17. Domingo-Almenara, X. et al. eRah: A computational tool integrating spectral Deconvolution and alignment with quantification and identification of metabolites in GC/MS-Based metabolomics. *Anal. Chem.* **88**, 9821–9829. https://doi.org/10.1021/acs.analchem.6b02927 (2016).

18. Horai, H. et al. MassBank: a public repository for sharing mass spectral data for life sciences. *J. Mass. Spectrom.* **45**, 703–714. https://doi.org/10.1002/jms.1777 (2010).
19. Pang, Z. et al. MetaboAnalyst 6.0: towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Res.* https://doi.org/10.1093/nar/gkae253 (2024).
20. Pang, Z., Chong, J., Li, S. & Xia, J. MetaboAnalystR 3.0: toward an optimized workflow for global metabolomics. *Metabolites* **10**, 186. https://doi.org/10.3390/metabo10050186 (2020).
21. Frost, G. The latency period of mesothelioma among a cohort of British asbestos workers (1978–2005). *Br. J. Cancer*. **109**, 1965–1973. https://doi.org/10.1038/bjc.2013.514 (2013).
22. Lamote, K. et al. Breath analysis by gas chromatography-mass spectrometry and electronic nose to screen for pleural mesothelioma: a cross-sectional case-control study. *Oncotarget* **8**, 91593–91602. https://doi.org/10.18632/oncotarget.21335 (2017).
23. Pinato, D. J. et al. Inflammation-Based prognostic indices in malignant pleural mesothelioma. *J. Thorac. Oncol.* **7**, 587–594. https://doi.org/10.1097/JTO.0b013e31823f45c1 (2012).
24. Schnabel, R. et al. Analysis of volatile organic compounds in exhaled breath to diagnose ventilator-associated pneumonia. *Sci. Rep.* **5**, 17179. https://doi.org/10.1038/srep17179 (2015).
25. Issitt, T. et al. Volatile compounds in human breath: critical review and meta-analysis. *J. Breath. Res.* **16**, 024001. https://doi.org/10.1088/1752-7163/ac5230 (2022).
26. Di Gilio, A. et al. Breath analysis for early detection of malignant pleural mesothelioma: volatile organic compounds (VOCs) determination and possible biochemical pathways. *Cancers (Basel).* **12**, 1262. https://doi.org/10.3390/cancers12051262 (2020).
27. Lamote, K. et al. Exhaled breath to screen for malignant pleural mesothelioma: a validation study. *Eur. Respir J.* **50**, 1700919. https://doi.org/10.1183/13993003.00919-2017 (2017).
28. Chapman, E. A., Thomas, P. S., Stone, E., Lewis, C. & Yates, D. H. A breath test for malignant mesothelioma using an electronic nose. *Eur. Respir J.* **40**, 448–454. https://doi.org/10.1183/09031936.00040911 (2012).
29. Dragonieri, S. et al. An electronic nose distinguishes exhaled breath of patients with malignant pleural mesothelioma from controls. *Lung Cancer*. **75**, 326–331. https://doi.org/10.1016/j.lungcan.2011.08.009 (2012).
30. Di Giuseppe, D. et al. Mineral fibres and asbestos bodies in human lung tissue: A case study. *Minerals* **9**, 618. https://doi.org/10.3390/min9100618 (2019).
31. Gualtieri, A. F., Pollastri, S., Bursi Gandolfi, N. & Gualtieri, M. L. In vitro acellular dissolution of mineral fibres: A comparative study. *Sci. Rep.* **8**, 7071. https://doi.org/10.1038/s41598-018-25531-4 (2018).
32. Nymark, P. et al. Gene expression profiles in asbestos-exposed epithelial and mesothelial lung cell lines. *BMC Genom.* **8**, 62. https://doi.org/10.1186/1471-2164-8-62 (2007).
33. Filetti, V. et al. Small RNA-Seq transcriptome profiling of mesothelial and mesothelioma cell lines revealed MicroRNA dysregulation after exposure to Asbestos-like fibers. *Biomedicines* **11**, 538. https://doi.org/10.3390/biomedicines11020538 (2023).

Declarations

Competing interests
The authors declare no competing interests.

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Additional information
Correspondence and requests for materials should be addressed to S.H.-S.