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PAPER

Headspace analysis of mesothelioma cell lines differentiates biphasic and epithelioid sub-types

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Abstract

Malignant mesothelioma (MM) is an incurable cancer. MM is often misdiagnosed, with a poor 5-year survival and limited treatment options. The discovery of endogenous volatile organic compounds (VOCs) is required in order to accelerate the development of a breath test as an alternative to conventional MM diagnosis. For the first time, this study used solid-phase microextraction and gas chromatography-mass spectrometry to identify VOCs released directly from the biphasic MM cell line MSTO-211H and the epithelioid MM cell line NCI-H28 as well as the non-malignant mesothelial cell line MET-5A. Multivariate statistical analysis showed separation between MSTO-211H, NCI-H28 and MET-5A results. 2-ethyl-1-hexanol was significantly increased in both MSTO-211H and NCI-H28 cells compared to MET-5A controls. In addition, ethyl propionate and cyclohexanol were significantly increased in MSTO-211H cells and dodecane was significantly increased in NCI-H28 cells. This is the first study reporting headspace analysis of these MM cell lines and the first to consider the effects of mesothelioma sub-type on VOC profile. Current results further highlight the potential for a diagnostic mesothelioma breath test as well as providing proof of concept for the differentiation between biphasic and epithelioid mesothelioma based on VOC profiles.

1. Introduction

Malignant mesothelioma (MM) is an extremely aggressive and incurable malignancy most commonly affecting the mesothelial cell lining of the pleura as well as other internal organs [1]. MM has a long-established causative link to asbestos exposure [2], a group of naturally occurring silicate mineral fibres that were widely used in UK manufacturing industries [3]. Despite the regulation of asbestos which was introduced in the mid-1980s, UK MM incidence has risen in that time due to a prolonged latency period of up to 60 years since initial exposure [4]. As well as this, approximately 80% of the global population live in countries without strict asbestos controls [1, 3], meaning that MM will be a worldwide public health issue for many years to come. In order to improve survival and treatment options, an increased number of patients—particularly at an early stage—must be identified to enrol onto clinical trials and allow for long-term monitoring of disease progression [5].

Currently MM diagnosis is ineffective; patients are often diagnosed at a very late stage leading to a poor prognosis of just 12–14 months following combined pemetrexed and platinum palliative therapy [6]. It is difficult to identify patients at an early stage due to the prolonged latency period and non-specific signs and symptoms such as chest pains and pleural effusions, which present only in the late stages of the disease [7]. MM tumours can often be mistaken for lung carcinomas and can be classified into epithelioid, sarcomatoid and biphasic sub-types, with differing prognoses [3]. Definitive MM diagnosis must be confirmed through a chest CT scan and invasive biopsies [8]. These issues highlight the requirement of a novel diagnostic method within MM; one capable of identifying patients at an earlier disease stage, differentiating between MM sub-types and avoiding invasive biopsy procedures.

Proteins, such as mesothelin, derived from blood and pleural effusions have been investigated as

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potential MM biomarkers but have failed to translate clinically due to a lack of sensitivity [9]. An alternative to protein biomarkers is the analysis of volatile organic compounds (VOCs) in exhaled breath, which has gained traction within disease diagnosis and monitoring [10]. Previous studies have used gas chromatography-mass spectrometry (GC-MS) to identify VOCs in MM patient breath samples, showing that VOC patterns could discriminate between MM patients and other clinical groups [11–13]. These initial studies provided evidence for the identification of MM patients based on a distinct profile of VOCs in exhaled breath—suggesting that it may be possible to diagnose mesothelioma non-invasively through a VOC-based breath test.

The use of VOC analysis within more well understood cancers, such as lung cancer, has seen further development than in MM. As such, previous studies have analysed the headspace gas above lung cancer cell lines as a model for patient breath analysis [14]. The use of *in vitro* cell lines allows for the exploration of the origins of VOC release through controlled molecular biology experiments targeting specific processes. These types of studies have assessed the impact of oxidative stress [15] and genetic mutations [16] on VOC output, something that would not be possible using patient-derived breath samples. To date there have been no such studies in MM—a cancer that is much rarer and less well understood than lung cancer, which therefore requires *in vitro* models in order to fully research it. Mesothelioma is also a cancer that stands to benefit greatly from VOC analysis due to the current issues faced with diagnosis and extremely poor 5-year survival rate [1]. Therefore, an *in vitro* model of MM VOC analysis would be a valuable tool in understanding this cancer and progressing towards a VOC-based diagnostic test.

For the first time, this study applied VOC analysis methods to a panel of mesothelioma cell lines. The cell lines were chosen to explore the differences in VOC production from biphasic and epithelioid MM sub-types, potentially using headspace to differentiate between these two groups. Biphasic mesotheliomas contain both epithelioid and sarcomatoid morphologies and are associated with a worse prognosis than the epithelioid sub-type alone [3]. Prognosis becomes even poorer as the percentage of sarcomatoid cells increases [3]. It is difficult to distinguish between MM sub-types with current methods meaning that patients are often mis-diagnosed [8]. MSTO-211H was used as a biphasic MM cell line and NCI-H28 as an epithelioid morphology—both cell lines are derived from metastatic pleural effusion [17]. These compounds were compared to a non-malignant mesothelial cell line control, MET-5A, in order to discover VOCs specifically associated with MM. Solid-phase microextraction (SPME) followed by GC-MS was used to analyse the headspace gas above MSTO-211H, NCI-H28 and MET-5A cell cultures, identifying VOCs released by the cells to establish a working model of MM VOC analysis.

2. Materials and methods

All reagents and materials were purchased from Sigma Aldrich (Gillingham, UK) unless otherwise stated.

2.1. Cell culture

MSTO-211H, NCI-H28 and MET-5A cell lines were purchased from American Type Culture Collection (ATCC). MSTO-211H and NCI-H28 cells were maintained in RPMI-1640 (Thermo Fisher; Loughborough, UK) supplemented with a final concentration of 10% volume/volume (v/v) foetal bovine serum (FBS) and 1% v/v penicillin/streptomycin. MET-5A cells were maintained in medium 199 with 10% v/v FBS, 1% v/v penicillin/streptomycin, 3.3 nM epidermal growth factor (Fisher Scientific; Loughborough, UK), 400 nM hydrocortisone, 870 nM zinc-free bovine insulin, 20 mM HEPES and 0.3% v/v Trace Elements B (VWR; Lutterworth, UK). All three cell lines were maintained at 37 °C in the presence of 5% CO_2 in air in a humidified incubator. Culture medium was replaced every 2–3 d and cells sub-cultured through Trypsin-EDTA (Thermo Fisher; Loughborough, UK) detachment when reaching 70%–80% confluence. All cell lines passed routine mycoplasma testing using the MycoAlert^{TM} Mycoplasma Detection Kit (Lonza Group Ltd, Switzerland).

2.2. Experimental design

MSTO-211H, NCI-H28 and MET-5A cell cultures were prepared for headspace analysis—all cell cultures were under passage number 25. 1.8×10^6 cells were seeded in standard T75 cell culture flasks and incubated at 37 °C with 5% CO_2 for 72 h. Control flasks containing complete RPMI-1640 and medium 199 only were also prepared and incubated at 37 °C with 5% CO_2 for 72 h. Control flasks were used to deduct background signals, caused by cell culture media, from the cell line profiles. After incubation, VOCs were extracted from the headspace gas of cell culture and control flasks and analysed using GC-MS.

2.2.1. VOC extraction

MSTO-211H, NCI-H28 and MET-5A cell confluence was recorded prior to VOC extraction. A 50/30 μm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (Supelco; Gillingham, UK) was used to extract VOCs from the headspace gas above MSTO-211H, NCI-H28 and MET-5A cell cultures and RPMI-1640 and medium 199 controls. A new SPME fibre was conditioned in the inlet of a GC-MS at 270 °C for 30 min according to manufacturer's instructions prior to initial use and cleaned for 10 min in the inlet of a GC-MS at 250 °C to remove residual compounds

before VOC extraction. For VOC extraction, the SPME fibre assembly was inserted directly through the filter cap of cell culture and control flasks and the fibre exposed to the headspace for 15 min. VOC extraction was performed at 37 °C with 5% CO_2. After VOC extraction, MSTO-211H, NCI-H28 and MET-5A cell number and viability was assessed via Trypan-Blue exclusion using a Countess automated cell counting device (Invitrogen; Loughborough, UK).

2.2.2. Gas chromatography-mass spectrometry

An Agilent 7890A with a Rtx-VMS capillary column (30 m × 0.25 mm × 1.4 μm; Restek; Saunderton, UK) and a MS-5975C triple axis detector was used for VOC analysis. The GC-MS inlet temperature was set to 250 °C. The oven temperature programming was as follows: 35 °C held for 5 min, ramped to 140 °C at 4 °C min^{-1} and held for 5 min, ramped again to 240 °C at 20 °C min^{-1} and held for 4 min. The total run time was 45.25 min. 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. Extracted VOCs were analysed through manual direct injection into the inlet of the GC-MS. The SPME fibre assembly was injected into the inlet of the GC-MS and the fibre was exposed for 10 min at the start of the oven temperature program to release VOCs and clean SPME fibre for further extractions.

2.2.3. Data analysis

Compounds were tentatively identified through mass spectral match to the NIST library database (National Institute of Health; V11). A pairwise analysis was performed in XCMS online (The Scripps Research Institute) to remove culture media background signals from cell line profiles. RPMI-1640 signals were deducted from MSTO-211H and NCI-H28 profiles and medium 199 signals were deducted from MET-5A profiles. Culture media background signals and signals from siloxane compounds were removed from further analysis. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS) were performed on MSTO-211H, NCI-H28 and MET-5A results using OpenChrom® [18]. T-tests were performed within XCMS online to compare the cell line groups, identifying significantly altered VOCs in MSTO-211H and NCI-H28 with comparison to the MET-5A group that also had a ≥80% spectral match to the NIST library database.

3. Results

3.1. Cell viability after VOC extraction

Cell viability was assessed to determine the effects of headspace SPME on cell cultures. Differences in the number of viable cells assessed from MSTO-211H, NCI-H28 and MET-5A cultures after VOC extraction was observed (figure 1). In contrast to this the number of viable cells as a percentage of total cells was at a consistently high level across MSTO-211H, NCI-H28 and MET-5A cultures (figure 1).

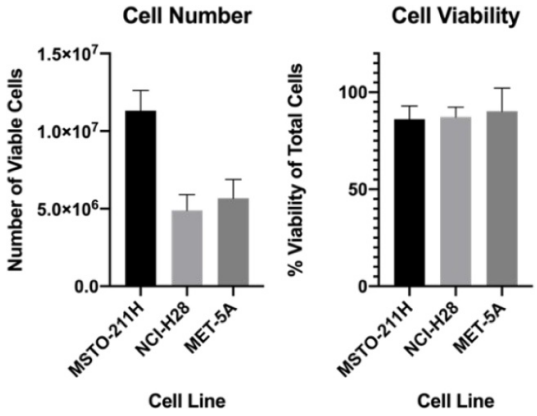

Figure 1. Cell number and percentage viability of MSTO-211H, NCI-H28 & MET-5A cell cultures after VOC extraction measured through Trypan-Blue exclusion. Six replicates of each cell line were measured (data represent mean ± standard deviation).

3.2. VOC profiles

Initial headspace analysis showed approximately 100–200 compounds in each MSTO-211H, NCI-H28 and MET-5A flask (figure 2). Chromatograms produced from SPME GC-MS of MSTO-211H, NCI-H28 and MET-5A groups appeared very similar, making it difficult to identify any observable differences in VOCs (figure 2).

3.3. Statistical analysis

Statistical analysis was performed to determine if there were differences between the groups. PCA was used to visualise the trends between the MSTO-211H, NCI-H28 and MET-5A groups, which were then further classified with OPLS. T-tests were performed in XCMS online to identify significantly different VOCs in the MSTO-211H and NCI-H28 groups with comparison to the MET-5A group.

3.3.1. PCA & OPLS score plots

Background culture media signals and signals from siloxane compounds were removed from analysis (tables S1–S3 (available online at stacks.iop.org/JBR/14/046011/mmedia)). Despite some overlapping results, some separation was observed between MSTO-211H, NCI-H28 and MET-5A groups using PCA (figure 3). When further classifying these results with OPLS, MSTO-211H and NCI-H28 groups were clearly separated from MET-5A results and MSTO-211H and NCI-H28 groups were distinct from each other (figure 3).

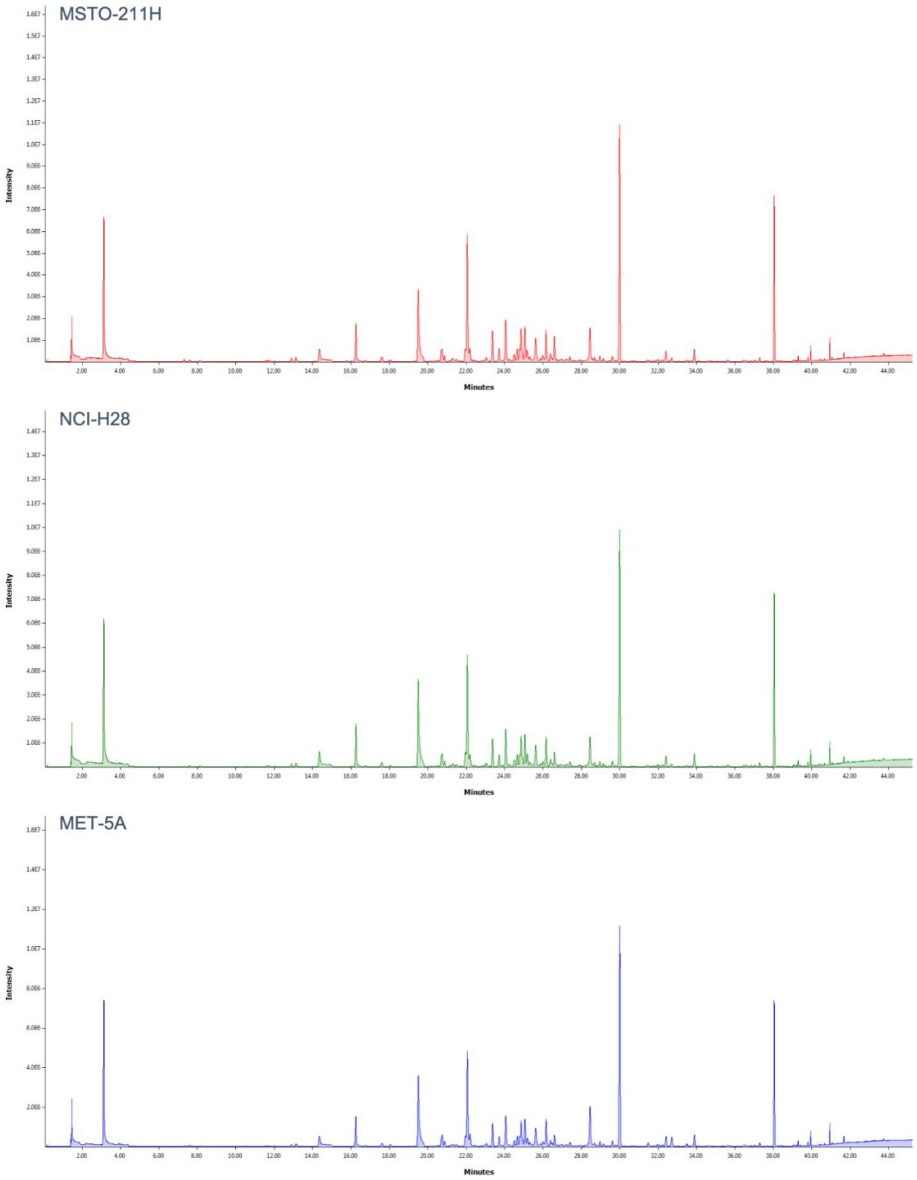

Figure 2. Exemplary GC-MS chromatograms showing VOC profiles of MSTO-211H, NCI-H28 and MET-5A cell culture headspace after 72 h incubation.

3.3.2. Mesothelioma specific VOCs

MSTO-211H and NCI-H28 specific VOCs were compared to MET-5A specific VOCs. Significant differences in several compounds were observed in the MSTO-211H and NCI-H28 groups with comparison to the MET-5A group (table 1). 2-ethyl-1-hexanol was significantly increased in both the MSTO-211H and NCI-H28 groups compared to the MET-5A group (table 1). In addition, ethyl propionate and cyclohexanol were significantly increased in MSTO-211H cells and dodecane was significantly increased in NCI-H28 cells (table 1).

4. Discussion

There is an urgent need to develop new diagnostic methods for MM; new approaches should be capable of identifying patients at an earlier disease stage, differentiating between MM sub-types and avoiding invasive biopsy procedures. Previous studies have explored breath analysis within mesothelioma. The current study applied VOC analysis to mesothelioma cell lines, identifying compounds *in vitro* and providing a model for the further study of mesothelioma. This is the first step in the development of *in vitro* VOC analysis within mesothelioma. Controlled *in vitro* studies such as this are useful to compare compounds found in patient breath and subsequently explore the biological origins of VOC production. Two MM cell lines; biphasic MSTO-211H and epithelioid NCI-H28; and the non-malignant mesothelial MET-5A were analysed, allowing for comparison between mesothelioma sub-types.

A change in metabolism during cell death and apoptosis has the potential to influence VOC profiles. The high viability of the cultures (figure 1) suggests

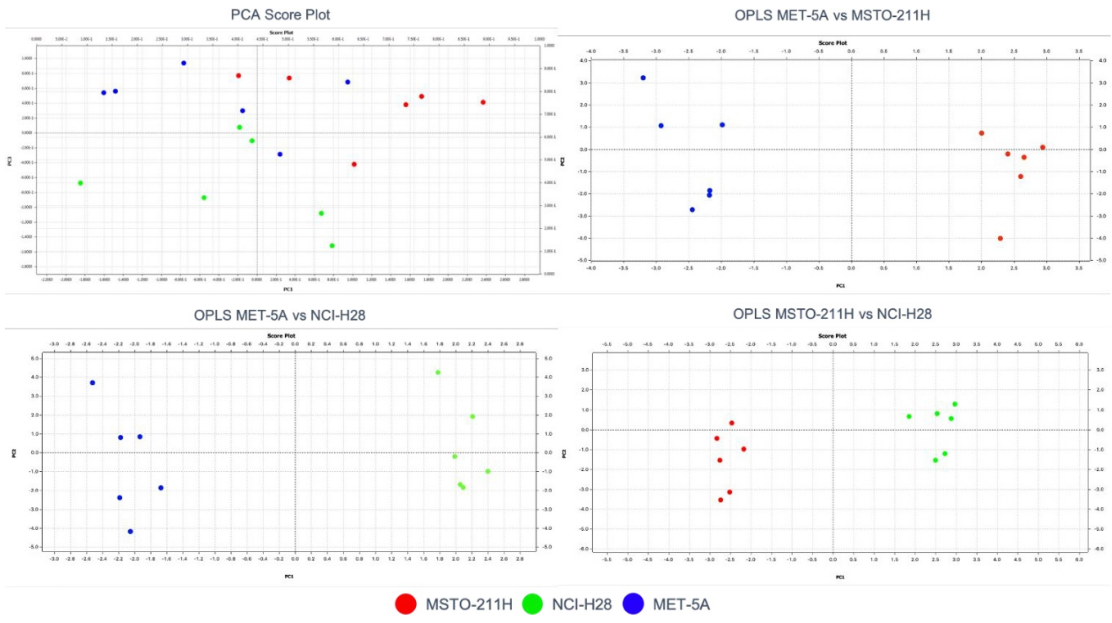

Figure 3. PCA and OPLS score plots of MSTO-211H, NCI-H28 and MET-5A results.

Table 1. Significantly altered VOCs in MSTO-211H and NCI-H28 groups with comparison to the MET-5A group that also had a ≥80% spectral match to the NIST library database.

Average RT (min)	Compound	Trend MSTO-211H	p-value	Trend NCI-H28	p-value
11.6	Ethyl propionate	↑	<0.01	—	—
20.8	Cyclohexanol	↑	<0.01	—	—
26.6	2-ethyl-1-hexanol	↑	<0.01	↑	0.01
30.0	Dodecane	—	—	↑	<0.01

that the cells did not undergo detrimental levels of cell death and apoptosis during the 72 h incubation time. Combined with the deduction of culture media background signals, it is therefore likely that the changes in VOCs observed between the three groups was caused by the different cell lines. Changes in cell number were also observed (figure 1), which was expected due to differences in size, morphology and growth rate between the three authenticated cell lines.

Previously, a number of VOCs identified in breath were used to discriminate samples obtained from MM patients to those those of healthy controls, as well as other clinical groups [11–13]. The current study used a panel of cell lines to act as a model for MM VOC analysis. MSTO-211H cells represented biphasic MM, NCI-H28 is considered to be epithelioid MM and MET-5A was selected as a non-malignant mesothelial cell type. Multivariate analysis revealed that the three cell lines produced distinct headspace VOC profiles (figure 3). The current results recapitulate that which has previously been reported *in vivo*—confirming that MM cells themselves produce a different VOC profile to that of non-malignant cells and showing that MM cell lines can act as a viable model for VOC analysis.

Mesothelioma tumours can consist of different cell morphologies producing the distinct clinical sub-types: epithelioid, sarcomatoid and biphasic MM [3]. Sarcomatoid MM has a worse prognosis than epithelioid, whilst biphasic mesotheliomas contain a combination of both cell types, with prognosis becoming increasingly poorer depending upon the percentage of sarcomatoid cells present [3]. At present, it is difficult to differentiate between MM sub-types at diagnosis meaning that patient stratification is not currently in clinical practice [3]. As well as this, previous MM breath analysis studies did not consider the impact of tumour sub-type on patient VOC profiles [11–13]. MSTO-211H and NCI-H28 VOC profiles were distinct enough to show separation between the two cell lines (figure 3) and compounds were identified that were specifically increased in either the MSTO-211H or NCI-H28 group with comparison to the MET-5A cells (table 1). This is the first time the effects of different mesothelioma cell subtypes on VOC profiles has been reported. Present results show that biphasic and epithelioid cell types can be distinguished *in vitro,* with further clinical work required to determine how this relationship occurs *in vivo*.

Several compounds were identified at a significantly increased level in the headspace of the mesothelioma cell lines compared to the non-malignant control (table 1). Of these compounds, ethyl propionate,

2-ethyl-1-hexanol and dodecane are present on the Volatilome Database [19], indicating that they have previously been reported to be present in human breath. 2-ethyl-1-hexanol is the only compound that was significantly increased in both MSTO-211H and NCI-H28 cell lines (table 1). Previously, 2-ethyl-1-hexanol was used statistically to differentiate MM patients from those exposed to a similar level of asbestos without mesothelioma development [12], as well as distinguishing MM patients from healthy controls [13]. The correlation between previous *in vivo* results and the current *in vitro* study suggest that 2-ethyl-1-hexanol is an endogenous VOC released directly from MM cells. Previous studies have also found that 2-ethyl-1-hexanol is a compound that has been commonly associated with other malignancies including lung [20], prostate [21] and colorectal cancer [22]. Significant differences in 2-ethyl-1-hexanol levels have been reported in urine [21], blood [22], pleural effusions derived from cancer patients [20] and released from *in vitro* tumour cell lines [14]. Recently, 2-ethyl-1-hexanol was found to be present in the sweat of lung, prostate, gastric, kidney, head and neck cancer patients but absent from healthy controls [23].

Dodecane is also a compound that has been identified in MM breath analysis literature, having previously been used in the discrimination between MM patients, asbestos exposed and healthy controls [11]. Again, this compound does not appear to be exclusively associated with mesothelioma. Using SPME, dodecane was found to be increased in cell lines derived from haematological malignancies with comparison to media-only controls [24]. Dodecane has also been identified in studies analysing cancer patient exhaled breath samples. Among other VOCs, dodecane was highlighted as a potential biomarker for oral squamous cell carcinoma [25]. In lung cancer dodecane has been suggested as a potential diagnostic biomarker [26, 27], particularly useful in identifying adenocarcinoma patients harbouring an epidermal growth factor receptor mutation [26]. Furthermore, dodecane was found at significantly increased levels in exhaled breath samples obtained from colorectal cancer patients with comparison to healthy controls [28].

The identification of altered 2-ethyl-1-hexanol and dodecane levels across a range of malignancies and biological matrices suggest that these are compounds that are ubiquitously associated with cancer or a specific cancer-related process. The production of reactive oxygen species (ROS) leading to an increase of oxidative stress is thought to play an important role in the production of VOCs [29]. During prolonged periods of oxidative stress, proteins and lipids are particurlarly susceptible to ROS oxidative attack, leading to lipid peroxidation and the production of VOCs [29]. An increase in ROS and oxidative stress is an important process in malignant tumourigenesis—particularly within MM [13]. Inhalation of asbestos fibres leads to rounds of frustrated phagocytosis and unresolved oxidative stress, creating an environment with high levels of ROS [30]. Due to the biopersistence of these fibres [30], it is likely that high levels of oxidative stress are maintained well into malignancy, with ROS causing damage to phospholipids, proteins and other macromolecules leading to the production of VOCs [13]. Within MM it has been speculated that an increased concentration of ROS could produce a high level of oxidated organic species [13]. Alkanes, such as dodecane, could arise from lipid peroxidation, with further metabolism of alkanes leading to alcohols such as 2-ethyl-1-hexanol [13]. This is reflected in the current study, with the two MM cell lines showing increases in these VOCs compared to the non-malignant control cell line, MET-5A. These results can be expanded further using the current *in vitro* system with experiments exploring the effects of ROS on VOC profiles. Inducing oxidative stress in a non-malignant cell line mimics what occurs during tumourigenesis and allows specific VOCs to be pinpointed to a particular biological process.

The identification of compounds across multiple malignancies is also a good example of how a single compound on its own lacks the specificity required to diagnose mesothelioma or any other cancer effectively. The range and variety of VOCs already identified thus far is vast. The considerable amount of compounds exhaled in a single breath means that crossover of specific VOCs between different pathologies is highly probable. It is therefore much more likely that a breath test in clinical practice would rely on the identification of multiple VOC signals, with changes in levels and patterns of compounds indicative of a disease, rather than the presence of a single compound. In contrast to 2-ethyl-1-hexanol and dodecane, this is the first time ethyl propionate and cyclohexanol have been reported to be associated with MM. Despite this, it is encouraging that 2-ethyl-1-hexanol and dodecane were associated with MM in breath and also at the cell line level, highlighting these two compounds as important targets that warrant further investigation.

5. Conclusion

MM breath analysis is still in its early stages—previous studies have provided a proof of concept for the identification of MM patients based on VOCs in exhaled breath. The current study aimed to identify VOCs directly from MM cells, in order to provide an *in vitro* model of this disease. 2-ethyl-1-hexanol and dodecane were identified, correlating with the previous literature. As well as this, an initial proof of concept for the distinction between epithelioid and biphasic MM sub-types using VOC analysis was shown. This pilot study is the first step in the development of *in vitro* MM VOC analysis. The use of cell

lines allows for subesequent experiments that would not be possible with patient breath samples, targeting the biological pathways behind these compounds. It unclear what a diagnostic breath test will look like in clinical practice, but this field of research has the potential to revolutionise mesothelioma diagnosis. It is vital that MM breath analysis research of all levels continues.

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Supplementary Material

Supplementary material for this article is available online

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