# Volatile metabolic biomarker discovery in breast cancer models

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# Abstract

This thesis contributes identification of volatile organic compounds (VOCs) released via metabolic mechanisms involved in disease and offers increased precision and accuracy in breath diagnostics. By quantifying VOC consumption and production in response to serum, glucose and oxygen starvation, more accurate identification of specific VOC signatures is made possible.

Chapter 1 explores previously published VOC research and identifies VOC chemical functional groups associated with disease. Chapter 2 applies novel methodology of VOC sampling over time in static head space chambers to establish VOC metabolic flux in a number of cell types and in the breath of mice. Chapter 3 develops the method from chapter 2 to study VOC response from breast cancer cells in low oxygen conditions. Chapter 4 develops the research of chapters 2 and 3 to link VOC fluctuations to cellular response to serum, glucose and oxygen starvation.

# Author's Declaration

I, Theo Issitt declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself. Contributions, as in-keeping with the journal style of this thesis, are presented at the end of each chapter.

The work in this thesis has either been published before submission or is ready for submission.

**Chapter 1: Volatile compounds in human breath: critical review and meta-analysis**

Theo Issitt, Laura Wiggins, Martin Veysey, Sean T. Sweeney, William J. Brackenbury and Kelly Redeker

Journal of Breath Research

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**Chapter 2: Sampling and analysis of low molecular weight volatile metabolites in cellular headspace and mouse breath**

Theo Issitt, Sean T Sweeney, William J Brackenbury and Kelly Redeker

Metabolites

**Published:** June 2022

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**Chapter 3: GC/MS analysis of hypoxic volatile metabolic markers in the MDA-MB-231 breast cancer cell line**

Theo Issitt, Matthew Reilly, Sean T. Sweeney, William J. Brackenbury, and Kelly Redeker

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Lots of thanks for my supervisors, Kelly, Sean, Will and other staff in the department who have supported my journey and given me the freedom to make mistakes and try out some crazy science! I think we have developed some brilliant platforms and have multiple grants in the works, clinical studies and brilliant worldwide collaborations. I hope we can make a difference to people's lives with the work we have done.

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# List of Abreviations

2-MP – 2 Methly Pentane

3-MP – 3 Methyl Pentane

5-AZA – 5 Azacytidine

ANOVA – Analysis of Variance

CFC – Chloroflourocarbon

cmuA – Chloromethane Utilising Enzyme A

DMEM - Dulbecco's Modified Eagle Medium

DMS – Dimethyl Suphide

DNMT – DNA Methyl Transferase

DOX – Doxorubicin

FBS – Faetal Bovine Serum

GCMS – Gas Chromatography Mass Spectrometry

HCY - Homocysteine

IBD – Inflammatory Bowel Disease

LDA – Linear Discriminant Analysis

MeBr – Methyl Bromide

MeCl – Methyl Chloride

MeI – Methyl Iodine

MeSH – Methanethiol

MET - Methioine

NAFLD – Non-Alcoholic Fatty Acid Liver Disease

nHex – Hexane

HPLC – High Purity Liquid Chromatography

PBS – Phosphate Buffered Saline

PCA – Principle Component Analysis

RF – Random Forest

RIPA – Radio-immunoprecipitation Assay

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

SAM – S-adenosyl-methioine

SAH – S-adenosyl-homocysteine

SIM – Select Ion Mode]

SNP - Sodium Nitroprusside

SPME - Solid Phase Microextraction

SRB - Sulphorhodamine B Assay

TD – Thermal Desorbtion

UGI – Upper Gastro Intestinal

VOC – Volatile Organic Compound

# Thesis Introduction

This thesis presents a collection of works focused on the development of a novel methodology for the detection and development of biomarkers of cellular processes and diseases – specifically analyzing volatile organic compounds and quantifying metabolic function in human cells, mainly of cancerous origin. On submission, chapters 1-3 are published, and chapter 4 is prepared for submission, but is currently being held back awaiting the outcome of an ongoing human clinical trial. Due to chapters being presented as journal publications there is a degree of repetition – for example volatile organic compounds (VOCs) are defined 4 times. However, I open this introduction with a brief overview of our current understanding of VOCs in diagnostics, which are the main concern of the research.

VOCs are small, carbon containing compounds which can exist as a gas at room temperature. They are of particular interest as they constitute odour, a powerful tool for diagnosis. Hippocrates, around 2000 years ago established the usefulness of disease-specific odours in his treatise on breath odour and disease identifying *Fetor hepaticus*, which are sulphur compounds, indicating poor liver function (Adams 1994). More recent diagnostic uses of smell include the sweet smell of acetone on the breath of diabetic patients, the ability of dogs to smell disease and many other examples (Di Francesco et al. 2005; Li and Duan 2015). These examples, which are the starting point for most reading around diagnosis by smell, formed the entry point for my research journey and presents the intent of the research: to discover VOC biomarkers of disease and develop diagnostic tools for improved early diagnosis of disease. While humans may recoil at the smell of rotting flesh or salivate from the smell of sweet cinnamon buns, our technological application of smell to describe complex processes, such as disease, on the whole, remains undeveloped.

Any fluctuations, or flux, in VOCs driven by organic processes can be said to reflect ‘metabolism’, whereby VOCs are consumed and produced because of active cellular mechanisms. VOCs make up the gaseous part of the metabolome and their study offers powerful diagnostic potential. An understanding of metabolomics is therefore required within the context of the disease pathology and frames the presented thesis.

Breath has been the main target for most of the research concerned with VOC diagnosis. The lungs are optimised for gas transfer in and out of the blood stream and so analysing the gases in breath provides information about metabolic processes occurring throughout the body. The human body is a very complex system, and of the large body of research so far conducted into breath VOCs the majority focuses on the detection of VOCs produced in large quantities rather than the many hundreds and thousands of VOCs actually found (Issitt et al. 2022b). The focus of my presented research is an exploration into less studied, or concentrated

compounds, inclusive of understanding and quantifying background, to accelerate the discovery of markers of metabolic processes involved in cell stress for early diagnosis of disease.

On day one of my PhD research, the question of which VOCs to investigate was raised. There are two main approaches for investigating VOCs when using gas chromatography mass spectrometry (GC/MS). The targeted approach, where you pick a 'suite' of specific molecules (VOCs) to investigate (optimising sensitivity and accuracy) or the non-targeted approach, where you look for everything within a size range (reduced sensitivity but you don't miss anything). My starting point was, as with most laboratory investigations, an extension of the resources and methodologies the lab has been working with and specialising in. Here, I started with halogenated compounds (compounds containing chlorine, iodine and bromine), some sulphur containing compounds and isoprene. Many of these are useful and powerful individual markers, however the 'suite' of VOCs with which we initially focussed, changed and evolved in the progress of the research. In chapter 1 I describe how we explored and drove a significant expansion of the suite of VOCs as research targets, particularly through an increase in the range of VOC functional groups, and which thereby informed the research methods and targets described in chapter 4 and the ongoing human study. Similarly, the work described in chapters 2 and 3 identifies VOCs that change or do not change under stress and thereby establish their utility as biomarkers – thus more accurately focussing the research of chapter 4 and further studies going forward.

The key method that we used to identify VOCs and their relative levels was to establish VOC changes by sampling the air above cells in a static headspace chamber, at multiple time points, via pre-evacuated electropolished stainless steel canisters. The samples were condensed using a liquid nitrogen trap and then loaded into a gas chromatograph mass spectrometer (GC/MS) to analyse gas constituents. Readings were taken at two time points allowing changes in VOCs to be measured.

The results presented in this thesis used GC/MS to quantify VOC metabolisms driven by cell stress types specific to diseases, which are represented in human breath, with the ultimate aim of providing more effective clinical diagnostic tools. The targets for the thesis, which are met in each chapter, are as follows:

1. To examine volatile biomarkers present in human breath, developing recommendations for VOC targets and research frameworks.
2. To test methodology of volatile metabolic flux in cellular and animal models.
3. To apply novel methods of volatile analysis to models of cellular stress.
4. To identify novel, volatile mechanistic pathways which are translatable to breath.

Chapter 1 of this thesis, as a meta-analysis and systematic review, provides context of the wider published research and recommends VOCs for targeted research in cellular or breath studies. Aim 1 is achieved in chapter 1: Volatile compounds in human breath: critical review and metanalysis, published in the Journal of Breath Research 2022 (Issitt et al. 2022a).

Aim 2 is concerned with the development and application of methods that investigate VOC flux, allowing comparisons of different cell types and the breath of mice. This is achieved in chapter 2: Sampling and analysis of low-molecular-weight volatile metabolites in cellular headspace and mice breath, published in Metabolites, 2022 (Issitt et al. 2022b)

Aim 3 applies the methods described in chapter 2 to a challenging environment of pathological relevance, hypoxia. Hypoxia is the state of low oxygen, experienced by cells in a range of disease but of note, in growing tumours. Hypoxia is a challenge to study because it requires controlled gaseous conditions and therefore investigating VOC flux of cells experiencing hypoxia is novel. This aim is achieved in chapter 3: GC/MS analysis of hypoxic volatile metabolic markers in the MDA-MB-231 cell line, published in Frontiers in Molecular Bioscience, 2023 (Issitt et al. 2023).

Aim 4 of the thesis is in part an accumulation of, and expansion from, all previous work and draws upon observations of VOC flux to inform mechanistic studies in cells under stress, to discover mechanisms of VOC consumption and production. This is achieved in chapter 4: Cellular response to starvation provides biomarkers for breast cancer through volatile metabolites of methylation and methionine metabolism.

At the inception of this work, which identified the above points, several other avenues were also explored which did not enter into this thesis but have developed into separate grant applications or masters theses. Of note, this included the study of volatile profiles of glial cells from rat brains, which, while being studied, revealed extensive changes as they aged. This was then investigated in other cells and over the course of the PhD and with several masters students a method was tested, much like the method presented in chapters 2-4, for the investigation of drosophila fruit flies pertaining to aging and neurodegenerative disease. Further studies using these methods which have been developed in this thesis have been applied to human cells derived from acute myeloid leukaemia and with animal models of parasite infection, with much success. This highlights how the thesis has changed and grown over four years, testing avenues of interesting research which link to metabolic pathology and our method.

The development of the presented work has been aided by a number of reviewers who have helped sculpt and drive the design of the finished manuscripts. For example, the section ‘application to COVID-19’ in chapter 1 was driven by reviewers’ comments about the

applicability to the COVID-19 pandemic in which this manuscript was submitted. This has helped keep the work current and looking forwards to progress understanding.

The following works will continue beyond the presented thesis and, with any luck, we shall see application of this research in clinics and beyond in the future.

# Summary of chapters

### Research aims

My PhD project has focused mainly on exploring cellular volatile response to stress. I developed a method for investigating volatile flux of cells in culture and in the breath of mice with the aim of volatile biomarker discovery for early diagnosis of disease. To this end, I chose to model cellular starvation as a model of the tumour microenvironment to describe volatile metabolisms which may translate to the breath of patients.

### Chapter 1: Volatile compounds in human breath: critical review and meta-analysis

In this chapter I aimed to cover the background of VOC research in disease, with a focus on VOC biomarkers of cancer, to set up for the research in the following chapters. I also spend some time considering methodological variations, patient and environmental factors which can generate conflicting and confounding information. During the process of researching the field, I noticed similarities in reported compounds for cancer, mainly in their chemical class. From this, I took all the reported molecules observed in the breath of cancer patients and compare it to the breath of patients with varying pathologies. This resulted in successful separation and classification of cancer from other diseases based on the functional group of the VOCs being reported. This then informed a research framework recommendation, which includes compounds from varying chemical functional groups to maximise diagnostic potential. In essence, this underpinned the subsequent research approaches, refining my biomarker discover approaches to consider diagnostic applications and translatability.

### Chapter 2: Sampling and analysis of low molecular weight volatile metabolites in cellular headspace and mouse breath

This chapter tests our methodology of static headspace analysis with two time points to determine volatile flux. This is performed for a range of cancerous cell types against appropriate non-cancerous cell controls. I demonstrate in this chapter that the method can distinguish cell type by the volatile flux of 12 select compounds. Further to this, I test the volatile response of those 12 compounds from cells treated with chemotherapeutic agent Doxorubicin and show clear response in the volatile flux. The method is shown to be effective for identification of volatile flux in the breath and faecal matter in immunocompromised mice.

**Chapter 3: GC/MS analysis of hypoxic volatile metabolic markers in the MDA-MB-231 breast cancer cell line**

In this chapter we apply our static headspace technique to cells in hypoxic conditions. I set out on this project thinking it would be simple, however maintaining low oxygen conditions is a challenge and required a redesign of the static headspace chamber. Here, I flush the chambers with 1% oxygen, carbon dioxide and nitrogen. This provided a challenge as measuring volatile flux requires volatiles of interest to be present at the first time point if active metabolic consumption is to be observed. Thusly, I was also able to demonstrate volatile flux in the cancer MDA-MB-231 cell using injected, known gases and able to demonstrate active metabolisms under low oxygen conditions.

**Chapter 4: Cellular response to starvation provides biomarkers for breast cancer through volatile metabolites linked to methylation and methionine metabolism**

In this chapter I demonstrate that volatile flux of cells in glucose, serum and oxygen starvation induce significant changes in volatile flux. From this I focus on methyl chloride, which has been a consistent biomarker throughout the previous two chapters. Consumption of methyl chloride by cells translates to the breath of tumour bearing mice. I establish rationale for mechanisms which may be responsible for the metabolisms in methyl chloride and show that production is link to cellular methylation activity and consumption is linked to methionine synthesis. The focus upon methyl chloride is driven partly because I observe lower levels in the breath of mice with tumours compared to control and that as tumour size increase, methyl chloride levels decrease.

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# Chapter 1:

## Volatile compounds in human breath: critical review and meta-analysis

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## Abstract

Volatile compounds contained in human breath reflect the inner workings of the body. A large number of studies have been published that link individual components of breath to disease, but diagnostic applications remain limited, in part due to inconsistent and conflicting identification of breath biomarkers. New approaches are therefore required to identify effective biomarker targets. Here, volatile organic compounds have been identified in the literature from four metabolically and physiologically distinct diseases and grouped into chemical functional groups (e.g. – methylated hydrocarbons or aldehydes; based on known metabolic and enzymatic pathways) to support biomarker discovery and provide new insight on existing data. Using this functional grouping approach, principal component analysis doubled explanatory capacity from 19.1% to 38% relative to single individual compound approaches. Random forest and linear discriminant analysis reveal 93% classification accuracy for cancer. This review and meta-analysis provides insight for future research design by identifying volatile functional groups associated with disease. By incorporating our understanding of the complexities of the human body, along with accounting for variability in methodological and analytical approaches, this work demonstrates that a suite of targeted, functional volatile biomarkers, rather than individual biomarker compounds, will improve accuracy and success in diagnostic research and application.

## Introduction

Human breath analysis offers a diagnostic tool that is non-invasive, rich in information, and low cost. Identification of the presence and abundance of gaseous biomarkers offers the potential for sensitive and accurate clinical diagnosis and long-term monitoring (Kwak and Preti 2011; Blanchet et al. 2017; Sagnik Das and Pal 2020). Our ability to 'translate' these signals into usable diagnostics currently lags behind the body of published research on captured breath compounds. Despite challenges faced in human breath research, quantification of individual compounds is already used to identify (mal)function of bodily processes in limited contexts. A major challenge of this developing field is aligning volatile compounds captured from breath with underlying (patho)physiologies. In particular, human breath-based clinical trials data is currently insufficiently integrated with our understanding of functional and mechanistic physiology. The focus here is on human breath, but gaseous biomarkers can be detected from skin, urine, blood, saliva and faeces (Amann et al. 2014; Drabińska et al. 2021).

The majority of breath-linked diagnostic research has targeted respiratory diseases. Attempts to identify volatile organic compound (VOC) biomarkers of lung cancer, both in vitro and in vivo (Jia et al. 2019; Gasparri et al. 2016; Wojciech Filipiak et al. 2016) are represented by a large body of work. Non-cancerous pulmonary diseases such as asthma (Holz et al. 2019; Smolinska et al. 2014), chronic obstructive pulmonary disease (COPD) (Gaida et al. 2016; Santini et al. 2016), cystic fibrosis (van Mastrigt et al. 2016; Paredi et al. 2000) and tuberculosis (Saktiawati et al. 2019; Beccaria et al. 2018) are also targets of research, but to a lesser extent.

In addition to pulmonary disease, VOC biomarkers from other cancers (Janfaza et al. 2019), cardiac disease (Bykova et al. 2019), liver (De Vincentis et al. 2019), gastrointestinal (Bannaga, Farrugia, and Arasaradnam 2019), and neurological conditions (Tiele et al. 2020; Tisch et al. 2013) have been studied and reported. The breadth of these studies offers an opportunity to compare how variable cellular states and pathophysiology correlate and/or differ in VOC profile. For example, diabetically linked VOCs (Souvik Das, Pal, and Mitra 2016) give insight into metabolic functions that may have implications for other disease-correlating phenotypes.

The diagnosis of infection is a promising field for breath research, in part because microorganisms often generate distinct VOCs, which can be discerned within human breath profiles (Berna and Odom John 2021; Ghosh et al. 2021). For example; tuberculosis (Michael Phillips et al. 2007; Beccaria et al. 2018) and Pseudomonas aeruginosa (Scott-Thomas et al. 2010; Shestivska et al. 2011), both infections of the lung, are metabolically distinct. Viral infections, separate from microbial infections, may also be detectable due to viral VOC

production, or modification of the human host metabolism. Canine detection by scent of COVID-19 has been demonstrated (Mendel et al. 2021; Jendrny et al. 2020) and several studies have reported on the potential for COVID-19 breath-based diagnosis (Ruszkiewicz et al. 2020; H. Chen et al. 2021; Grassin-Delyle et al. 2021; Berna et al. 2021; Wintjens et al. 2021; de Vries et al. 2021; Shan et al. 2020), with varying accuracies (Subali et al. 2022).

Differences between disease states can increase the power of diagnostic tests. Bayes' Theorem links probability of disease to prevalence within a population as well as the presence or absence of clinical markers (Miettinen, Steurer, and Hofman 2019). This paper aims to highlight the need for diagnostic research frameworks that include VOC biomarkers which act as comparative controls to increase diagnostic precision and accuracy.

## VOCs - The complex pathway from cell to breath

Human breath contains hundreds of volatile organic compounds. Metabolic processes within the human body both consume and generate VOCs, also referred to as the 'volatilome'- which is defined as the volatile fraction of the metabolome (Amann et al. 2014). As a fraction of the metabolome, VOCs are recognized to directly reflect gene transcription and protein expression. Illness, which is often linked to altered metabolisms and local environmental changes, is therefore expected to alter 'volatilome' profiles. The available human 'volatilome' consists of gaseous, low concentration (<1x10-4 %), low molecular weight (<350 amu molecular weight), and high-vapour pressure compounds extant within the gas phase at human temperatures and ambient pressures.

The primary target of most breath research are endogenous (internally generated) VOCs however, human breath consists of a mixture of both endogenous and exogenous VOCs. Exogenous VOCs arise from sources external to the body which include local air volatiles (e.g.- car exhaust) as well as metabolic by-products from diet and/or medications. Exogenous compounds that persist continually in the environment (i.e. the clinic, or urban streets) must be characterised, quantified and separated in order to clarify which endogenous compounds are produced or metabolised by the patient. Quantifying metabolism of exogenous VOCs can be a powerful diagnostic tool in its own right- for example in organ function, where metabolism of limonene (e.g. - produced by air fresheners and various plants) can be used to assess liver function (O'Hara et al. 2016). This technology can be applied to preoperative and postoperative assessment of liver function, and drug-induced liver damage (Molina-Molina et al. 2021). Alternatively, through utilisation of easily detectable, stable isotopically labelled molecules, such as 13-Carbon labelled hydrocarbons, specific bodily processes can be monitored and assessed through breath (Gaude et al. 2019) including measurement of gastric

emptying through labelled CO2 levels (Sangnes et al. 2019) or labelled Urea in the breath, indicative of H. pylori (Savarino, Vigneri, and Celle 1999; Modak 2007). Similarly, levels of hydrogen in the breath can accurately assess malabsorption in the gastric tract through bacterial processing of administered fructose (Born et al. 1995; Helwig et al. 2019).

Further to the use of exogenous compounds as molecular probes, some of the most impactful and fundamental breath research has focused upon the effect of exogenous VOCs on human health, increasing understanding of volatile dynamics (Westhoff et al. 2019). For example, the effects of cigarette smoke and carcinogenic VOCs (Capone et al. 2018) or exposure to VOCs in firefighters (Wallace et al. 2019). Some of the VOCs outlined here as biomarkers of disease have been identified as toxic to human health, such as benzene, 1,3-butadiene, styrene and isoprene, the most abundant VOC in human breath (Li, Pal, and Kannan 2021). Therefore concentration and context is an important factor when investigating volatile dynamics.

Microbial emissions also produce quasi-exogenous volatiles that may be revealing of pathological conditions or confound diagnosis. Cells of non-human origin outnumber the body's cells by far (Proctor 2011) and their metabolisms form a considerable fraction of the VOCs released and metabolised by the human body (Amann et al. 2014; Bos et al. 2016). For example, VOCs like acetone can be produced by anaerobic and aerobic bacteria (Sohrabi et al. 2014; Seesaard et al. 2020) and residual levels of ethanol and methanol can be either exogenous or microbial in origin (Dorokhov et al. 2015). Usefulness of volatile biomarkers is therefore defined by pathophysiology and comorbidities since altered microbiomes may be a significant and defining source of VOCs. This is especially likely in disorders of the bowel (Bannaga, Farrugia, and Arasaradnam 2019; Bodelier et al. 2015).

Breath analysis therefore requires systemic approaches for successful diagnostic application, accounting for both patient variability and environmental effects. Increased understanding of volatile metabolic processing in the body will aid in contextualising the qualitative and quantitative effects of stress, age, time of day, gender, activity, disease status, and/or transport of VOCs to the site of detection, all of which affect VOCs in breath (Wojciech Filipiak et al. 2016; Blanchet et al. 2017).

VOCs produced or metabolised by cellular processes, which are not subject to direct diffusion to exhaled air, must travel around the body through the bloodstream. They will i) pass tissues with varying constitutions and affinities to that volatile, ii) be metabolised through enzymatically independent and dependent pathways (the majority of these enzymes are expressed in the liver), and iii) diffuse from the bloodstream into the lung air space across the alveolar wall (Figure 1). In the lung, VOCs released from the blood mix with all local metabolites and

metabolising agents prior to exhalation. In the mouth volatiles from the lung, mouth, nose, upper gastrointestinal tract and stomach mix prior to sampling.

The primary physicochemical properties governing VOC movement within the body are blood:air and lipid:air partitioning coefficients, representing how likely a volatile is to solubilize in aqueous solutions (e.g. - blood) or dissolve into fat (Kramer et al. 2016). These basic thermodynamic properties are governed in human tissues by a molecule's size and polarity. For most cells in the body, volatiles initially move between the blood and the cell rather than directly diffusing into alveolar airspace (Figure 1). Once a volatile compound enters the bloodstream it must pass through the organs and tissues of the body for which it may have variable affinities (J. King et al. 2012; Kramer et al. 2016). The relative distribution of tissue types is a major source of variability between individuals. Lipophilic volatiles can accumulate in fat tissues, while compounds with low affinity for fat drain more efficiently into the blood, highlighting body mass index effects on VOCs in breath (Blanchet et al. 2017).

Further metabolism of released VOCs within the body can substantively modify the available volatilome. For instance, altered metabolisms (e.g.- the state of ketosis or fasting) have been shown to alter breath VOC profile (Statheropoulos, Agapiou, and Georgiadou 2006). In diseases such as diabetes, changes in acetone can be indicative of diabetic ketoacidosis (Galassetti et al. 2005). This global change, affecting all cells, contrasts with VOC sinks and/or sources that are site specific such as tumours, whereby the local microenvironment may present alterations in pH (Strambi and De Milito 2015), hypoxia (Petrova et al. 2018) and cellular ion concentrations (Leslie et al. 2019).

It is important to note that individual VOC biomarkers linked to cellular state may not be able to differentiate between causative agents or symptoms. For example; cellular iron overload (Issitt et al. 2019), senescence (He and Sharpless 2017) or cell death (Gaschler and Stockwell 2017) may all produce mitochondrial dysfunction and oxidative stress, producing similar VOC biomarkers. Therefore, VOC research should aim to identify differences in volatile metabolic outcomes between these states that may translate to the breath.

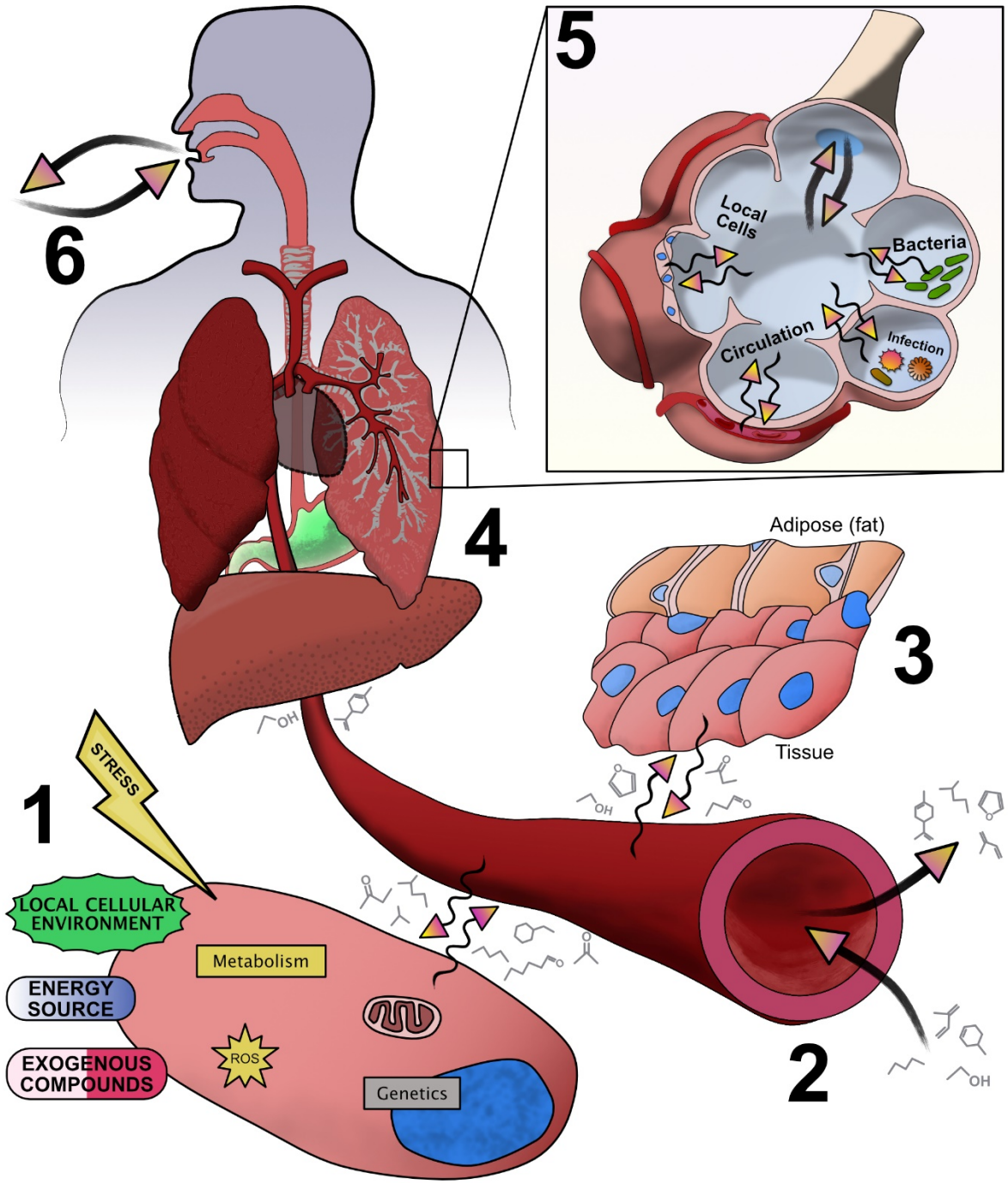


Figure 1. **The journey of volatile compounds: from cell to breath.**

Compounds detected in the breath can be traced back to local cellular changes. Volatiles interact with tissues, organs and other compounds as they move around the body, influencing their expression in the breath.1) Local environmental changes and stimulating factors elicit cellular response which in turn alters volatiles both given out and taken up. 2) Volatile compounds diffuse in and out of the blood stream to move around the body. 3) As compounds move around the body they diffuse in and out of tissues dependent upon saturation and their affinity for blood, fat or tissue. 4) Volatile compounds can be metabolised by enzymes such

as CYP450s, highly expressed in the liver. 5) Gases diffuse in and out of the blood in the lung across the alveolar wall. Volatiles from the blood mix with those released by local lung and immune cells, the lung microbiome and infectious bodies. 6) Compounds are inhaled and exhaled breath is a mixture of alveolar, lung and mouth air with volatiles from the stomach and upper gastrointestinal tract.

## Challenges in VOC biomarker comparison and collection

Volatile-focused biomarker research is confounded by varying behaviours and metabolisms between individuals (Blanchet et al. 2017). Volatile biomarkers may well be indicative of an isolated cell, in vitro, but within a body, may be subject to further metabolism (Figure 1). Some VOCs may therefore be a direct readout of enzymatic activity while others reflect multiple enzymatic processes. For example; limonene, which is not produced by human metabolism, can be measured in breath to monitor liver function as a read out of cytochrome P450 (CYP450) activity (O'Hara et al. 2016; De Vincentis et al. 2019). Whereas, peroxidation of lipids, hypothesised to be a source of aldehydes and hydrocarbons in the breath (Souvik Das, Pal, and Mitra 2016; Amann et al. 2014; M. Phillips, Greenberg, and Awad 1994; Hakim et al. 2012), can be mediated by enzymes, such as lipoxygenase, cyclooxygenases or cytochrome P450 (Massey and Nicolaou 2011) or non-enzymatic peroxidation through oxygen-radical oxidative routes (Esterbauer, Schaur, and Zollner 1991).

The direct processing of functional VOC groups, such as aldehydes, makes several enzymes both sources of VOC biomarkers and potential confounding elements in the processing of VOCs produced from other cellular mechanisms. Some of these enzymes should be considered as confounding factors affecting translation of research, as they may break down primary targets. Of this wide array of enzymes, Alcohol Dehydrogenases (ADHs), Aldehyde Dehydrogenases (ALDHs), Aldehyde Oxidases (AOX), Aldo-keto reductases (AKRfs) and Short-chain dehydrogenases/reductases (SDRs) are examples of classes that directly influence commonly detected and targeted VOCs.

Once endogenous metabolism and associated differences in form/behaviour have been addressed there are still methodological approaches which can bias reported outcomes. The pro's and con's of various techniques have been reviewed previously in the context of human breath (Jia et al. 2019; Kim, Jahan, and Kabir 2012). Briefly, variability in reported information can occur through analytical approach (i.e.- the instrument through which the data is quantified) (Kim, Jahan, and Kabir 2012) or in sampling approach. Sampling modifies reported results through changes in temperature, humidity, phase of breath (alveolar vs whole breath) or expiratory flow rate (Jia et al. 2019).

Analytical and collection methodologies vary across published studies (Table S1). In studies where breath samples are taken and concentrated prior to analysis, most studies collect breath into specialist polymer bags or use chemical traps. Collection methods should be considered when collecting and interpreting as they can affect the VOCs which are collected. For example, Tedlar® bags can affect breath VOCs through compound degradation and interaction with the bag product (Beauchamp et al. 2008; Ghimenti et al. 2015; Liangou et al. 2021; Ibrahim, Carr, et al. 2021). On the whole, most studies use some form of thermal desorption tube (TD) containing a specialized sorbent or solid phase microextraction (SPME) fibre (Tables S1/S2). Each available suite of methods results in compound bias. While researchers attempt to counter such biases, methodological variability inevitably generates inconsistency in published VOC outcomes.

Variation in reported human breath outcomes, and associated biomarkers, therefore results from;

1. Variability inherent in, and between, sampling methodologies;
2. Inherent human variability;
3. Complex interactions between compounds in breath; and
4. Confounding signals from comorbidities.

Like any diagnostic tool, precise and accurate interpretation of results depends on our ability to statistically link detectable changes to outcomes. Due to the complexities that arise from varying individual metabolisms and variability derived from methodological approaches, volatile biomarkers have been inconsistently reported, in terms of both presence/absence and quantity, for a range of diseases. For example, propanol, isoprene, acetone, pentane, hexanal, benzene, ethylbenzene and toluene have individually been reported to be lung cancer identifiers in 6 or more studies (Jia et al. 2019). However, increases in isoprene (as one example) from lung cancer patient breath compared to control groups (X. Chen et al. 2005; Ma et al. 2014; Ulanowska et al. 2011; Poli et al. 2005) conflicts with reports where isoprene decreased in lung cancer patients (Wehinger et al. 2007; Bajtarevic et al. 2009). To date, published diagnostic compounds from human breath appear to demonstrate little continuity with in vitro models (Jia et al. 2019).

Having outlined the challenges faced by researchers in identification of volatile biomarkers in breath, in this paper, we perform a comparative analysis that will allow researchers to identify and target biomarkers linked to pathophysiology and to consider their work in the context of a range of human diseases. Through considering how disease location, VOC interaction, and systemic variability affects end-point breath profiles, research efforts can be more clearly focussed and optimised.

## Methodological approach and rationale

Breath research is varied and multiple tools and approaches for research exist. Some of the most active areas of breath research include; lung cancer, breast cancer, cancers of the mouth, throat and upper gastrointestinal tract, diabetes, liver disease and inflammatory bowel disease (IBD). Our collation of this data is based on available studies, with lung cancer studies outnumbering all other breath research.

### Volatile biomarker comparison and data collection

To demonstrate the challenges researchers face in deriving VOC biomarkers from breath research we collected data from four metabolically and physiologically distinct diseases for which there exist a number of available studies (Figure 2, Table S1). Several systematic and comprehensive reviews; for lung, breast and other cancers (Hanna et al. 2019; Jia et al. 2019), irritable bowel studies (Van Malderen et al. 2020; Markar et al. 2015), diabetes (Souvik Das, Pal, and Mitra 2016) and liver disease (De Vincentis et al. 2019) were cross referenced to widen scope and inclusion of studies (see PRISMA flow chart, supplementary figure 1). Detailed exclusion criteria, workflow and transparent methodology can be seen in supplementary and in the PROSPERO database (Issitt, TJ, Redeker K 2021). Systematic searches of title and abstract were performed for each disease using boolean operators AND and OR using the Embase and Medline databases through the OVID platform. Detailed information on systematic search terms and results are provided in supplementary methods along with PRISMA flow chart (Page et al. 2021). This research can also be found on the PROSPERO data base (Issitt, TJ, Redeker K 2021) where clear inclusion criteria, methodology and data extraction are given. Risk of bias and data analysis can also be found in supplementary materials.

It is important to note that a range of important studies into the breath of patients with pulmonary disease such as asthma (Holz et al. 2019; Smolinska et al. 2014), COPD (Gaida et al. 2016; Santini et al. 2016; Christiansen et al. 2016), cystic fibrosis (van Mastrigt et al. 2016; Paredi et al. 2000) and tuberculosis (Saktiawati et al. 2019) as well as many other diseases, infectious or otherwise have been conducted. However, for these diseases, there do not yet exist sufficient studies fitting the selection criteria for inclusion here. This is highlighted by a systematic review into breath analysis and COPD (Christiansen et al. 2016), which identifies 12 papers, many of which use smokers as a control group, and highlights the lack of clinical breath biomarkers (Issitt, TJ, Redeker K 2021). Neurodegenerative disease also shows promise as a breath diagnostic application, but it is still a developing field and more biomarker research into breath needs to be conducted. Infectious diseases also suffer from

this same problem with the added element of many different types being investigated, making them incompatible for this meta-analysis. Asthma, COPD and parkinsons disease have also been searched using our methodology, and the results discussed in the supplementary.

Reviewer T.I. screened outcomes from electronic searches, their inclusion was based on criteria outlined in supplementary methods. This was double checked by reviewer K.R.

## Separation of studies based on methodology

Pilot studies are often employed when investigating VOCs in human breath of diseases that have not been investigated before (Hicks et al. 2015; Tiele et al. 2020; Khalid et al. 2013; Patel et al. 2014; M. Phillips et al. 1999; Sahota et al. 2016; Alkhouri et al. 2015). These studies examine compounds in breath using a more untarged or scanning approach (identified here as SCAN). Compared to control groups, statistically significant increases in VOCs often form the basis and rationale for investigating identified compounds at more depth in future studies.

Non-targeted (discovery), or scanning, approaches to gas analyses are useful for identifying where signals are substantially altered when compared to control groups. However, the large number of compounds in breath (>1000 (Kuo et al. 2020; Amann et al. 2014; Drabińska et al. 2021)) and the (usually) single temporal sampling approach often means that only compounds that exceed substantial signal-to-noise ratios, constrained by sampling and analysis methods, and that overcome complexities associated with individual and population variability are reported. Informative compounds that exist in smaller quantities or compounds that are absorbed and metabolised by the body are often missed from these types of scanning studies and therefore subsequent targeted selective-ion mode (SIM) analyses may be searching in the wrong place.

Of the studies utilised in this meta-analysis, only those studies where compounds were reported as increased, when compared to control group, have been used. Where studies have reported VOC uptake, they have not been included due to the rarity of this approach. This is a significant lapse in the published literature as volatile uptake may form a very important avenue for biomarker discovery. When combined with longitudinal studies in diagnostic applications, this approach may help to overcome systemic issues affecting cohort variability.

Targeted, or Selected/Selective Ion Monitoring (SIM), MS analyses can provide a more sensitive, targeted approach to quantifying volatiles in breath. By focusing on individual compounds, researchers achieve substantially greater methodological sensitivity in detection and quantification. Monitoring targeted VOCs can provide in-depth information about complex

processes, such as the citric acid cycle (Tejero Rioseras et al. 2018) but it is important to focus on correct VOCs for accurate diagnosis.

In the first stage of data collection, papers were considered regardless of detection method (Table S1). For further analysis, studies were reduced to SCAN studies as well as those studies which searched for a suite of volatiles that were representative of multiple functional groups (Table S2).

## Categorical variables

Of the 84 studies retained after selection criteria, 43 focus on lung cancer, five breast cancer, 13 Diabetes, 13 liver disease and eight IBD. Five further studies that focused on cancers of the stomach, mouth, larynx and upper gastrointestinal region, are grouped as upper gastrointestinal (UGI) cancers. It is outside the scope of this research to consider pathological variability within each group, due to limited study numbers, therefore diseases have been grouped. For example; the liver disease group includes studies investigating liver cirrhosis in adults and non-alcoholic fatty acid liver disease (NAFLD) in children. Variability in pathology has been noted in Table S2. Furthermore, diagnosis and separation of pathologies may cause inaccuracies when using breath volatiles, for example, separation of IBD conditions: Crohn's disease and Ulcerative colitis from control groups can be accurate but separating the two pathological profiles is less accurate (Tiele et al. 2019).

Studies investigating limited numbers of compounds generate uninformative outcomes when compared with studies investigating different, targeted compounds or studies employing a non-targeted approach (Table S1). However, some variability is likely to be due to the range of instrumental and collection techniques employed. Most studies listed here utilised Gas Chromatography Mass-Spectrometry (GC/MS) as their analytical platform, but other methods include Proton-Transfer Reaction Mass-Spectrometry (PTR-MS), Selected Ion Flow-Tube (SIFT-MS), Ion-Mobility-Spectrometry (IMS) (Table S1). There may also be further subdivisions, for example, standard GC/MS or GCxGC TOF, all of which will have an impact upon the observation of compounds (Beale et al. 2018). These methods should be considered when comparing reported VOCs between studies.

## Meta-Analysis and compound nesting

For each study, reviewer TI extracted data of VOCs which were identified as increased/enhanced in concentration. Volatiles reported from these studies were compared

through Principal Component Analysis (PCA) using a binary function - present (1) or not (0) in a matrix (Table S2) using R-studio and ggplot2. This data was then used to train two classification models, random forest (RF) and linear discriminant analysis (LDA), with predictions and classification accuracy scores obtained through leave-one-out cross validation. All classification was performed in R-studio, using the randomForest package for RF, and the MASS package for LDA. Complete equal weighting of individual compounds and/or equal consideration of all possible individual VOCs (i.e when considering each possible compound) led to uninformative PCA outcomes. Compound nesting (combining similar/related compounds under one heading) was applied to clarify PCA outcomes. As an example of compound nesting; monomethylated alkanes, such as methylated variants of undecane (of which 4 isomers exist), have been considered as one VOC biomarker in Figure 2. The nesting categories can be seen in supplementary tables.

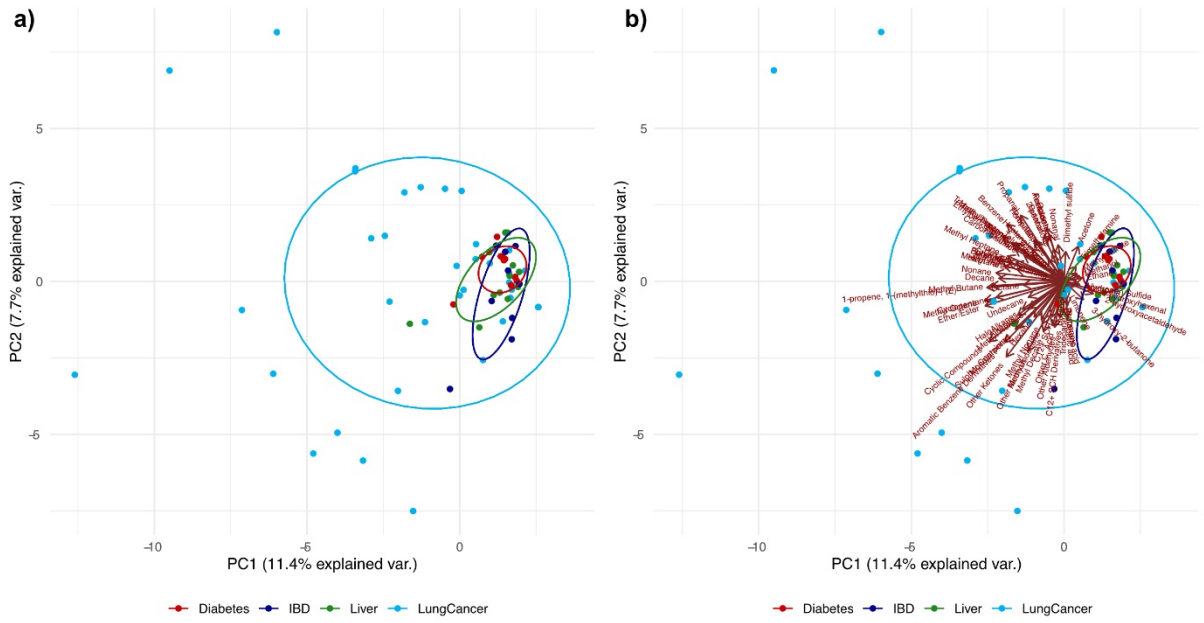


**Figure 2.** PCA plots of volatiles released by patients for; diabetes (n13), IBD (n9), liver disease (n12), lung cancer (n41). **a, b** show same data, axis/compound identifiers shown in **b**. Ellipses represent 95% CI.

## Results

By considering every reported biomarker across a wide variety of studies and methodologies, including targeted single biomarker studies, no single or suite of VOC compounds show diagnostic potential for lung cancer (Figure 2). Primary PCA axes explain very little of the observed variance across all studies (only 19.1% of the variation within the data can be explained by PCA axes 1 and 2). IBD, diabetes and liver disease are inseparable (within the 95% confidence intervals (CI) assigned by the PCA) while lung cancer VOCs overlap all groups with several outlying studies (Figure 2). The lack of definitive outcomes when comprehensively including all reported data, as represented in the PCA analysis (Figure 2), is expected when considering comorbidities, systemic variability and methodological differences.

Alternative grouping of compounds may be more informative of processes underlying production of individual compounds. It may be more appropriate to consider, for example, all five carbon alkanes (e.g. pentane or methylated butanes), regardless of methylation or ethylation, as indicative of a functional process, inclusive of modification events. These aggregated 5 carbon compounds may therefore be more descriptive of specific metabolisms than individual compounds, reducing the impacts of individual variability in compound metabolism or derivatization.

We hypothesised that applying a nesting approach (combining compounds with similar functional grouping) would reveal distinctive trends between pathologies in VOC data. For example, altered levels of alcohols in the breath have been reported often in liver disease, including ethanol, methanol and propanol (Hanouneh et al. 2014). Similarly, a number of aldehydes have been reported for several cancer types, with hexanal being the most commonly reported (Janfaza et al. 2019).Assuming that hexanal (in cancer) or ethanol (in liver disease) are the critical, important breath biomarkers and not aldehyde/alcohol metabolisms more generally may reduce the information that can be gleaned from single biomarkers. While individual VOC biomarkers may increase specificity, there is a need to perform further analysis to identify how they relate to functional chemical groups and disease/stress-based metabolisms.

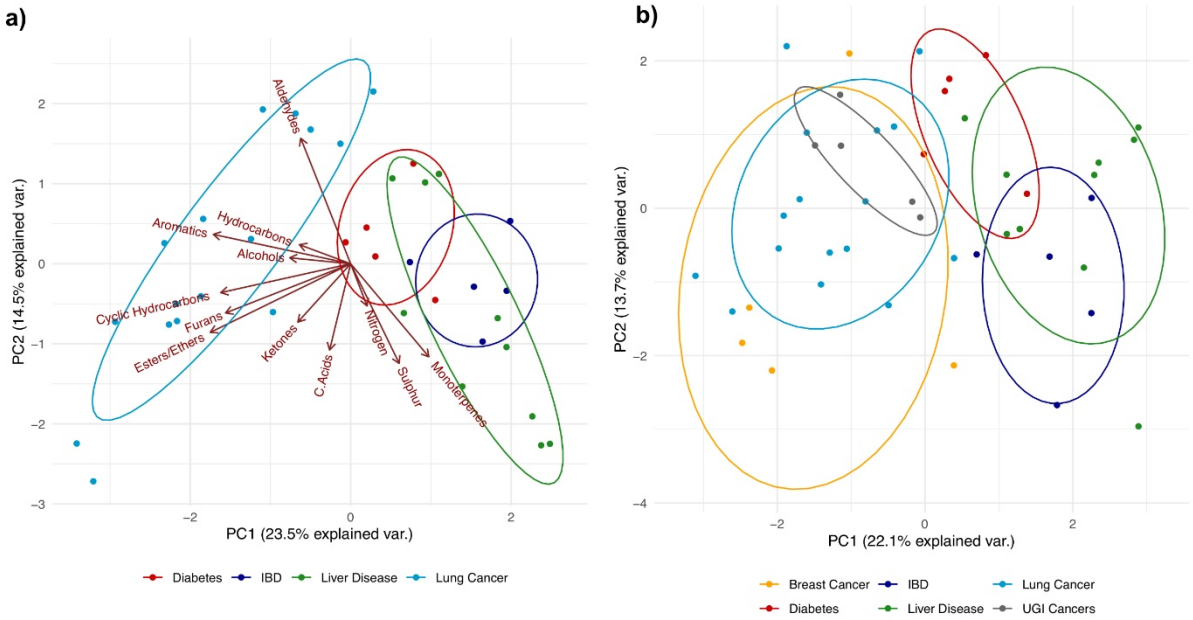


**Figure 3.** PCA plot of volatiles released by patients, arranged by functional group as shown by axis in **(A)** for; diabetes (n6), IBD (n5), liver disease (n10) and lung cancer (n18). **B** as in A, with additional groups; breast cancer (n5) and UGI cancers (n5). All studies shown are non-exclusionary analytical approaches. Ellipses represent 95% CI.

## Functional grouping of Volatiles

Data (barring targeted studies) was grouped into chemical functional groups, as defined in table 1 (Hanson 2001). PCA analysis using functional groups was able to explain substantially more of the data presented (38.0% from axes 1 and 2, Figure 3) and created a clear separation between lung cancer and all other disease states (Figure 3). Primary functional groups which separate lung cancer from other diseases include hydrocarbons (notably six carbon compounds and above, irrespective of saturation or branching), aldehydes, furans, cyclic hydrocarbons and aromatics (Figure 3). Benzene derivatives (aromatics) were reported in the breath in every lung cancer study. Isoprene, a commonly reported biomarker for cancer (Hanna et al. 2019), has not been included in this analysis due to high variability of published outcomes (Julian King et al. 2010). Its inclusion however did not significantly alter PCA outcomes.

Most diabetic studies were defined by the appearance of ketones in the breath, notably and unsurprisingly acetone, a volatile commonly associated with diabetes(Souvik Das, Pal, and Mitra 2016) as well as alcohols, including butanol, methanol and ethanol.

IBD was defined by the presence of hydrocarbons (notably, shorter compounds, eight carbons or less), nitrogen and sulphur compounds. The pathophysiology of IBD, such as Crohn's disease is characterized by periodic inflammation (linked to oxidative stress and subsequent hydrocarbon release (M. Phillips et al. 2000; Ratcliffe et al. 2020)) and an altered microbiome (linked to alterations in sulphur metabolism and nitrogen compounds (Hanouneh et al. 2014; De Vincentis et al. 2019)).

Studies investigating forms of liver disease, including NAFLD and cirrhosis, were strongly defined by the presence of monoterpenes in the breath, notably limonene and pinene. However, this was slightly skewed in the PCA analysis as only 4 out of the 10 included studies reported monoterpenes (Table S2). Ketones, nitrogen and sulphur compounds were also seen in patients suffering from liver disease. Interestingly, only 4 studies reported alcohols in the breath of liver disease patients (one focusing on NAFLD (Hanouneh et al. 2014), one comparing between NAFLD and cirrhosis (Netzer et al. 2009), and two investigating cirrhosis only, Table S2) and this was less defining of liver disease as a group than other functional groups, despite impaired alcohol processing being a hallmark of liver disease and therefore purportedly a breath biomarker (De Vincentis et al. 2019).

## Cancer comparisons

To investigate the possibility that grouped lung cancer breath VOC outcomes (Figure 3a) were the result of proximity to pulmonary architecture, facilitating direct diffusion of VOCs to lung airspace rather than systemically processed VOCs (Figure 1), we compared VOCs reported in the breath of breast cancer patients and cancers of the upper GI tract and mouth (UGI) to the groups already presented (Figure 3B). Addition of breast and UGI cancer studies reveals close correlation of all cancer groups (breast, lung and UGI) along similar axes, while retaining significant separation from diabetes, liver disease and IBD outcomes. To clearly demonstrate this separation, these subgroups were grouped into cancer vs other (supplementary figure 2A). The PCA biplot is also provided to show which elements were identified as most discriminatory. (supplementary figure 2B). This suggests that PCA separation from other diseases is not due to relative location within the pulmonary architecture, mouth and oesophagus. Associations of functional VOCs are more consistent between cancer pathologies relative to other disease states, suggesting that these signals are disease correlated.

## Further Analyses

To further expand on the use of PCA and to acknowledge the presence of potential 'voodoo correlations' in the data (Miekisch, Herbig, and Schubert 2012) we performed random forest and LDA for classification of cancerous vs. non-cancerous (other) diseases (figure 4). This demonstrated that the functionally grouped VOCs can be used in combination to classify cancer with high accuracy. Random forest determined 93% accuracy for cancer and 100% for 'other'. LDA determined 93% accuracy for cancer and 81% for 'other'.

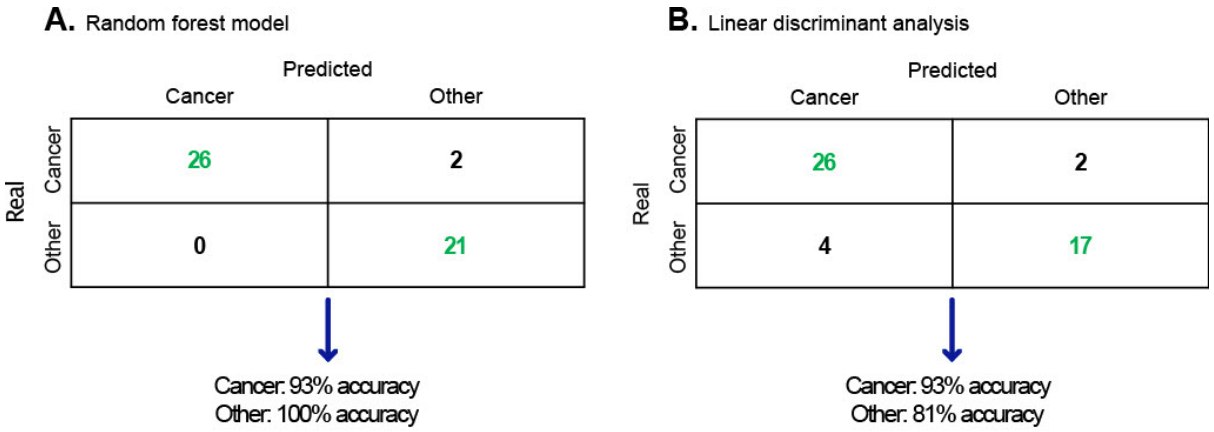


**Figure 4.** Confusion matrices to summarise supervised classification prediction for **(A)** Random forest model and **(B)** Linear discriminant analysis. Classification accuracy scores for each model are also provided.

## Discussion

The difficulties faced by breath researchers exploring released VOCs is highlighted by multiple reviews investigating lung cancer and pulmonary disorders (Miekisch, Schubert, and Noeldge-Schomburg 2004; Jia et al. 2019; Hakim et al. 2012; Mazzone 2008; Amann et al. 2011; Hanna et al. 2019; Zhou, Liu, and Duan 2012), diabetes, liver disease and IBD (Souvik Das, Pal, and Mitra 2016; Minh, Blake, and Galassetti 2012; De Vincentis et al. 2019; Van Malderen et al. 2020; Markar et al. 2015). Within these reviews, limited consensus has been reached regarding the efficacy of individual compounds to identify specific diseases and/or disease-based metabolisms. These are reviewed in Table 1. In spite of this ongoing and dedicated research, sufficiently precise and accurate breath biomarkers for diagnostic application have continued to elude researchers for cancer (Jia et al. 2019), liver disease (De Vincentis et al. 2019), IBD (Markar et al. 2015) or diabetes (Minh, Blake, and Galassetti 2012). We have re-affirmed the challenges in biomarker identification through our introductory discussion on sources of breath VOC variability and through the lack of descriptive potential, as shown in Figure 2.

Functional group analysis, which we show clarifies existing, previously disparate, studies (Figure 3), are not to be taken as recommendations for singular biomarker approaches even when these single biomarkers exist as components of a larger functional group. Clearly, singular volatile approaches are not effective (Figure 2) and this has been recognised by researchers previously (Miekisch, Herbig, and Schubert 2012). Functional group analysis does, however, provide a guide for further research, described here as a ‘breath print and research framework’. We propose that analysis of multiple volatile biomarkers from a range of functional classes will provide increased discriminatory power.

### Functional Groups of Volatile Biomarkers

We have improved disease separation within our PCA analyses (Figure 3a), affirmed that location of disease does not drive reported outcomes (Figure 3b), and highlighted trends in volatiles discovered in human breath by using a functional grouping approach. Successful application of functional groups to biomarker discovery implies that functional groups are more defining of process or disease (Figure 3), than single volatile markers (Figure 2). A recent review has surveyed volatiles released by humans and highlighted functional groups (Drabińska et al. 2021).

Further to PCA analyses, both random forest and LDA confusion matrices revealed high accuracy in recognising cancer and ‘other’ (Figure 4). While it should be recognised that

grouping very distinct diseases together in this way is confounding within itself, it demonstrates the power of this approach. The classification results obtained here are suggestive that VOCs could prove a powerful tool for cancer diagnostics, with many providing good discrimination between cancerous and non-cancerous diseases.

A number of metabolic pathways and key characteristics of functional groups associated with VOCs in breath have been reviewed (Hakim et al. 2012; Souvik Das, Pal, and Mitra 2016; Amann et al. 2014) and some of these pathways, pertinent to diseases investigated here, have been highlighted (Table 1). Understanding remains limited and further research into targeted cellular metabolisms is needed to disentangle common functional outcomes. We present here a brief analysis of exogenous sources and endogenous metabolisms for several functional groups with predictive power in our analysis.

Only 4 disease outcomes are included here, due to reasons discussed in the methodology section. For this reason we have included possible pathophysiological sources of compounds, for cross reference to other pathologies in table 1. For example, aldehydes have been linked to inflammatory linked stress and subsequent lipid peroxidation in a range of diseases, such as COPD (Zhou, Liu, and Duan 2012) and can be seen here in cancer (table 1). Possible endogenous sources may then be cross referenced to develop a suite of diagnostic compounds dependent upon disease.

| Class | Example Compounds | Prevalent In | Possible Endogenous Source |
|---|---|---|---|
| Hydrocarbon | Butane, Heptane | All | Lipid peroxidation (Riely, Cohen, and Lieberman 1974; Negre-Salvayre et al. 2008; Sobotka et al. 1994; Kneepkens, Lepage, and Roy 1994; Hakim et al. 2012; Ratcliffe et al. 2020), ethanol Metabolism (Müller and Sies 1982) |
| Alcohol | Ethanol, Propanol | Cancer, diabetes, liver Disease | Alcohol Metabolism, Ketone Metabolism (Miekisch, Schubert, and Noeldge-Schomburg 2004; Bornhorst and Mbughuni 2019; Davis, Dal Cortivo, and Maturo 1984), Hydrocarbon Metabolism (Kneepkens, Lepage, and Roy 1994; Dadamio et al. 2012) |
| Ketone | Acetone, Butanone | Cancer, diabetes, liver Disease | Amino acid metabolism to acetone (Pedersen 1929; Janfaza et al. 2019; López-Soriano, Alemany, and Argilés 1985; Ruzsányi and Péter Kalapos 2017), isopropanol to acetone (Janfaza et al. 2019; Nordmann et al. 1973), fatty acid metabolism and oxidation (Erhart et al. 2009; Hakim et al. 2012; Janfaza et al. 2019; M. Ye et al. 2015) |
| Aldehyde | Hexanal, Acetaldehyde | Cancer | Lipid Peroxidation (Esterbauer, Schaur, and Zollner 1991; Shahidi 2001; Ayala, Muñoz, and Argüelles 2014; Ratcliffe et al. 2020), alcohol metabolism (Wickramasinghe et al. 1981), enzymatic function (Mellick 2006) (Janfaza et al. 2019; Nordmann et al. 1973) |
| Carboxylic Acids | Propanoic acid | Cancer (breast) | Aldehyde oxidation (Larkin 1990), Lipid peroxidation (Callol-Sanchez et al. 2017; Jareño-Esteban et al. 2017; Ratcliffe et al. 2020), Microbial (Dryahina et al. 2017) |
| Ester/Ether | Butyl Acetate, Dimethyl-Ether | Cancer | Enzymatic action i.e. esterases (Hakim et al. 2012; Fukami and Yokoi 2012) |
| Isoprenoids | Limonene, Pinene | Liver disease | CYP450 activity (O'Hara et al. 2016; De Vincentis et al. 2019) |
| Nitrogen | Trimethylamine, Ammonia | IBD, liver Disease | Amino acid metabolism (Miekisch, Schubert, and Noeldge-Schomburg 2004), Microbial (W. Ye et al. 2019; Snel et al. 2011) |
| Furan | Furan | Cancer | Unclear, microbial action (Trefz et al. 2013) |
| Sulphur | Dimethyl Sulphide, Hydrogen Sulphide | Cancer (lung), IBD, liver Disease | Urea cycle (Shimamoto, Hirata, and Katsu 2000), Microbial (Hanouneh et al. 2014; De Vincentis et al. 2019) |
| Aromatic | Benzene, Xylene | Cancer | Released from fatty tissue (Haick et al. 2014), CYP450 (Guengerich, Peter Guengerich, and Shimada 1991), unknown endogenous creation |
| Cyclic Hydrocarbons/Ketones | Cyclopentane, Cyclohexanone | Cancer | Unclear |

**Table 1. Functional groups of volatile compounds seen in breath research studies and possible endogenous sources of variance. The data presented here links studies (presented in figure 3) to prevalent functional groups of volatiles to cancer types, irritable bowel disease (IBD), diabetes and liver disease.**

## Aromatics, furans and cyclic hydrocarbons

Cyclic compounds, such as aromatics, cyclic hydrocarbons, and furans, act as important compounds in differentiating between lung cancer and other disease states in our analysis (Figure 3a). Cyclic compounds have, however, generally been regarded as contaminants in breath research (Hakim et al. 2012; Jia et al. 2019) and, because of this, their diagnostic power has often been dismissed. Due to common exposure as exogenous compounds, the use of these aromatic compounds as diagnostic tools should be taken with caution and use as a single compound diagnostic would not be recommended. They retain diagnostic power however, in part as a negative marker, within our approach.

Benzene (and derivatives) and furans are present in cigarette smoke and higher in the breath of smokers (Buszewski et al. 2009), a particular consideration for lung cancer breath profiles. Studies which addressed this by contrasting VOC screens from smokers/non-smokers suffering from lung cancer have found that benzene derivatives and furans were still present (Rudnicka et al. 2011). Furthermore, studies have shown that cultured, in vivo cancer cells release a range of benzene derivatives (Thriumani et al. 2018; Silva et al. 2017; Hanai et al. 2012; Jia et al. 2018; Serasanambati et al. 2019; Peled et al. 2013; Kwak et al. 2013). Human fibroblasts (Wojciech Filipiak et al. 2010) and human mammary epithelial cells (Silva et al. 2017) also produce aromatics when grown in culture. This highlights how false positives from exogenous sources can confound separation of functionally useful markers from contamination.

Furans have been associated with smoking and these compounds are not associated with endogenous origin (W. Filipiak et al. 2012). Appearance in heated food suggests an association with diet (Zoller, Sager, and Reinhard 2007). Furan appears in the breath of healthy, non-smoking individuals in addition to smoking and non-smoking cancer patients and individuals (Rudnicka et al. 2011; Kushch et al. 2008). Furans, have been reported in lung cancer, and one study into laryngeal cancer (Fielding et al. 2020) (Table S2). As this compound was not seen in breast cancer or other diseases investigated it suggests that there might be either a) a pulmonary diffusion aspect to detection, b) a smoking component or c) both.

Cyclic hydrocarbons, such as cyclopentane and cyclohexane, have not been investigated with respect to metabolic cellular function but their appearance in the headspace of cell lines such as mesothelioma (Little et al. 2020) and their effective use diagnosing cancer patients from breath for colorectal (Bhattacharyya et al. 2017), lung (M. Phillips et al. 1999; Handa et al. 2014) and breast cancer (Barash et al. 2015) suggests that they retain diagnostic capacity irrespective of exogenous contaminant sources. Cyclohexanone and other cyclic

hydrocarbons are by-products of plastic and fuel combustion (Wahl et al. 1999), and are unlikely to be contaminants in cellular headspace analysis. Cyclohexane has been shown to be descriptive of malignant pleural mesothelioma when contrasted with subjects with similar professional asbestos exposure (de Gennaro et al. 2010). However, oxygenation of cyclohexane produces cyclohexanone, thought to be a result of fatty acid oxidation and weight loss (Liu et al. 2014).

Throughout the data presented here, furans, cyclic hydrocarbons, aromatic compounds and benzene derivatives have been consistent markers of cancer, irrespective of lung cancer, breast cancer or cancers of the mouth and upper GI tract (Figure 3 and Table S2) (X. Chen et al. 2007). While these compounds all have exogenous sources, this work highlights their diagnostic potential. While in many instances, they may be confounded with smoking related diseases, their absence from IBD, liver disease and diabetes studies, may allow diagnosticians to remove these diseases from consideration, providing a powerful combination of VOC biomarkers and a starting point for comprehensive ‘breath print’ analyses.

## Developing a ‘breath-print’ and research framework

The identification of a single volatile biomarker for diagnosis of complex pathologies, appears unlikely considering the unsuccessful outcomes of more than three decades of research on diseases such as lung cancer. It seems more likely that multiple biomarkers will provide maximum diagnostic accuracy and this has been recognised by breath researchers (Leopold et al. 2015; Miekisch, Herbig, and Schubert 2012; Khoubnasabjafari et al. 2021; Politi et al. 2021). For example, acetone has been a target for diabetic breath research since the 1960s (Tassopoulos, Barnett, and Fraser 1969), linked to ketoacidosis (Minh, Blake, and Galassetti 2012) and characteristic of the sweet smell on the breath (Guo et al. 2012) and found in greater concentrations in the breath of diabetics. However, as a single marker it does not optimise diagnostic potential, due to concentration variability linked to insulin resistance, lipolytic activity, exercise, fasting status and gender (Souvik Das, Pal, and Mitra 2016). Other VOC markers can therefore be utilised in tandem to build up a ‘breath-print’, increasing diagnostic power and overcoming systematic variability and comorbidities.

In addition to multiple volatile biomarkers increasing diagnostic accuracy, a ‘breath-print’ may potentially include wider breath dynamics and pulmonary function such as flow rate, pressure, and gas transfer. Combined, this can build an accurate picture of lung function (Pleil et al. 2020) and these tools are used frequently in the clinic for assessing patients with COPD, asthma or any restriction to breathing (Sylvester et al. 2020; Pleil et al. 2021). Lung function

impacts testing and collection of volatiles, creating variability between individuals and so consideration of this will increase the power of diagnosis by VOCs.

Recommendations for volatile compounds as disease diagnostic markers have not yet been made for many disease states. This is, in part, due to the variation in approaches (Table S1) and systemic complications (Figure 1). In this research we have arranged reported markers from non-exclusionary studies into functional groups and substantially improved disease separation, generating greater correlation across primary PCA axes (Figure 3). We have also shown that cancer studies generate similar outcomes, irrespective of location, lending credence to the idea that our reported outcomes are independent of bodily location and, therefore, due to common metabolic action. This work agrees with systematic and prospective reviews which have identified correlations between disease compounds such as aldehydes for cancer diagnosis (Janfaza et al. 2019; Hanna et al. 2019; Jia et al. 2019). We, therefore, recommend that research targets consist of a suite of markers that encompass a range of functional groups.

Application of functional group analysis is limited as it can remove specificity. For example, butanone and acetone are both ketones but butanone is highly present in the lung cancer group but not in the diabetic group (Table S2). Therefore, when selecting compounds for investigation, a selection of compounds from several functional groups (i.e. 3 ketones, 3 aldehydes, 3 hydrocarbons, 3 sulphur compounds etc) may optimise descriptive and diagnostic potential.

Accordingly, a suite of VOCs (a ‘breath-print’) can be utilised to account for variability within individuals. However, an understanding of functional groups and how they relate to metabolic processes will allow for more effective identification of volatile compounds to serve as biomarkers within the ‘breath-print’. Based on group separation in figure 3b, IBD, liver and diabetes are separable from lung, breast and OG cancer, but each of these sub-groups (cancers versus gut/liver diseases) have a number of overlapping compounds. A suite of VOCs for targeted cancer diagnosis would include both positive and negative markers. The following outlines a framework for developing cancer diagnostic targets for breath where a study might focus on 16 to 20 VOCs.

Positive markers of cancer would include;

I. Aldehydes, such as; pentanal, hexanal and heptanal;
II. Multiple hydrocarbons above 6 carbons such as heptane, octane and decane (there appears to be no preference for branched chained hydrocarbons in the data)
III. Aromatic and cyclic compounds, such as ethyl benzene, furan, cyclopentane and cyclohexanone.

The ketone, butanone, was also highly reported for cancer studies. Presence of each biomarker individually is not confirmation of diagnosis but acts to increase diagnostic accuracy.

Negative markers might include;

I. Monoterpenes, either limonene or pinene.
II. Nitrogen-containing compounds such as trimethylamine and methyl nitrate.
III. Ketones; specifically acetone
IV. Alcohols such as ethanol or methanol (isopropanol and propanol are common in cancer patients).

For sulphur compounds; dimethyl sulfide was reported by cancer studies while hydrogen sulfide appears indicative of liver disease.

Interpretation of volatile compounds from human breath is multifaceted and complex. Likely markers of cellular processes can be identified through knowledge of dominant metabolisms and considering systemic alterations and interactions. By considering markers of contrasting processes and pathophysiologies, the power of diagnosis will increase. Functional group targeting can help overcome variability within individuals and cohorts when looking for breath biomarkers of particular cellular functions. The ‘breath-print’ approach takes into account variability of biomarker metabolisms, conflicting comorbidities and physiological variations within individuals.

## Application to COVID-19

A primary goal of this work is to provide contextual VOC targets, so that future research may target compounds with increased likelihood of diagnostic power. We may speculate upon how this work may relate to a critical topic in contemporary breath research: the diagnosis of viral lung infection, namely COVID-19. At this junction, with the limited published data available, we consider the underlying processes involved and compare this with research into other infections of the lung.

Several studies have explored whether COVID diagnosis via breath, using sensors and enose approaches (H. Chen et al. 2021; Wintjens et al. 2021; de Vries et al. 2021; Shan et al. 2020), is plausible. Currently, 5 studies have been published which a) fit the criteria for inclusion in this article and ii) identify specific VOCs as candidates for diagnosis. Substantial variability in COVID status exists within the studies undertaken, most notably, age of patients and disease severity at the point of breath collection (Berna et al. 2021; Liangou et al. 2021; Ruszkiewicz

et al. 2020; Ibrahim, Cordell, et al. 2021; Grassin-Delyle et al. 2021). Severity of disease influences VOCs seen in the breath (as shown in table 1). Severely ill patients, including those presenting with Acute Respiratory Distress syndrome (ARDs), will have impaired VOC diffusion into the lung space, due to the presence of fluid in the lungs. Furthermore, they may present with a range of disease complications outside of pulmonary ailments (Grasselli et al. 2020; Arentz et al. 2020).

Published reports that fulfil our selection criteria report COVID representative functional groups as: aldehydes (notably C7 and over), carboxylic acids, oxygenated species, monoterpenes and halocarbons (Ruszkiewicz et al. 2020; Grassin-Delyle et al. 2021; Berna et al. 2021; Ibrahim, Cordell, et al. 2021; Liangou et al. 2021). With an awareness of the limitations outlined and the large variability in collection and analysis methodology, we compared the functional outcomes from these papers against cancer and all other studies grouped (supplementary figure 3). COVID-19 revealed clear separation from cancer studies and sat within ‘other’ grouped studies with explained variance of 34.7% for PC1 and PC2 combined. One outlier for the COVID-19 group was identified as Ruszkiewicz et al 2020 due predominantly to the lack of hydrocarbons detected.

Pathophysiology of COVID-19 infections includes inflammatory response, characterised by oxidative stress (table 1) which has been linked to aldehydes and hydrocarbons (Ratcliffe et al. 2020). Aldehydes are present in all 5 COVID-19 breath studies presented here and hydrocarbons are present in 4 studies. In comparison, studies investigating Influenza, a virus of the lung, revealed increased hydrocarbons in patients' breath following Influenza A vaccination (Michael Phillips et al. 2010) and pigs infected with Influenza revealed aldehydes in their breath (Traxler et al. 2018).

As the volume of research around viral pathogens and volatile profiles grows, targets specific to pathogens will increase and the application of targets for early diagnosis aside from those targets linked to secondary and tertiary effects of infection will aid early application.. We have demonstrated that researchers can consider targets from different functional groups and varying disease states

## Conclusion

In conclusion, while mechanistic studies continue to be reported, and collections of cellular VOCs compiled (Wojciech Filipiak et al. 2016) and contrasted with human breath databases (Kuo et al. 2020; Jia et al. 2019; Agarwal, Sharma, and Fatima 2016; Janfaza et al. 2017), we contend that further information can be gained from comparing and contrasting breath profiles

already reported within targeted metabolic and physiological contexts and that this approach will help inform further research. We have demonstrated that commonality exists in a suite of volatiles present in the breath of patients across a range of diseases and that these volatiles can also separate disease groups.

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## Author contributions

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## Supplementary Material

A transparent method is provided for clarity of data collection and processing. A review protocol is available at https://www.crd.york.ac.uk/PROSPERO - Registration number CRD42021234660.

Papers were collected based on an electronic search (title and abstract) using the Embase and MEDLINE databases through the OVID platform.

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The search terms lung cancer, volatile organic compound, VOC, breath, and exhaled were used in combination with the Boolean operators AND and OR.

Abstract - Lung cancer, Breath or exhaled, VOC or volatile organic compound
Title - lung cancer and breath

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The search terms Diabetes, volatile organic compound, VOC, breath, and exhaled were used in combination with the Boolean operators AND and OR.

Abstract - diabetes, Breath or exhaled, VOC or volatile organic compound
Title - diabetes and breath

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The search terms Liver, volatile organic compound, VOC, breath, and exhaled were used in combination with the Boolean operators AND and OR.

Abstract - liver, Breath or exhaled, VOC or volatile organic compound
Title - liver disease or liver cirrhosis and breath

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The search terms IBD, Inflammatory bowel disease, volatile organic compound, VOC, breath, and exhaled were used in combination with the Boolean operators AND and OR.

Abstract - IBD, Breath or exhaled, VOC or volatile organic compound
Title - inflammatory bowel disease or IBD and breath

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Results from these searches are shown in PRISMA workflow (supplementary figure 1).

Inclusion was dependent on patients of the disease group having their breath analysed for volatile organic compounds compared with a healthy control group. These volatiles are required to be identified for comparison of biomarkers.

Healthy controls defined In-line with the diagnosis of pathology by trained physician, biopsy or appropriate, are included if they reflect comparable controls in demographic. This includes age, sex, comorbidities, smoking status and BMI. The control group should reflect the test group and if it does not, it is not included. Each study represents some variability in cohort recruitment and demographic

Results from these searches were compared to results from other systematic reviews of breath volatiles pertaining to reviews. Applicability was assessed for additional reviews under the same criteria. for lung, breast and other cancers (Hanna et al. 2019; Jia et al. 2019), irritable bowel studies(Van Malderen et al. 2020; Markar et al. 2015), diabetes(Das, Pal, and Mitra 2016) and liver disease(De Vincentis et al. 2019).

Many studies have been conducted looking at the breath of patients with diseases of the respiratory tract, predominantly COPD and asthma. We also conducted literature searches following the above guidelines. For Asthma 197 articles were identified of which 3 offered breath volatile investigation. COPD searches resulted in 75 articles, and 1 paper was identified as appropriate (Van Berkel et al. 2010). Parkinsons disease offered 9 results.

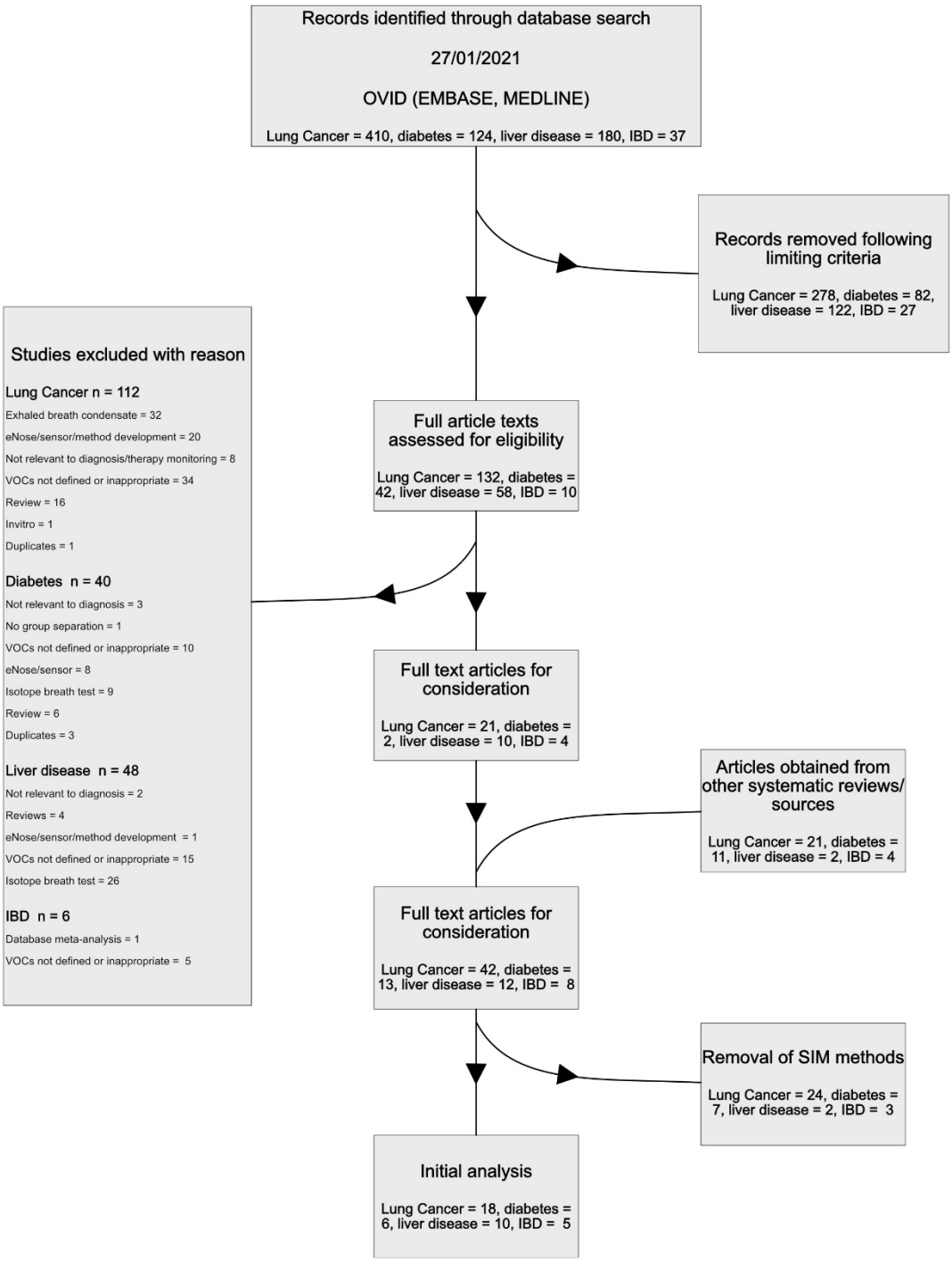
**Supplementary search strategy**

Example search strategy through the OVID system. Underlined words represent variables for search terms.

1. ((((((inflammatory bowel disease and volatile organic compound) or VOC) and exhaled) or breath).ab. and inflammatory bowel disease.ti.) or IBD.ti.) and breath.ti.
2. Limit 1 to abstracts
3. limit 2 to cochrane library [Limit not valid in Ovid MEDLINE(R); records were retained]
4. limit 3 to english language
5. limit 4 to human
6. limit 6 to (embase or medline) [Limit not valid in Ovid MEDLINE(R); records were retained]
7. limit 6 to abstracts
8. limit 7 to cochrane library [Limit not valid in Ovid MEDLINE(R); records were retained]
9. limit 8 to english language
10. limit 9 to human
11. limit 10 to (article or article in press or adaptive clinical trial or case reports or clinical study or clinical trial, all or clinical trial or comparative study or controlled clinical trial or introductory journal article or journal article or pragmatic clinical trial or randomized controlled trial) [Limit not valid in Embase,Ovid MEDLINE(R); records were retained]
12. limit 10 to (embase or medline) [Limit not valid in Ovid MEDLINE(R); records were retained]

Duplicates were removed if they were single markers used with the same analytical platform.

Risk of bias: For the purposes of this study application of bias is based primarily upon analytical platform. Selective Ion Modes (SIM)/targeted analytical approaches reveal information pertaining to only the targeted ions. This bias has been split into SIM-narrow, SIM-wide and SCAN. SCAN or non exclusionary methods are un bias analytical approaches that do not focus on VOCs. SIM WIDE have been designated as a medium bias for this study, focusing on importance of functional groups, as such these studys target specific VOCs but do so from a range of functional groups. Therefore their application for like-like VOC marker comparisons are limited but within functional comparisons, they are representative. SIM narrow studies are the most bias and may look at either a selection of volatiles from one functional group, a single marker or a very restricted selection. More information is available in the methodology section in the main text.




**Supplementary Figure 1.** PRISMA flow chart for literature search

| Author | Collection | Analysis | Disease | Analytical Method |
|---|---|---|---|---|
| Bajtarevic 2009 | SPME | GCMS | Lung Cancer | SCAN |
| Bousamra 2014 | ATM | FTICR-MS | Lung Cancer | SIM - Narrow |
| Buszwki 2011 | SPME | GCMS | Lung Cancer | SCAN |
| Buszwki 2012 | SPME | GC-TOF-MS | Lung Cancer | SCAN |
| Callol-Sanchez 2017 | SPME | GCMS | Lung Cancer | SIM - Narrow |
| Capuano 2015 | SPME | GCMS | Lung Cancer | SCAN |
| Chen 2005 | SPME | SAW | Lung Cancer | SCAN |
| Corradi 2015 | SPME | GCMS | Lung Cancer | SIM - Wide |
| Crohns 2009 | Sorbent | GCMS | Lung Cancer | SIM - Narrow |
| Deng 2004 | SPME | GCMS | Lung Cancer | SIM - Narrow |
| Feinburg 2016 | Direct | PTR-MS | Lung Cancer | SIM - Narrow |
| Filipak 2013 | TD | GCMS | Lung Cancer | SCAN |
| Fu 2014 | ATM capture | FTICR-MS | Lung Cancer | SIM - Narrow |
| Fuchs 2010 | SPME OFD | GC-MS | Lung Cancer | SIM - Narrow |
| Gaspar 2009 | HS-SPME | GC-MS | Lung Cancer | SIM - Narrow |
| Handa 2014 | Direct | IMS | Lung Cancer | SCAN |
| Kiskel 2010 | SPME | GCMS | Lung Cancer | SCAN |
| Li 2015 | ATM | FTICR-MS and GC-MS | Lung Cancer | SIM - Narrow |
| Ligor 2009 | SPME | GCMS | Lung Cancer | SCAN |
| Ligor 2015 | SPME | GCMS | Lung Cancer | SIM - Narrow |
| Ma 2014 | SPME | GCx GCFID | Lung Cancer | SIM - Narrow |
| Ma 2015 | TD | GCMS | Lung Cancer | SIM - Narrow |
| Peled 2012 | SPME | GCMS | Lung Cancer | SIM - Narrow |
| Peng 2009 | SPME | GCMS | Lung Cancer | SCAN |
| Peng 2010 | SPME | GCMS | Lung Cancer | SIM - Narrow |
| Philips 1993 | TD | GCMS | Lung Cancer | SIM - Narrow |
| Philips 2003 | TD | GCMS | Lung Cancer | SCAN |
| Philips 2006 | TD | GCMS | Lung Cancer | SCAN |
| Phillips 2007 | TD | GCMS | Lung Cancer | SCAN |
| Poli 2005 | SPME | GCMS | Lung Cancer | SIM - Narrow |
| Poli 2010 | SPME | GCMS | Lung Cancer | SIM - Narrow |
| Rudnicka 2011 | SPME | GCTOF/MS | Lung Cancer | SCAN |
| Rudnicka 2019 | SPME | GCMS | Lung Cancer | SCAN |
| Schallschmidt 2016 | SPME | GCMS | Lung Cancer | SIM - Wide |
| Schumer 2015 | ATM | Silicon Chip MS | Lung Cancer | SIM - Narrow |
| Schumer 2016 | ATM | Silicon Chip MS | Lung Cancer | SIM - Narrow |
| Skeldon 2006 | Direct | TDLS | Lung Cancer | SIM - Narrow |
| Song 2010 | SPME | GCMS | Lung Cancer | SIM - Narrow |
| Sukumra 2017 | Direct | GCMS | Lung Cancer | SIM - Narrow |
| Ulonwaski 2011 | SPME | GCMS | Lung Cancer | SCAN |
| Wang 2012 | SPME | GCMS | Lung Cancer | SCAN |
| Wehinger 2007 | Direct | PTR-MS | Lung Cancer | SIM - Narrow |
| Zou 2014 | SPME | GCMS | Lung Cancer | SIM - Narrow |

| Author | Collection | Analysis | Disease | Analytical Method |
|---|---|---|---|---|
| K. Yan 2014 | Direct | Sensor Array | Diabetes | SIM - Narrow |
| Fan 2014 | Direct | HT-GCMS | Diabetes | SIM - Narrow |
| Grieter 2010 | Direct | PTRMS | Diabetes | SCAN |
| Halbritter 2012 | Direct | PTRMS | Diabetes | SCAN |
| Lee 2009 | Direct | GCMS | Diabetes | SIM - Wide |
| Minh 2011 | Direct | GCMS | Diabetes | SCAN |
| Novak 2007 | Direct | GCMS | Diabetes | SIM - Narrow |
| Saasa 2019 | HS-SPME | GCMS | Diabetes | SIM - Narrow |
| Saidi 2018 | SPME | GC/Q-TOFMS | Diabetes | SIM - Wide |
| Trefz,Oberman2019 | Direct | PTR-TOFMS | Diabetes | SIM - Wide |
| Trefz,Schmidt 2019 | Direct | PTR-TOFMS | Diabetes | SIM - Wide |
| Turner 2009 | SIFT | GCMS | Diabetes | SIM - Narrow |
| Y. Yan 2014 | SPME | GCMS | Diabetes | SCAN |
| FernándezDelRio2015 | Direct | PTRMS | LD | SCAN |
| Dadamio 2012 | TD | GCMS | LD | SCAN |
| Eng 2015 | Direct | SIFTMS | LD | SCAN |
| Haneouh 2014 | Direct | SIFTMS | LD | SIM - Wide |
| Morisco 2013 | Direct | PTR-TOF-MS | LD | SCAN |
| Naim 2014 | Direct | SIFTMS | LD | SIM - Wide |
| Naim 2015 | Direct | SIFTMS | LD | SCAN |
| Netzer 2009 | Direct | IMRMS | LD | SCAN |
| Pijls 2016 | TD | GC-TOF-MS | LD | SCAN |
| Shimamoto 2000 | Direct | Sensor Array | LD | SIM - Narrow |
| Solga 2006 | TD | GCMS | LD | SIM - Narrow |
| Tangerman 1983 | TD | GCMS | LD | SIM - Narrow |
| Van de Velde 2008 | TD | GCMS | LD | SIM - Wide |
| Verdam 2013 | TD | GCMS | LD | SIM - Narrow |
| Hicks 2015 | Direct | SIFTMS | IBD | SCAN |
| Bodlier 2015 | Direct | GC-TOF-MS | IBD | SIM - Narrow |
| Dryahina 2017 | Direct | SIFTMS | IBD | SIM - Wide |
| Kokoszka 1993 | Direct | GCMS | IBD | SIM - Narrow |
| Monasta 2017 | Direct | IMRMS | IBD | SIM - Wide |
| Patel 2014 | Direct | SIFTMS | IBD | SIM - Wide |
| Pelli 1999 | Direct | GCMS | IBD | SIM - Narrow |
| Reider 2016 | Direct | SIFTMS | IBD | SIM - Wide |
| Sedghi 1994 | Direct | GCMS | IBD | SIM - Narrow |
| Amal 2013 | TD | GCMS | OG Cancers | SCAN |
| Bouza 2017 | SPME | GCMS | OG Cancers | SCAN |
| Chin 2018 | TD | GCMS | OG Cancers | SCAN |
| Fielding2020 | Direct | GCMS | OG Cancers | SCAN |
| Garcia 2014 | SPME | GCMS | OG Cancers | SCAN |
| Basrash 2015 | TD | GCMS | Breast Cancer | SCAN |
| Peng 2010 | TD | GCMS | Breast Cancer | SCAN |
| Phillips 2006 | TD | GCMS | Breast Cancer | SCAN |
| Wang 2014 | SPME | GCMS | Breast Cancer | SCAN |
| Zhang 2020 | SPME | GCMS | Breast Cancer | SCAN |

Abbreviations:
ATM: 2-(aminooxy)-N,N,N-trimethylethanammonium iodide
FID: Flame Ionisation Detector
FTICR: Fourier Transform Ion Cyclotron resonance
GCMS: Gas Chromatography Mass Spectrometry
HS-SPME: Headspace SPME
HT-GCMS: High Temperature GCMS
IMRMS: Ion Molecule Reaction MS
PTRMS: Proton Transfer Reaction MS
SAW: Surface Acoustic Wave
SIFTMS: Selected Ion Flow Tube MS
SPME: Solid Phase Micro Extraction
TD: Thermal Deabsorption
TOF:Time Of Flight

**Supplementary Table 1 (Table S1):** Studies collected for initial analysis by PCA. Analytical bias is show by SCAN (low) in green, SIM - Wide (medium) in yellow or SIM - Narrow (high) in red.

| Author | Collection | Analysis | Disease | Notes | Scan or SIM | Alcohols | Aldehydes | CAcid | Ester/ Ether | Hydrocarbons | Furan | Aromatics | Mono-terpenes | Ketones | Nitrogen | Sulphur | Cyclic Hydrocarbons |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bajtarevic 2009 | SPME | GCMS | Lung Cancer | | SCAN | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| Buszwki 2011 | SPME | GCMS | Lung Cancer | | SCAN | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 |
| Buszwki 2012 | SPME | GC–TOF–MS | Lung Cancer | | SCAN | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 |
| Capuano 2015 | SPME | GCMS | Lung Cancer | | SCAN | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 |
| Chen 2005 | SPME | SAW | Lung Cancer | | SIM WIDE | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Corradi 2015 | SPME | GCMS | Lung Cancer | NSCLC | SCAN | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Filipak 2014 | TD | GCMS | Lung Cancer | | SCAN | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| Handa 2014 | Direct | IMS | Lung Cancer | | SCAN | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Ligor 2009 | SPME | GCMS | Lung Cancer | | SCAN | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| Peng 2009 | SPME | GCMS | Lung Cancer | | SCAN | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| Philips 2003 | TD | GCMS | Lung Cancer | | SCAN | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| Philips 2007 | TD | GCMS | Lung Cancer | | SCAN | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| Phillips 2008 | TD | GCMS | Lung Cancer | | SCAN | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Rudnicka 2011 | SPME | GC–TOF–MS | Lung Cancer | | SCAN | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 |
| Rudnicka 2019 | SPME | GCMS | Lung Cancer | | SCAN | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| Schallschmidt 2016 | SPME | GCMS | Lung Cancer | | SIM WIDE | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| Ulonwaski 2011 | SPME | GCMS | Lung Cancer | | SCAN | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 |
| Wang 2012 | SPME | GCMS | Lung Cancer | | SCAN | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| Amal 2013 | TD | GCMS | OG Cancers | Gastric | SCAN | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| Bouza 2017 | SPME | GCMS | OG Cancers | Oral Cavity | SCAN | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Chin 2018 | TD | GCMS | OG Cancers | Oesophageal–gastic | SCAN | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| Fielding2020 | Direct | GCMS | OG Cancers | Laryngeal | SCAN | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 |
| Garcia 2014 | SPME | GCMS | OG Cancers | Laryngeal | SCAN | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| Basrash 2015 | TD | GCMS | Breast Cancer | Comparative mutations | SCAN | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Peng 2010 | TD | GCMS | Breast Cancer | | SCAN | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| Phillips 2006 | TD | GCMS | Breast Cancer | | SCAN | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| Wang 2014 | SPME | GCMS | Breast Cancer | | SCAN | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| Zhang 2020 | SPME | GCMS | Breast Cancer | | SCAN | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 |
| Grieter 2010 | Direct | PTRMS | Diabetes | Type 2 | SCAN | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| Lee 2009 | Cold Trap | GCMS | Diabetes | Hyperglycemia | SIM WIDE | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| Minh 2011 | Cold Trap | GCMS | Diabetes | Type 1 | SCAN | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| Trefz, Oberman 2019 | Direct | PTR–TOF–MS | Diabetes | Type 1, Paediatric | SIM WIDE | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Trefz, Schmidt 2019 | Direct | PTR–TOFMS | Diabetes | Type 1, Paediatric | SIM WIDE | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Yan 2014 | SPME | GCMS | Diabetes | Type 2 | SCAN | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| Dadamio 2012 | TD | GCMS | Liver Disease | Cirrhosis | SCAN | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 |
| Fernández Del Rio 2015 | Direct | PTRMS | Liver Disease | Cirrhosis | SCAN | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| Eng 2015 | Direct | SIFTMS | Liver Disease | Cirrhosis, Paediatric | SCAN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| Haneouh 2014 | Direct | SIFTMS | Liver Disease | AHLD | SIM WIDE | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| Morisco 2013 | Direct | PTR–TOF–MS | Liver Disease | Cirrhosis | SCAN | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| Naim 2014 | Direct | SIFTMS | Liver Disease | Mixed | SCAN | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 |
| Naim 2015 | Direct | SIFTMS | Liver Disease | NAFLD, Paediatric | SIM WIDE | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| Netzer 2009 | Direct | IMRMS | Liver Disease | Mixed | SCAN | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Pijls 2016 | TD | GC–TOF–MS | Liver Disease | Cirrhosis | SCAN | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| Van de Velde 2008 | TD | GCMS | Liver Disease | Cirrhosis | SIM WIDE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| Dryahina 2017 | Direct | SIFTMS | IBD | UC, CD | SIM WIDE | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Hicks 2015 | Direct | SIFTMS | IBD | UC, CD | SCAN | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| Monasta 2017 | Direct | IMRMS | IBD | UC, CD, Paediatric | SIM WIDE | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| Patel 2014 | Direct | SIFTMS | IBD | Paediatric | SIM WIDE | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| Reider 2016 | Direct | SIFTMS | IBD | UC, CD, OGD | SIM WIDE | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |

**Supplementary Table 2 (Table S2)** : Studies collected for secondary analysis by PCA. All studies collected here use SCAN (Scanning, non ion selective) or SIM (selective ion mode) WIDE approaches. Presence of reported volatile within functional group is shown as 1. NAFLD: Non-alcoholic fatty liver disease, AHLD: Alcoholic hepatitis, UC: Ulcerative colitis, CD: Crohns disease, OGD: other inflammatory gastrointestinal diseases, NSCLC: non

**Supplementary table 3. QUADAS assessment.**

| | | | | | Risk of bias | | | | Applicability concerns | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Author | Collection | Analysis | Disease | Analytical Method | Patient selection | Index test | Reference standard | Flow and timing | Patient selection | Index test | Reference standard |
| Bajtarevic 2009 | SPME | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Bousamra 2014 | ATM | FTICR-MS | Lung Cancer | SIM-Narrow | L | ? | L | L | L | ? | H |
| Buszwki 2011 | SPME | GCMS | Lung Cancer | SCAN | L | L | L | L | L | ? | L |
| Buszwki 2012 | SPME | GC-TOF-MS | Lung Cancer | SCAN | L | ? | L | L | L | L | H |
| Callol-Sanchez | SPME | GCMS | Lung Cancer | SIM - Narrow | L | L | L | L | L | L | L |
| Capuano 2015 | SPME | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Chen 2005 | SPME | SAW | Lung Cancer | SCAN | L | H | L | L | L | L | L |
| Corradi 2015 | SPME | GCMS | Lung Cancer | SIM - Wide | H | L | L | L | ? | L | ? |
| Crohns 2009 | Sorbent | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Deng 2004 | SPME | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Feinburg 2016 | Direct | PTR-MS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Filipak 2013 | TD | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Fu 2014 | ATM capture | FTICR-MS | Lung Cancer | SIM-Narrow | L | ? | L | L | L | L | H |
| Fuchs 2010 | SPME OFD | GC-MS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Gaspar 2009 | HS-SPME | GC-MS | Lung Cancer | SIM Narrow | L | L | L | L | L | L | H |
| Handa 2014 | Direct | IMS | Lung Cancer | SCAN | L | H | L | L | L | ? | L |
| Kiskel 2010 | SPME | GCMS | Lung Cancer | SCAN | L | L | H | L | L | L | L |
| Li 2015 | ATM | FTICR-MS and GC-MS | Lung Cancer | SIM-Narrow | L | ? | L | L | L | ? | H |
| Ligor 2009 | SPME | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Ligor 2015 | SPME | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | & Modelling | | | | | | | |
| Ma 2014 | SPME | GCx GCFID | Lung Cancer | SIM-Narrow | L | H | L | L | L | ? | H |
| Ma 2015 | TD | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Peled 2012 | SPME | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Peng 2009 | SPME | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Peng 2010 | SPME | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Philips 1993 | TD | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | ? | H |
| Philips 2003 | TD | GCMS | Lung Cancer | SCAN | L | L | L | L | L | ? | L |
| Philips 2006 | TD | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Phillips 2007 | TD | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Poli 2005 | SPME | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Poli 2010 | SPME | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Rudnicka 2011 | SPME | GCTOF/MS | Lung Cancer | SCAN | L | L | L | L | L | ? | L |
| Rudnicka 2019 | SPME | GCMS | Lung Cancer | SCAN | L | L | L | L | L | ? | L |
| Schallschmidt 2016 | SPME | GCMS | Lung Cancer | SIM - Wide | L | L | L | L | L | ? | ? |
| Schumer 2015 | ATM | Silicon Chip MS | Lung Cancer | SIM-Narrow | L | L | L | L | L | H | H |
| Schumer 2016 | ATM | Silicon Chip MS | Lung Cancer | SIM-Narrow | L | L | L | L | L | H | H |
| Skeldon 2006 | Direct | TDLS | Lung Cancer | SIM-Narrow | L | L | L | L | L | ? | H |
| Song 2010 | SPME | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Sukumra 2017 | Direct | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| Ulonwaski 2011 | SPME | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Wang 2012 | SPME | GCMS | Lung Cancer | SCAN | L | L | L | L | L | L | L |
| Wehinger 2007 | Direct | PTR-MS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Zou 2014 | SPME | GCMS | Lung Cancer | SIM-Narrow | L | L | L | L | L | L | H |
| K. Yan 2014 | Direct | Sensor Array | Diabetes | SIM - Narrow | L | L | L | L | L | H | H |
| Fan 2014 | Direct | HT-GCMS | Diabetes | SIM - Narrow | L | L | L | L | L | ? | H |
| Grieter 2010 | Direct | PTRMS | Diabetes | SCAN | H | L | L | L | ? | L | L |
| Halbritter 2012 | Direct | PTRMS | Diabetes | SCAN | L | L | L | L | L | L | L |
| Lee 2009 | Direct | GCMS | Diabetes | SIM WIDE | H | L | L | L | H | L | ? |
| Minh 2011 | Direct | GCMS | Diabetes | SCAN | L | L | L | L | L | L | L |
| Novak 2007 | Direct | GCMS | Diabete | SIM - Narrow | L | L | L | L | L | L | H |
| Saasa 2019 | HS-SPME | GCMS | Diabetes | SIM - Narrow | L | L | L | L | L | L | H |
| Saidi 2018 | SPME | GC/Q-TOFMS | Diabetes | SIM WIDE | L | L | L | L | L | H | ? |
| Trefz, Oberman 2019 | Direct | PTR-TOFMS | Diabetes | SIM WIDE | H | L | L | L | H | ? | ? |
| Trefz, Schmidt 2019 | Direct | PTR-TOFMS | Diabetes | SIM WIDE | H | L | L | L | H | ? | ? |
| Turner 2009 | SIFT | GCMS | Diabetes | SIM - Narrow | L | L | L | L | L | L | H |
| Y. Yan 2014 | SPME | GCMS | Diabetes | SCAN | L | L | L | L | ? | L | L |
| Fernández Del Rio 2015 | Direct | PTRMS | LD | SCAN | L | L | L | L | L | L | L |
| Dadamio 2012 | TD | GCMS | LD | SCAN | L | L | L | L | L | L | L |
| Eng 2015 | Direct | SIFTMS | LD | SCAN | H | L | L | L | H | L | L |
| Haneouh 2014 | Direct | SIFTMS | LD | SIM - Wide | L | L | L | L | L | L | ? |
| Morisco 2013 | Direct | PTR-TOF-MS | LD | SCAN | L | L | L | L | L | ? | L |
| Naim 2014 | Direct | SIFTMS | LD | SIM - Wide | H | L | L | L | H | L | ? |
| Naim 2015 | Direct | SIFTMS | LD | SCAN | L | L | L | L | L | L | L |
| Netzer 2009 | Direct | IMRMS | LD | SCAN | L | L | L | L | L | H | L |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pijls 2016 | TD | GC-TOF-MS | LD | SCAN | L | L | L | L | L | H | L |
| Shimamoto 2000 | Direct | Sensor Array | LD | SIM-Narrow | L | L | L | L | L | H | H |
| Solga 2006 | TD | GCMS | LD | SIM-Narrow | L | L | L | L | L | L | H |
| Tangerman 1983 | TD | GCMS | LD | SIM - Narrow | L | L | L | L | L | L | H |
| Van de Velde 2008 | TD | GCMS | LD | SIM - Wide | L | L | L | L | L | L | ? |
| Verdam 2013 | TD | GCMS | LD | SIM - Narrow | L | L | L | L | L | L | L |
| Hicks 2015 | Direct | SIFTMS | IBD | SCAN | L | L | L | L | L | L | L |
| Bodlier 2015 | Direct | GC-TOF-MS | IBD | SIM Narrow | L | L | L | L | L | ? | H |
| Dryahina 2017 | Direct | SIFTMS | IBD | SIM WIDE | L | L | L | L | L | L | ? |
| Kokoszka 1993 | Direct | GCMS | IBD | SIM Narrow | L | L | L | L | L | L | H |
| Monasta 2017 | Direct | IMRMS | IBD | SIM WIDE | H | L | L | L | H | H | ? |
| Patel 2014 | Direct | SIFTMS | IBD | SIM WIDE | H | L | L | L | H | L | ? |
| Pelli 1999 | Direct | GCMS | IBD | SIM Narrow | L | L | L | L | L | L | H |
| Reider 2016 | Direct | SIFTMS | IBD | SIM WIDE | L | L | L | L | L | L | ? |
| Sedghi 1994 | Direct | GCMS | IBD | SIM Narrow | L | L | L | L | L | L | H |
| Amal 2013 | TD | GCMS | OG Cancers | SCAN | H | L | L | L | H | ? | L |
| Bouza 2017 | SPME | GCMS | OG Cancers | SCAN | H | L | L | L | H | L | L |
| Chin 2018 | TD | GCMS | OG Cancers | SCAN | H | L | L | L | H | L | L |
| Fielding2020 | Direct | GCMS | OG Cancers | SCAN | H | L | L | L | H | L | L |
| Garcia 2014 | SPME | GCMS | OG Cancers | SCAN | H | L | L | L | H | L | L |
| Basrash 2015 | TD | GCMS | Breast Cancer | SCAN | L | L | L | L | L | L | L |
| Peng 2010 | TD | GCMS | Breast Cancer | SCAN | L | L | L | L | L | L | L |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phillips 2006** | **TD** | **GCMS** | **Breast Cancer** | **SCAN** | **L** | **L** | **L** | **L** | **L** | **?** | **L** |
| **Wang 2014** | **SPME** | **GCMS** | **Breast Cancer** | **SCAN** | **L** | **L** | **L** | **L** | **L** | **?** | **L** |
| **Zhang 2020** | **SPME** | **GCMS** | **Breast Cancer** | **SCAN** | **L** | **L** | **L** | **L** | **L** | **L** | **L** |

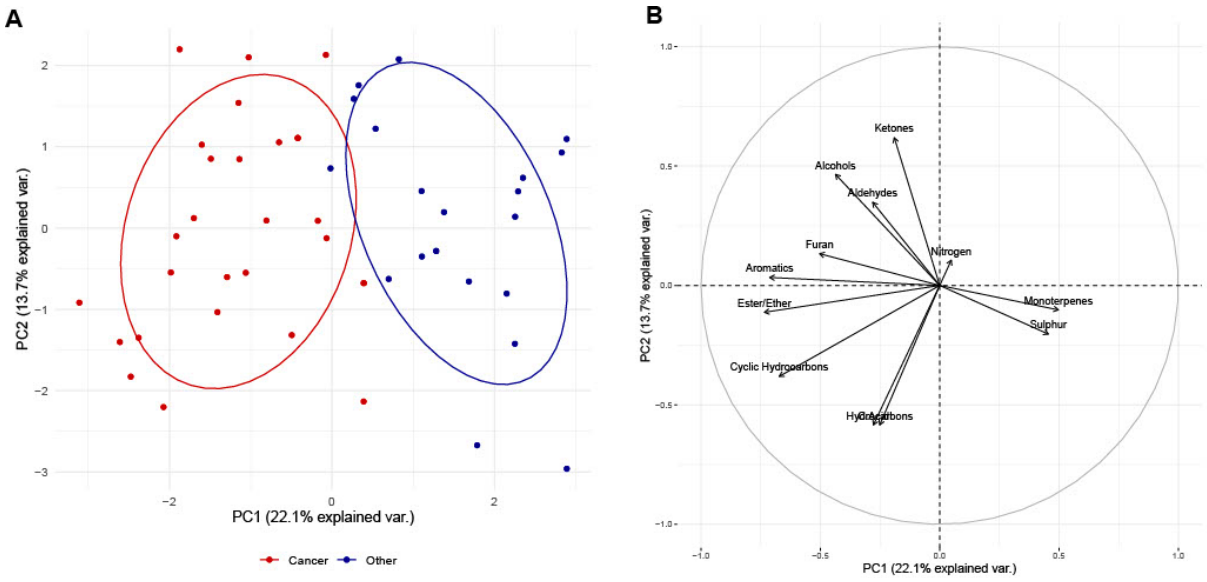
**Supplementary table 4.** Modified QUADAS-2

| | | QUADAS-2 | QUADAS-2 Modified | |
|---|---|---|---|---|
| Risk of bias | Patient selection | Was a consecutive or random sample of patients enrolled? | Were patients sampled representative of population? | Am |
| | | Was a case-control design avoided? | Does the study include positive and negative (healthy) populations for comparison? | Am |
| | | Did the study avoid inappropriate exclusions? | Did the study avoid inappropriate exclusions | Un |
| | Index test | If a threshold was used, was it prespecified? | Validation of results performed? | Am |
| | | Were the index test results interpreted without knowledge of the results of the reference standard? | Was the index test and interpretation of data performed in standardised and reproducible fashion? | Am |
| | Reference standard | Is the reference standard likely to correctly classify the target condition? | Is the reference standard likely to correctly classify the target condition? | Un |
| | | Were the reference standard results interpreted without knowledge of the results of the index test? | - | Om* |
| | Flow and timing | Was there an appropriate interval between index test and reference standard? | Was there an appropriate interval between index test and reference standard? | Un |

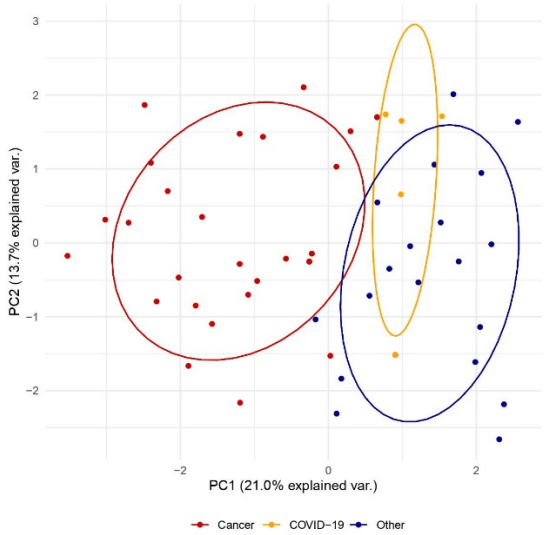
| | | | | |
|---|---|---|---|---|
| | | Did all patients receive the same reference standard? | Did all patients receive the same reference standard? | Un |
| | | Were all patients included in the analysis? | Were all patients included in the analysis? | Un |
| | | | | |
| Applicability concerns | Patient selection | Are there concerns that the included patients and setting do not match the review question? | Are there concerns that the included patients and setting do not match the review question? | Un |
| | Index test | Are there concerns that the index test, its conduct, or interpretation differs from the review question? | Are there concerns around suitable reproducibility and sensitivity of the chosen index test? | Am |
| | Reference standard | Are there concerns that the target condition as defined by the reference standard does not match the question? | Are there concerns that the target condition as defined by the reference standard does not match the question? | Un |

Am = amendment. Om = omitted. Ad = addition. Un = unchanged. *Criteria omitted as not applicable in the case of phase 1 biomarker discovery studies.

REVIEW QUESTION Test population: human subjects Index test(s): VOC analysis within exhaled breath only. Reference standard: the accepted standard for diagnosis of disease in that field. Target condition: cancer/diabetes/Inflammatory bowel disease/liver disease Setting: hospital, medical centre Intended use of the index test: diagnostic Patient presentation: routine investigation for symptoms of malignancy/disease Prior testing: not applicable




**Supplementary Figure 2.** PCA plot of volatiles released by patients, arranged by functional group **(A)** for; cancer (n27) vs other (n38). **B**. Compounds to the left of the image are increased in cancer. All studies shownare non-exclusionary analytical approaches. Ellipses represent 95% CI.




**Supplementary Figure 3.** PCA plot of volatiles released by patients, arranged by functional group for; cancer (n27), COVID-19 (n5) vs other (n38). Ellipses represent 95% CI.

Lung Cancer: (Bajtarevic et al. 2009; Bousamra et al. 2014; Buszewski et al. 2011, 2012; Chen et al. 2005; Corradi et al. 2015; Crohns et al. 2009; Deng, Zhang, and Li 2004; Feinberg et al. 2016; Filipiak et al. 2014; Fu et al. 2014; Fuchs et al. 2010; Gaspar et al. 2009; Handa et al. 2014; Kischkel et al. 2010; Li et al. 2015; M. Ligor et al. 2009; T. Ligor, Pater, and

Buszewski 2015; H. Ma et al. 2014; W. Ma et al. 2015; Peled et al. 2012; Gang Peng et al. 2009; G. Peng et al. 2010; Michael Phillips et al. 2003, 2007, 2008; Poli et al. 2005, 2010; Rudnicka et al. 2011; Sakumura et al. 2017; Schallschmidt et al. 2016; Schumer et al. 2015, 2016; Skeldon et al. 2006; Song et al. 2010; Ulanowska et al. 2011; Y. Wang et al. 2012; Wehinger et al. 2007; Zou et al. 2014; Capuano et al. 2015; M. Phillips et al. 1999; Callol-Sanchez et al. 2017)

Diabetes: (K. Yan et al. 2014; Fan et al. 2014; Turner et al. 2009; Minh et al. 2011; Lee et al. 2009; Greiter et al. 2010; Trefz, Obermeier, et al. 2019; Y. Yan et al. 2014; Saasa et al. 2019; Trefz, Schmidt, et al. 2019; Halbritter et al. 2012; Saidi et al. 2018; Novak et al. 2007; Stevens et al. 2013)

Liver: (Fernández Del Río et al. 2015; Pijls et al. 2016; Eng et al. 2015; Dadamio et al. 2012; Verdam et al. 2013; Shimamoto, Hirata, and Katsu 2000; Solga et al. 2006; Alkhouri et al. 2014; Hanouneh et al. 2014; Morisco et al. 2013; Netzer et al. 2009; O'Hara et al. 2016; Sehnert et al. 2002; Tangerman, Meuwese-Arends, and van Tongeren 1983; Alkhouri et al. 2015; Van den Velde et al. 2008)

IBD: (Hicks et al. 2015; Dryahina et al. 2017; Patel et al. 2014; Bodelier et al. 2015; Monasta et al. 2017; Rieder et al. 2016; Baranska et al. 2016; Kokoszka et al. 1993; Sedghi et al. 1994; Pelli et al. 1999)

Breast Cancer: (Barash et al. 2015; Michael Phillips et al. 2006; G. Peng et al. 2010; Zhang et al. 2020; C. Wang et al. 2014)

Upper gastro-intestinal (UGI) cancers: (Bouza et al. 2017; Chin et al. 2018; Amal et al. 2013; García et al. 2014; Fielding et al. 2020)

Alkhouri, Naim, Frank Cikach, Katharine Eng, Jonathan Moses, Nishaben Patel, Chen Yan, Ibrahim Hanouneh, David Grove, Rocio Lopez, and Raed Dweik. 2014. "Analysis of Breath Volatile Organic Compounds as a Noninvasive Tool to Diagnose Nonalcoholic Fatty Liver Disease in Children." European Journal of Gastroenterology & Hepatology 26 (1): 82–87.

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Buszewski, Bogusław, Agnieszka Ulanowska, Tomasz Kowalkowski, and Krzysztof Cieśliński. 2011. “Investigation of Lung Cancer Biomarkers by Hyphenated Separation Techniques and Chemometrics.” Clinical Chemistry and Laboratory Medicine: CCLM / FESCC 50 (3): 573–81.

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# Chapter 2:

## Sampling and analysis of low molecular weight volatile metabolites in cellular headspace and mouse breath

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## Abstract

Volatile compounds, abundant in breath, can be used to accurately diagnose and monitor a range of medical conditions. This offers a non-invasive, low-cost approach with screening applications; however, uptake of this diagnostic approach has been limited by conflicting published outcomes. Most published reports rely on large scale screening of the public, at single time points and without reference to ambient air. Here, we present a novel approach to volatile sampling from cellular headspace and mouse breath that incorporates multi-time point analysis and ambient air subtraction revealing compound flux as an effective proxy of active metabolism. This approach to investigating breath volatiles offers a new avenue for disease biomarker discovery and diagnosis. Using gas chromatography mass spectrometry (GC/MS), we focus on low molecular weight, metabolic substrate/by-product compounds and demonstrate that this non-invasive technique is sensitive (reproducible at ~1 μg cellular protein, or ~500,000 cells) and capable of precisely determining cell type, status and treatment. Isolated cellular models represent components of larger mammalian systems and we show that stress and pathology-indicative compounds are detectable in mice, supporting further investigation using this methodology as a tool to identify volatile targets in human patients.

## Introduction

Volatile Organic Compounds (VOCs) are small, carbon containing compounds that are found at least partially in the gaseous state at standard temperature and pressure. The human 'volatilome' describes the VOCs that are produced and metabolised within the human body (Amann et al. 2014). These compounds provide valuable insights into metabolic processes and can be detected from the breath, skin, urine, faeces and saliva (Amann et al. 2014; Drabińska et al. 2021), providing an opportunity to diagnose and monitor treatment as well as measure bodily functions.

A large amount of research has been conducted upon human breath with a range of VOCs linked to disease (Issitt et al. 2022). However, In the field of breath and 'smell' diagnostics, more human research (e.g. sampling individually and directly from breath) has thus far been conducted than research that tests volatile outcomes from preclinical, pathogenically representative, cellular models, limiting mechanistic understanding of VOC metabolism. There is a paucity of published research linking cellular processes and VOC metabolisms to identify diagnostically powerful and translatable VOC biomarkers of cellular and disease processes (Issitt et al. 2022). We focus here upon breath as it provides insights into systemic, internal bodily processes via diffusion between the lungs and blood.

Many methodological approaches for breath VOC collection have been described (Blanchet et al. 2017; Lawal et al. 2017; Bruderer et al. 2019; Hanna et al. 2019) and some metabolic processes have been linked to the volatilome, such as reactive oxygen species production of aldehydes and alkanes (Shibamoto 2006; Liu, Li, and Duan 2019; Amann et al. 2014) and microbial function linked to sulphur compounds like dimethyl sulphide (Hanouneh et al. 2014; Issitt et al. 2022). Diagnostic applications of VOCs remain limited in the clinic, in part due to conflicting and confounding results (Issitt et al. 2022).

Useful VOC biomarkers should be descriptive of a condition while overcoming environmental, individual and methodological variabilities. Reported breath VOC variability accrues from individual comorbidities and variations in analytical and collection methods, leading to reduced sensitivity and lack of recognition of potentially useful biomarker compounds. Commonly used methodological approaches also rely on single time point sampling and do not take into account the ambient volatile environment, allowing environmental variability to influence and reduce reported outcome precision, relying instead upon substantial deviations from the norm and reducing the utility of breath volatiles through loss of information. New approaches and perspectives are therefore needed to contextualise the valuable research done so far and to identify robust volatile biomarkers to provide fast, non-invasive, low-cost diagnostics.

Metabolism of VOCs, defined here as flux (reported in grams compound per gram organism weight per time, i.e. g g-1 s-1), considers both release and consumption. Production of compounds can be an expression of metabolic products, for example; acetone release in the breath from alterations in metabolism (Das, Pal, and Mitra 2016) and carbon dioxide release from glucose metabolism. Emissions of VOCs may also be caused by release from saturated tissues, such as muscular release of isoprene in human breath during exercise (Hori et al. 2020). Consumption of VOCs can also be observed through active metabolism, such as with CYP450 enzymes in the human liver (O'Hara et al. 2016) or consumption of oxygen. Quantifying and understanding healthy human metabolism and its impact on VOCs is a developing field and is necessary to define population variability and compound-specific standard ranges in human breath.

Uptake of compounds is not reported as often as release (Issitt et al. 2022) and so volatile 'sinks', the use of VOCs by cells as substrates, may be overlooked, as a result of collection methodology and analytical focus using non targeted gas chromatography mass spectrometry techniques. Non-targeted approaches primarily detect relatively concentrated material (ppbv) whereas targeted approaches are generally capable of quantifying at much lower concentrations (pptv).

In the case of disease, understanding systemic uptake/release is critical in development of biomarkers for clinical application. Disease metabolism outcomes depend upon compound reactivity, transportation time spent within active metabolic regions or saturated tissues, and active metabolic by-products and interactions with the disease pathology (Issitt et al. 2022). Alterations in VOC flux stem from cellular environmental changes which influence metabolic response, either as a result of dysfunction or as the result of normal processes, such as exercise. Identification and separation of these processes in the volatilome is challenging because many cellular processes, dysfunctional or otherwise, produce similar changes in environmental and physiological state. For example, a shift towards glycolysis in cancer (Feinberg et al. 2017; Sreedhar and Zhao 2018) or mitochondrial dysfunction (Issitt et al. 2019), may result in similar global/tissue alterations in pH and reactive oxygen species, producing VOCs associated with this change. Breath volatiles can also be seen to change as a result of normal metabolisms, such as with fasting and eating (Statheropoulos, Agapiou, and Georgiadou 2006; Krilaviciute et al. 2019) or circadian rhythms (Wilkinson et al. 2019). It is therefore important to be able to identify and characterise variation in cellular type, status (disease) and response to environmental stress.

To investigate if volatile metabolism of different cell type and status (disease) can be detected, we quantified volatile signatures (12 discrete compounds via SIM) of cells derived from two

tissues and disease pathologies. We further reveal how environmental and cellular changes elicit detectable alterations in the healthy cell volatilome, through treatment with chemotherapy drug, doxorubicin. These volatile metabolisms, linked to phenotype and pathophysiology, provide potential targets for diagnostic research. We demonstrate how these cellular models are applicable in mammalian analysis through quantification of mice and human breath volatiles, targeting the specific compounds which have shown most promise in these early analyses.

These analyses rely upon a novel, non-invasive volatile sampling method, which allows multi-time point analysis of VOC consumption and production from cellular headspace and can be used in an ethically appropriate manner with mice volatile sampling. In this work we use targeted mass spectrometry, or 'selective ion mode' (SIM), and multiple time points to observe VOC metabolisms.

# Materials and Methods

## Cell Culture and Treatment Conditions

Breast cancer cell lines MDA-MB-231 and MCF7 and kidney-derived cell lines; HEK-293t and RCC4 were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Scientific, Waltham, MA, USA), 25 mM glucose, supplemented with L-glutamine (4 mM) and 5% foetal bovine serum (Thermo Scientific, Waltham, MA, USA). The nontransformed human epithelial mammary cell line MCF10A was grown in DMEM/F12 (Thermo Scien-tific, Waltham, MA, USA) supplemented with 5% FBS, 4 mM L-glutamine (Thermo Scien-tific, Waltham, MA, USA), 20 ng/mL EGF (Sigma-Aldrich, Roche; Mannheim, Germany), 0.5 mg/mL hydrocortisone (Sigma-Aldrich, Burlington, MA, USA), 100 ng/mL cholera toxin (Sigma-Aldrich, Burlington, MA, USA) and 10 µg/mL insulin (Sigma-Aldrich, Bur-lington, MA, USA). All cell culture media was supplemented with 0.1 mM NaI and 1 mM NaBr (to model physiological availability of iodine and bromide). All cells were grown at 37 °C with 5% CO2.

MDA-MB-231 and MCF7 cells were a gift from Dr Mustafa Djamgoz. MCF10A were a gift from Dr. Norman Maitland, while HEK293t were a gift from Dr. Jared Cartwright and RCC4 were a gift from Dr. Dimitris Lagos.

To initiate the volatile collection, the procedure cells were trypsinised, and ~500,000 cells were seeded into 8 mL complete media. Cells were then allowed to attach for 3 h, washed with warm PBS 2× and an 8 ml treatment media was applied. Volatile headspace sampling was performed 24 h later.

Doxorubicin was dissolved in DMSO. Doxorubicin treatment was applied in DMEM 25 mM glucose, supplemented with L-glutamine (4 mM) and 5% FBS for the MDA-MB-231 cells and treatment medium for MCF10A. Appropriate doxorubicin concentration was determined using MTT and SRB assays, which assess metabolic activity and protein concentration as a measure of growth, respectively. Concentrations for doxorubicin treatment were chosen based on no less than 25% reduction in growth of metabolic activity following 24 h of treatment and supporting evidence in the literature of similar concentrations, eliciting senescent and maintaining growth [61, 56, 57]. This was determined by SRB, MTT and trypan blue exclusion assays (Figure S3). An amount of 750 nM was chosen to induce chronic cell stress over this time period while reducing the amount of cell death.

## Headspace and Breath Sampling

### Cellular Headspace Sampling

Following the incubation period (24 h), 5 mL of supernatant medium was removed and plates, with lids removed, were placed into specially constructed chambers (Figure 1B) on a platform rocker on its slowest setting. Medium was equilibrated with lab air by flushing the chamber for 20 min using a Yamitsu air pump with a flow rate of 750 mL per min. Time zero (T0) samples were taken using an evacuated 500 mL electropolished stainless steel canister (LabCommerce, San Jose, USA) through Ascarite® and Drierite® traps (Redeker et al., 2007). The chamber headspace was then isolated by closing the lid valves and the chamber itself was left on the rocker for 120 min, at which point another air sample (T1) was collected. Cells were removed from the chamber, washed with PBS twice and lysed in 500 µl RIPA buffer (NaCl (5 M), 5 mL Tris-HCl (1 M, pH 8.0), 1 mL Nonidet P-40, 5 mL so-dium deoxycholate (10 %), 1 mL SDS (10%)) with protease inhibitor (Sigma-Aldrich, Roche; Mannheim, Germany). Protein concentration of lysates were determined using the Bradford assay [62]. Background (medium only) readings were taken for all medium types and treatments, cell free and DMSO (vehicle), following 24 h incubation at 37 °C and 5% CO2 (Figure S1). DMSO concentration was used equivalent to the highest equivalent dose of doxorubicin; 0.000008%. These readings had no significant differences (determined by ANOVA) and were therefore pooled and the averages subtracted from each individual cell reading.

### Mouse Headspace Sampling

Nine week old female Rag2−/− Il2rg−/− mice were selected for sampling. This mouse strain is an immunocompromised model. Experimental replicates were 2 mice from a cage across 3 separate litters/cages: 6 mice in total. Experiments have been reported inline with the ARRIVE guidelines.

Using tube handling methods, mice were gently placed with a cardboard tube and blue paper into the custom chambers. Flushing the chamber for 10 min using a Yamitsu air pump with a flow rate of 750 mL per min in undisturbed conditions, mice were allowed to acclimatise. T0 samples were then taken, and as with cellular headspace, the chambers were sealed for 20 min and T1 samples were then taken.

### GC-MS, Calibration and Peak Analysis

Collected canister samples were transferred to a liquid nitrogen trap through a pressure differential. Pressure change between beginning and end of “injection” was measured,

allowing calculation of the moles of gas injected. Sample in the trap was then transferred, via heated helium flow, to a Restek© (Bellefonte, PN, USA) PoraBond Q column (25 m length, 0.32 mm ID, 0.5 µm diameter thickness) connected to a quadrupole mass spectrometer (Aglient/HP 5972 MSD, Santa Clara, CA, USA). All samples here were analysed with a select ion mode (SIM) targeting the selected compound's greatest detected mass unit. All samples were run within 6 days of collection. The oven program was as follows: 35 °C for 2 min, 10 °C/min to 115 °C, 1 °C/min to 131 °C and 25 °C min to 250 °C with a 5 min 30 sec hold. The quadrupole, ion source and transfer line temperatures were 280, 280 and 250 °C, respectively.

Calibration was performed using standard gases (BOC Specialty Gases, Woking, UK) and injections of various volumes, equal to different total amounts of compound. Linear regression analyses of calibration curves confirmed strong linear relationships between the observed SIM peak areas and moles of gas injected for each VOC ($r2 > 0.9$ in all cases). For compounds not purchased as speciality gases with ppbv concentration, 1–2 mL of compound in liquid phase was injected into a butyl sealed Wheaton style glass vial (100 mL) and allowed to equilibrate for 1 h. An amount of 1 mL of headspace air was then removed using a gas tight syringe (Trajan, SGE) and injected into the headspace of a second 100 mL butyl sealed Wheaton style glass vial. This was then repeated, and 1 mL of the 2nd serial dilution vial was injected into the GCMS system with 29 mL of lab air. This was performed for methanethiol (MeSH (SPEXorganics, St Neots, UK)), isoprene (Alfa Aesar, Ward Hill, MA, USA), acetone (Sigma-Aldrich, Burlington, MA, USA), 2- & 3-methyl pentane and n-hexane (Thermo Scientific, Waltham, MA, USA).

Nearly all reported compounds detected by the GC-MS were confirmed by matching retention times and mass–charge (m/z) ratios with known standards. This is in addition to a compound with retention time of 27.3, with masses 57 and 43 (M57), which, by relative distribution pattern, was determined, tentatively, to be 2-butanone from the NIST library and the human metabolome database (Wallace 2020).

Concentrations were calculated using peak area. Peak area/moles injected were calculated from previously generated calibration curves. Sample VOC concentrations were then normalised to CFC-11 concentrations (240 parts-per-trillion by volume (ppt)). CFC-11 was used as an internal standard, per sample standard for normalisation as atmospheric concentrations of CFC-11 are globally consistent and stable (K. R. Redeker, Davis, and Kalin 2007).

To account for differences in rates of proliferation (MCF10a cells proliferate at a higher rate than both MCF7 and 231 cells), results from GCMS analysis were normalised to protein content at time of sampling per plate using a Bradford assay (Bradford 1976).

## Molecular Assays

### Sulphorhodamine B Assay

To determine cell growth, SRB assay was performed. The SRB assay measures cell density based on protein content [65]. Following incubation, cell monolayers were fixed with 10% (wt/vol) trichloroacetic acid (TCA) and stained for 30 min, after which the excess dye was removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein bound dye was dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader (Vichai and Kirtikara 2006).

### MTT Assay

MDA-MB-231 and MCF10A cells were seeded onto 96 well plates at a density of 8000 cells per well. Serial dilutions across the plate were performed once the cells had attached to the plate (4 h). Cells were then placed in cell culture incubation conditions. A total of 24 h later, 20 µL of MTT solution was added to each well and incubated for 3 h. Medium was removed, and precipitates solubilised in 100 µL DMSO. Absorbance was then measured at 570 nm using a Clariostar Plus microplate reader (BMG Labtech, Offenburg, Germany).

### Trypan Blue Exclusion Assay

Trypan blue exclusion assay was performed on MDA-MB-231 and MCF10A cells following treatment with DOX or DMSO. Following a published protocol [66], trypsinised cells were mixed with 0.4% Trypan blue solution and counted to determine the number of unstained (viable) and stained (nonviable) cells.

## Data Analysis

Figures were arranged and statistical analyses were performed with GraphPad (Prism). Specific statistical analysis can be seen in figure legends. ANOVA with Bonferroni or Tukey post hoc analysis was performed for each data set to determine statistical significance.

## Ethical Approval

Approval for all animal procedures was granted by the University of York Animal Welfare and Ethical Review Body. All procedures were carried out under authority of a UK Home Office Project Licence and associated Personal Licences.

## Results

### Volatile Flux in Cellular Headspace

The methodological approach is outlined in Figure 1a. Headspace sampling from custom chambers (Figure 1b) from multiple time points allows calculation of cellular volatile fluxes (pg/ug/h−1).

Headspace analysis was conducted for media only and all supplementation (dimethyl sulfoxide (DMSO) and doxorubicin) controls (Figures 1 and S1). No significant variation was observed between Dulbecco's Modified Eagle's Medium (DMEM), DMEM:F12 media (Figure S1E–G) or with the addition of DMSO (Figure S1E–G). Because no variation was observed between DMEM and DMEM:F12 with the DMSO addition, DMSO values represent a combination of DMEM (n = 3) and DMEM:F12 (n = 3) with the DMSO addition.

Headspace above cells had appropriate media controls (average) deducted, demonstrated in Figures 1C and S1A,B. This was then normalised to protein content (Figures 1D and S1C,D) to give the ug of the compound per hour per ug of protein. This is shown for MDA-MB-231 cells, but the media subtraction process was repeated for each cell line and treatment.

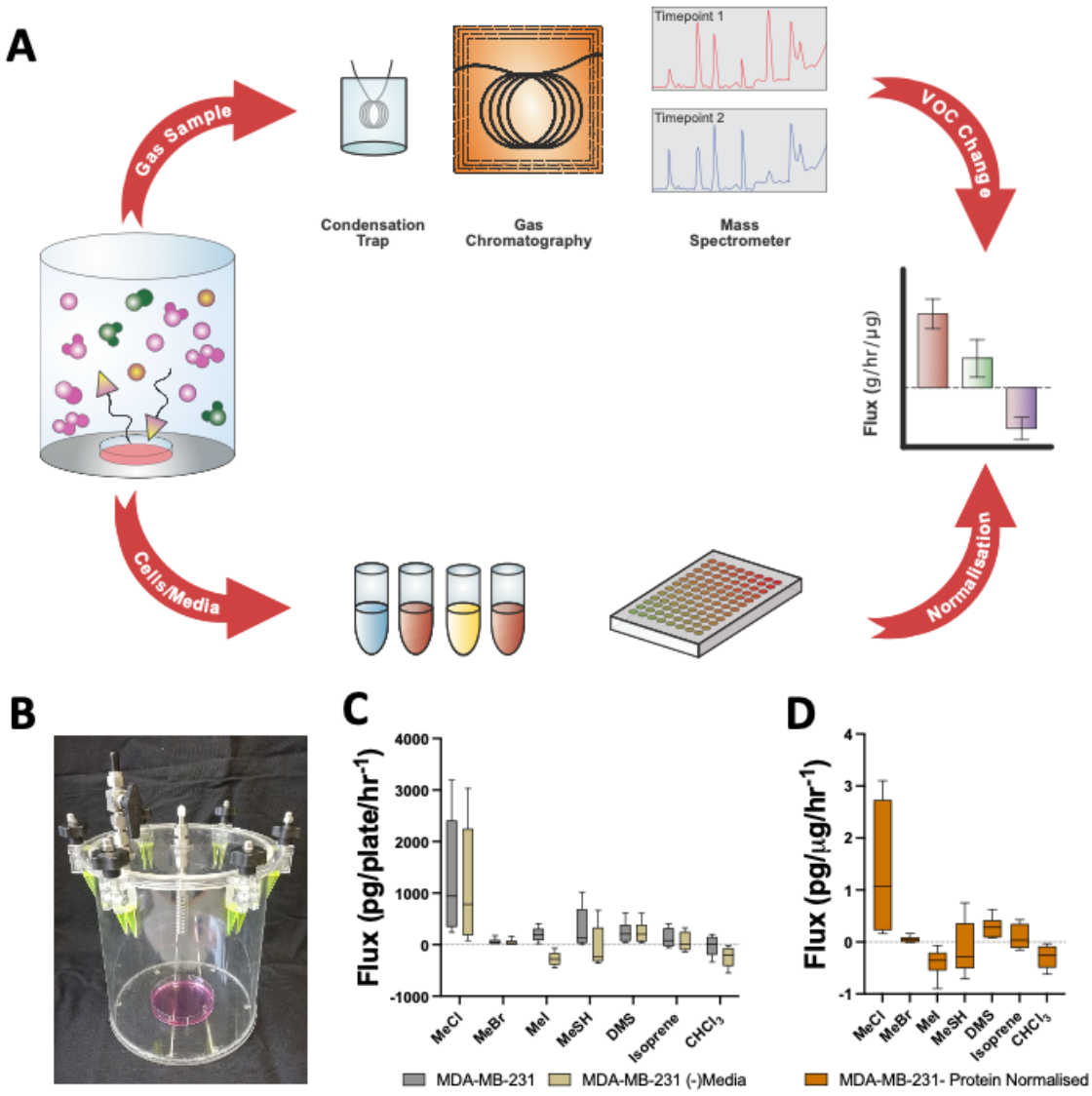


**Figure 1.** Direct volatile sampling of cellular headspace. **(A)** Schematic overview for methodological approach; headspace sampling and generation of VOC flux. **(B)** Image of collection chamber**. (C)** Selected volatile fluxes (g/h/plate) for 10 cm dishes containing DMEM media control only vs. plate containing MDA-MB-231 (mean ± SEM; n = 6). **(D)** Media subtracted and protein normalised VOC flux for MDA-MB-231 cells (mean ± SEM; n = 6). ANOVA followed by Bonferroni post hoc test was performed.

## Volatile Profiles by Cell Type

Comparison of cells growing at basal capacity (i.e., in fully supplemented, optimum media) within a laboratory setting revealed differences in selected volatiles in the headspace. Methyl chloride (MeCl), isoprene and acetone significantly differ between cell lines. Cancer cell lines show consistently higher levels of MeCl and acetone compared to non-cancer cell lines.

## Headspace Volatiles Differ between Breast and Kidney Derived Cells

For noncancer cells (Figure 2A), HEK293t cells show significant uptake of MeCl compared to MCF10A and a significant release of isoprene (Figure 2A). HEK293T cells consumed significantly more acetone than MCF10a, and M57 uptake was also increased (Figure 2A). In contrast, 2-methyl pentane (2-MP) production appears increased in HEK293T cells compared to MCF10A (Figure 2A).

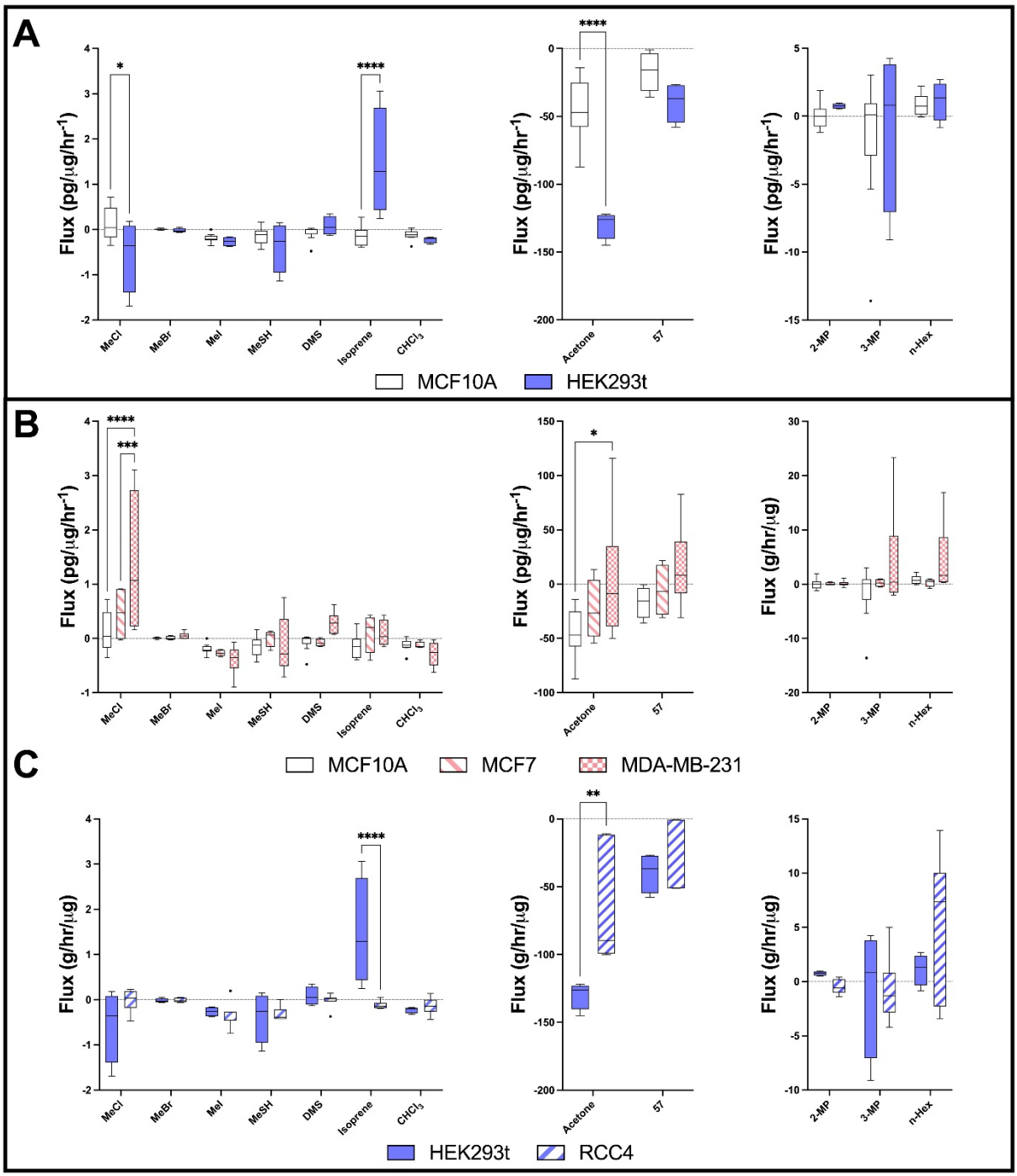


**Figure 2.** Cellular volatile profiles of breast and kidney derived cell lines. **(A)** Volatile flux (g/hr/µg) for noncancerous derived cell lines, from breast; MCF10a and kidney; HEK293t. **(B)** Volatile flux for cancerous breast derived cell lines, MCF7 and MDA-MB-231. **(C)** Volatile flux for cancerous kidney derived cell line RCC4. Media subtracted and protein normalised VOC flux for MCF10a (n = 9); MCF7 (n = 4); MDA-MB-231 cells (n = 6). CHCI3 = Chloroform, DMS = Dimethyl sulphide, MeBr = Methyl bromide, MeCl = Methyl Chloride, MeI = Methyl iodide, MeSH = Methanoethiol. Boxplot whiskers show median ± Tukey distribution. ANOVA followed

by Bonferroni post hoc test was performed; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

## Headspace Volatiles Differ between Cancer and Noncancer Breast Epithelial Cells

When comparing the headspace samples from breast cancer MCF7 and MDA-MB-231 to those of noncancer MCF10A cells derived from breast tissue (Figure 2A–C), MeCl levels were enhanced over MCF7 and were significantly enhanced over MDA-MB-231 cells compared with MCF10A. Methyl bromide (MeBr) and dimethyl sulphide (DMS) levels were increased over MDA-MB-231 cells compared to both MCF7 and MCF10A. MCF7 cells exhibited significantly increased production of isoprene compared to MCF10A, which exhibited isoprene uptake. MDA-MB-231 cells also revealed the production of isoprene rather than consumption. Acetone uptake is reduced in MCF7 cells compared to MCF10A and MDA-MD-231 and show significant changes in the production of acetone; however, the range is large (Figure 2B). (Figure 2E). M57 was increased in MDA-MB-231 cells compared with MCF10A.

## Headspace Volatiles Differ between Cancer and Noncancer Kidney Derived Cells

For cells derived from kidney (Figure 2C), HEK293T cells showed the uptake of MeCl, which is unique when compared to all other untreated cells lines. RCC4 cells showed little production or consumption of MeCl. Isoprene was significantly more concentrated in the headspace of HEK293T cells compared to RCC4, which showed a metabolic uptake. Acetone consumption was significantly reduced in RCC4 cells compared to HEK293t (Figure 2C). RCC4 cells showed some uptake of 2-MP vs. HEK293T production, with increased production of n-hexane vs. HEK293T (Figure 2C).

## Effects of Chemotherapeutic Agent, Doxorubicin, upon Cellular Volatile Profiles

Doxorubicin treatment produced significant alterations in the volatile profile of both MCF10A and MDA-MB-231 cells, as shown in Figure 3. The treatment of MDA-MB-231 with 250 nM and 750 nM revealed consistent trends with increasing concentrations (Figure S2A). For the MDA-MB-231 cells, MeCl switched significantly from production to uptake with increasing concentrations of doxorubicin. Methanethiol (MeSH) also showed increased uptake, while DMS was significantly increased in its release. The uptake of acetone by the MDA-MB-231 cells was observed, but it was nonsignificant. Significant uptake by MDA-MB-231 cells was observed for M57, with no change in MCF10A. Doxorubicin also produced significant

increases in 3-methyl pentane (3-MP) and provoked n-hexane release in MDA-MB-231 cells. MCF10A cell volatiles changed in a similar manner as MDA-MB-231 in response to the doxorubicin treatment. MeCl showed a similar shift to uptake from production, where DMS production was increased, and chloroform ($CHCl_3$) was produced.

MTT assay was performed as an indication of metabolic activity. MCF10A cells show greater metabolic activity than the MDA-MB-231 cells. Treatment with doxorubicin increased the metabolic activity by this assay compared to vehicle (Figure S2A). The sulphorhodamine B (SRB) assay revealed no significant variations for cell growth at 24 h between treatments. At 48 h, the doxorubicin treatment suppressed growth in both cell lines (Figure S3). Trypan blue exclusion revealed a nonsignificant reduction in cell viability at 370 and 740 nM doxorubicin for MDA-MB-231 cells and a similar but significant reduction in cell viability in MCF10a cells exposed to 740 nM doxorubicin (Figure S3).

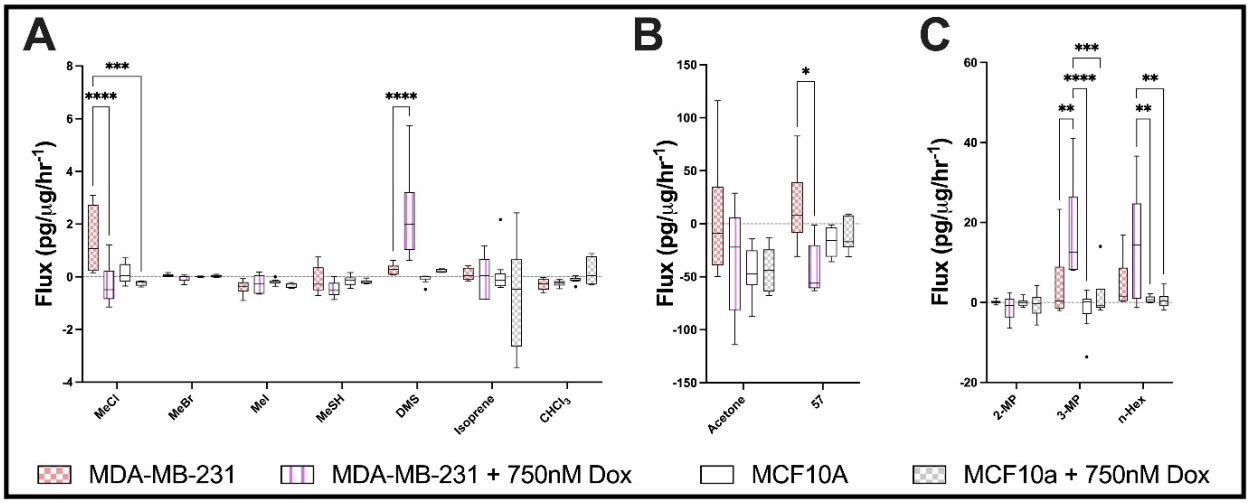


**Figure 3.** Doxorubicin induces volatile response in breast cell lines. **(A-C)** Boxplot for select volatile organic compounds (median ± Tukey distribution; n = 6). ANOVA followed by Tukey post hoc test was performed; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Doxorubicin has been abbreviated to Dox.

## Breath and Faecal Volatiles from Mice

Collection of breath from 9 week old female Rag2−/− Il2rg−/− mice using the sampling chambers (Figure 1B) reveals metabolic interaction with several volatile compounds (Figure 4). Because the mice were allowed to behave normally in the chambers for 20 min following 10 min of acclimatisation, the presence of mouse (in white, Figure 4) is representative of both

mouse breath and faecal volatiles, whereas faecal (in orange, Figure 4) indicates faecal material volatiles only.

Mice show significant positive production of MeCl compared to faecal material, as well as the production of isoprene (Figure 4A). The 3-MP uptake by mice is significant, although the uptake is reduced by the presence of faecal matter (which generally produced 3-MP) (Figure 4C).

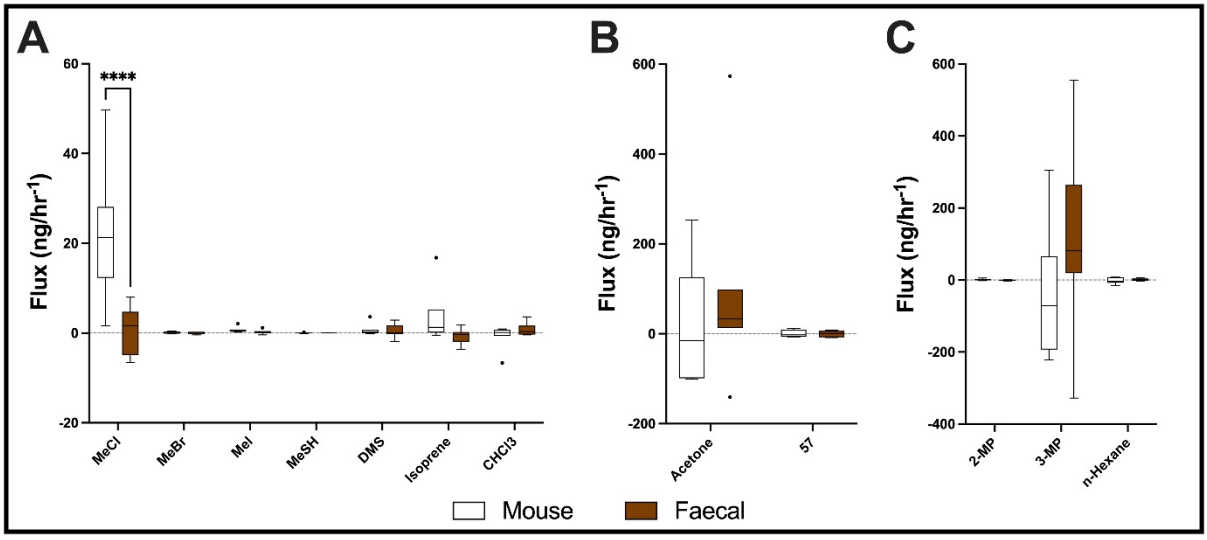


**Figure 4.** Volatile organic compounds from mouse breath and faecal material. **(A-C)** Boxplot for select volatile organic compounds from chambers with single mice vs. chambers with mice removed and faecal material. Flux in g/h (median ± Tukey distribution; n = 6 mice across 3 separate cages). ANOVA followed by Bonferroni hoc test was performed; **** $p < 0.0001$.

## Discussion

This research demonstrates that volatile analysis is capable of separating cellular models by cellular type, disease status and response to chemically induced stress. Furthermore, we have shown that representative, discrete indicator compounds are found in mouse breath and are actively produced or metabolised. A selection of these compounds, including methyl halides have also recently been reported in human breath (Shahi et al. 2022). These outcomes support further research into their potential use as biomarkers of disease.

### Cellular Volatiles and metabolisms

There are limited data on cellular headspace volatile concentrations, and less on volatile metabolites. Headspace volatiles for MCF10A, MCF7 and MDA-MB-231 cells have previously been investigated (Silva et al. 2017; Lavra et al. 2015). HEK293T cells have also had some limited investigation (Li et al. 2016). This is the first time that RCC4 cell headspace volatiles have been reported. In this work we have focused on a novel approach to describing the dynamics of 12 select VOCs, reflective of cellular metabolisms, not discovery of new volatiles using non targeting approaches. This allows greater precision and resolution in assessment of select VOC dynamics, which is well suited to a longitudinal approach.

A further challenge in volatile breath research is the paucity of data regarding metabolic processes and alterations dependent upon compound and/or cellular type/state. For example, while chloroform exposure is well documented and the compound is broken down in the liver by CYP450 enzymes (Constan et al. 1999), its (normal) metabolic consumption and production in mammalian systems has not previously been described.

Likewise, human erythrocytes contain a glutathione-s-transferase isoenzyme that metabolises methyl halides (Redford-Ellis and Gowenlock 1971; Hallier, Deutschmann, et al. 1990) but this is not present in all humans (Peter et al. 1989). Methyl halide metabolism remains unidentified and undescribed in human systems. All plants and fungi measured to date produce methyl halides but the functional reason for this metabolism remains unclear (Manley 2002). A role for active metabolism of methyl halides in mammalian systems is presented in this paper, as we have shown active production and consumption of MeCl, MeBr and MeI in varying situations. Their potential as disease biomarkers however, requires further research.

In our tested cellular systems, metabolism of MeCl is descriptive of cellular type with cancer cells exhibiting increased release relative to their healthy controls. Under treatment of doxorubicin, MeCl uptake is seen in response. Furthermore, this compound can be quantified in the breath of mice and humans. The association of methyl halides with mammalian systems

has been limited, overexposure of MeCl in rats was not linked to DNA adducts where MeI and MeBr have been shown to cause systemic DNA methylation (Bolt and Gansewendt 1992). Long term exposure of MeCl at high concentrations (1000ppm) produced renal tumours in male rats and glutathione depletion (Hallier, Jaeger, et al. 1990).

MeSH and DMS are linked as sulphur containing compounds and are metabolites for each other, with MeSH serving as a precursor to DMS (with a methylating agent) and DMS serving as a precursor to MeSH (with a demethylating agent) (Carrión et al. 2017, 2019). Glutathione (GSH) based metabolism of MeCl can result in formation of MeSH (Arts et al. 2019). Both MeSH and DMS have been linked to bacterial processing (Hanouneh et al. 2014; De Vincentis et al. 2019). HepG2 (hepatocarcinoma cells) and TBE have been shown to produce DMS (Schivo et al. 2014; Mochalski et al. 2013), whereas we have only shown production in MDA-MB-231 cells and in MCF10a and MDA-MB-231 cells following treatment with Doxorubicin. Sulphur containing VOCs have been shown in human breath for a variety of diseases and processes (Issitt et al. 2022). Sulphur is also a dietary requirement (World Health Organisation 1985) which suggests that diet will impact sulfur volatile metabolism, and breath volatile concentrations, in individuals.

Isoprene and isoprenoids, as endogenous biomarkers, have been shown to be linked in patients with muscular dystrophy and are outputs of the mevalonate pathway (King et al. 2012). Monitoring their levels may be important in a variety of diseases, such as cancer, as isoprenoids have been shown to be important compounds in tumour biology (Mo et al. 2019). However, large variability between individuals, as demonstrated here and in a recent review (Issitt et al. 2022) show that this volatile, while the most abundant VOC in human breath, is a challenging biomarker for individual/cohort diagnoses. Longitudinal and metabolic approaches, like those described here may prove able to utilise biomarkers with high variability between individuals but further research is required.

Alkanes have been associated with oxidative stress and reactive oxygen species induced lipid peroxidation, linked to a range of diseases (Calenic et al. 2015). 2- and 3-methyl pentane have been identified as potential markers of cancer (Phillips et al. 2003; Kischkel et al. 2010) as has hexane (Corradi et al. 2015). 2-MP has been shown to be produced by the lung cancer cell line NCI-H2807 (Sponring et al. 2009) whereas we have only shown production by HEK293t cells. 3-MP uptake has been demonstrated in the lung cancer cell line A549 (Schallschmidt, Becker, Jung, et al. 2015; Schallschmidt, Becker, Zwaka, et al. 2015), Alkanes are found in the breath of patients with a range of diseases, but prevalent in cancer (Issitt et al. 2022). Methylated alkanes are also descriptive of oxidative stress in transplant rejection (Amann et al. 2014; Phillips et al. 2004). However, the interplay between methylated

and straight chain alkanes is less understood and so 6 carbon alkanes were targeted here. MCF-7 cells have been shown to release alkanes in response to oxidative stress (Liu, Li, and Duan 2019)which is supported by the release of 3-MP and hexane in response to Doxorubicin

Of the compounds reported here, acetone is one of the most well documented, and has been identified as a volatile compound associated with altered metabolisms and the development of ketosis (Das, Pal, and Mitra 2016). Therefore its dynamics are of interest in models of cancer which show altered energy processing. Uptake of acetone has been shown in the headspace of A549 and TBE cells (Schallschmidt, Becker, Jung, et al. 2015; Schivo et al. 2014) but emissions have been shown by VGP (vertical growth phase melanoma cells) (Kwak et al. 2013) and A549 cells (Filipiak et al. 2010). We have not shown consistent acetone production in any cell lines here but varying levels of consumption across all cells. HEK293t cells consumed the most acetone and cancerous cells showed relatively less consumption against non cancerous cells.

We have shown both novel VOC targets and targets previously identified in cellular headspace and breath. We propose that characterization of volatiles relative to cell type and status will allow utilization of a “breath-print” approach, where multiple volatiles indicative of specific healthy states or pathologies are combined to provide accurate and specific disease indicators. Refinement of target VOCs will increase with further research and we have recommended research frameworks previously (Issitt et al. 2022).

## Mouse Volatiles

Our approach minimises stress in animals, which directly influences the breathing profile (Lim et al. 2014; Noble et al. 2017). This longitudinal approach also allows us to view the compounds which are being metabolised/absorbed by mice and/or their faecal matter. Like humans, these mice show release of MeCl, however it is of note that these mice are immunocompromised and their breath volatiles may differ from standard wild type mice models.

Identified active metabolisms of VOCs in mice provide targets for future disease mouse models and translates well into the breath of humans (Shahi et al. 2022). Here, we show variability over time and individual variability in mouse breath. With further research the expected and average human range for each compound may be understood, to produce standards for medical application. However, individual variability over time supports a longitudinal approach to diagnosis as direct comparisons between individuals may confound results.

## Conclusions

Here, we have shown a new approach to VOC headspace sampling from cells in culture and mice. We present novel compound metabolisms, not observed in cell lines or mice previously, notably, methyl halides and direct, quantified metabolic response due to drug treatment. We have demonstrated quantified fluxes (both consumption and production), in contrast to measurement of presence versus absence (Filipiak et al. 2016; Jia et al. 2019; Issitt et al. 2022).

Using this technique, we can identify cells from different tissues and if cells from that tissue are cancerous or not. Furthermore, the response to cellular stress, from the chemotherapeutic doxorubicin, is clearly defined in the volatile profile of both MDA-MB-231 breast carcinoma cells and non-cancer MCF10A cells. However, the cancer cell line MDA-MB-231 revealed more significant alterations for MeCl, DMS, M57, 3-MP and n-Hexane. This may have implications for monitoring chemotherapeutic treatments.

Our approach to investigating volatiles considers ambient environmental compounds and the processing of those compounds by the body. Ambient compounds which are taken up by cells or the body may be active metabolic substrates or accidentally metabolised, however these reported metabolisms require further investigation. Volatile metabolisms in mammalian systems is an emerging field and the processing of environmentally available VOCs takes into consideration the use of these compounds as potential substrates or chemical interactants.

Using this approach may allow researchers to investigate volatile compounds in a new way for volatile biomarker discovery and diagnostic procedures. The compounds investigated here, including methyl halides present an opportunity to explore metabolisms as they are processed by cells and present in cellular headspace and breath. Methyl chloride is consistently enhanced in mammalian breath and cellular headspace and its significant alterations in response to cellular stress may translate well into breath. Several compounds presented here show similar promise for human diagnosis and further research is required to refine and describe the representative conditions that create specific metabolic outcomes.

## Author Contributions

Conceptualization, T.I., W.J.B. and K.R.; Data curation, T.I.; Formal analysis, T.I.; Funding acquisition, S.T.S., W.J.B. and K.R.; Investigation, T.I.; Methodology, T.I. and K.R.; Project administration, T.I. and S.T.S.; Resources, W.J.B.; Visualization, T.I.; Writing—original draft, T.I.; Writing—review and editing, S.T.S., W.J.B. and K.R. All authors have read and agreed to the published version of the manuscript.

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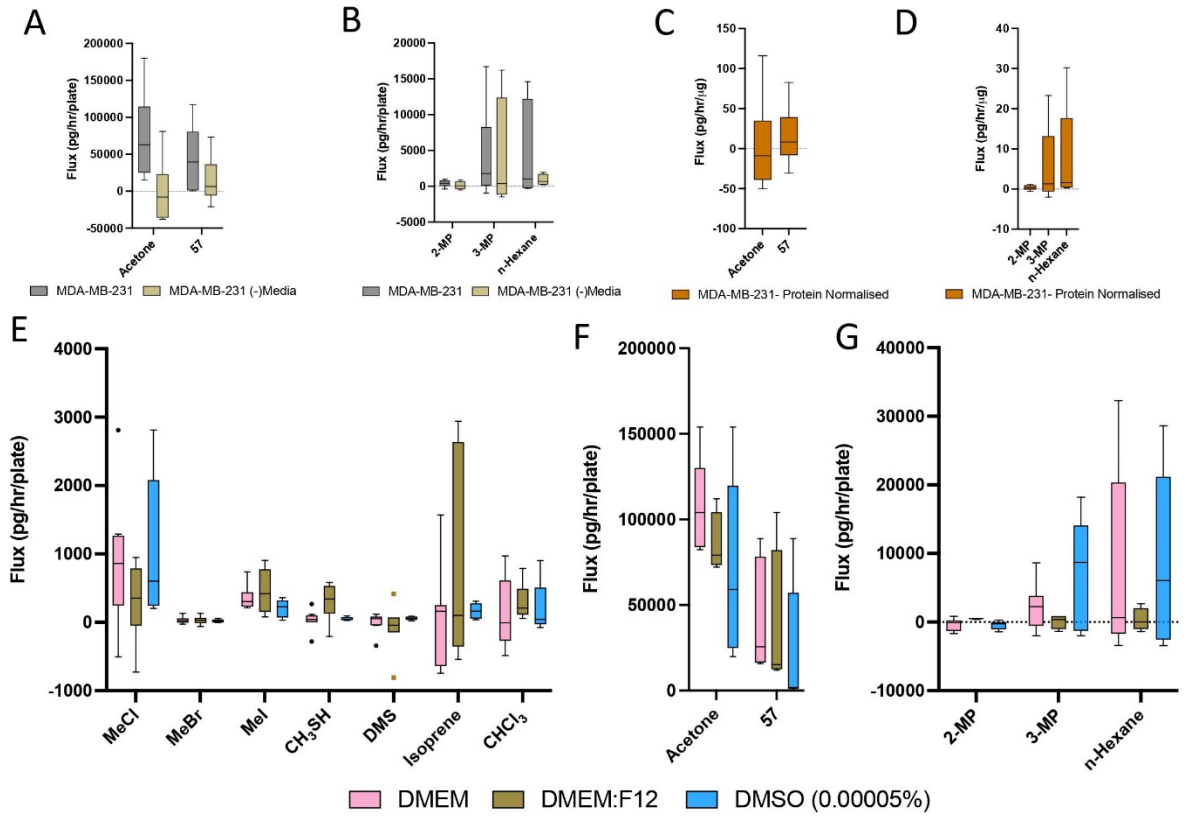
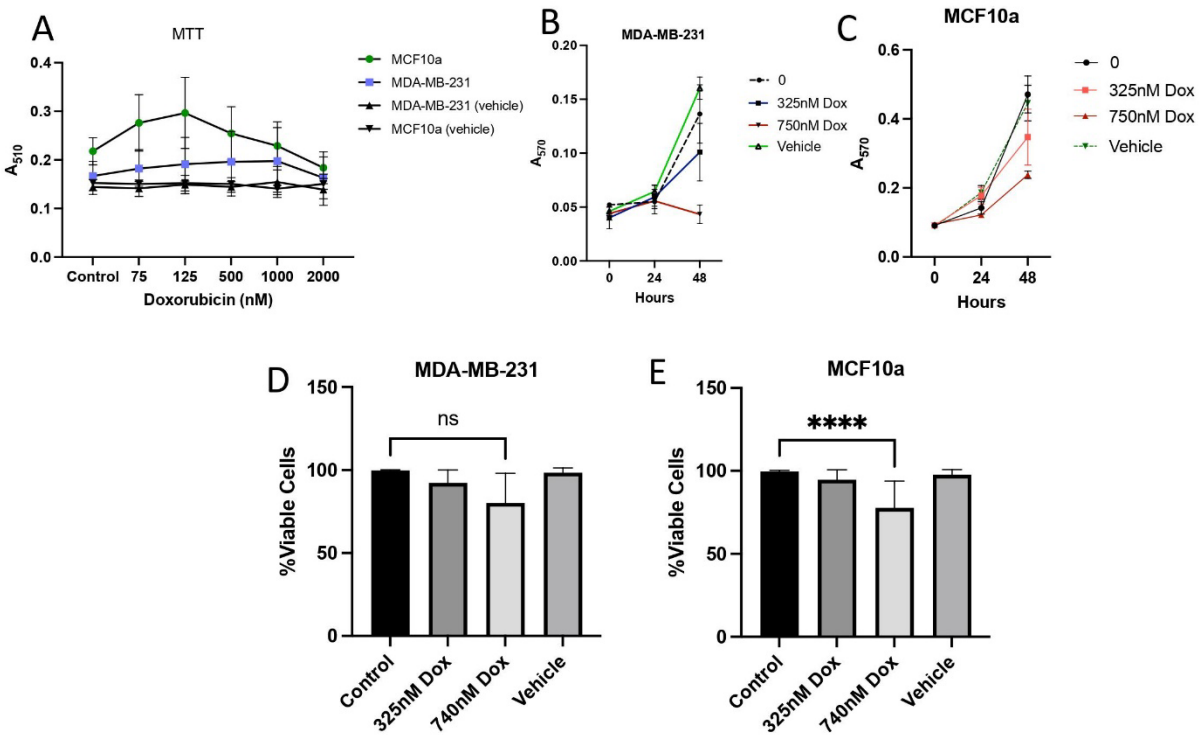
Supplementary Material




**Supplementary figure 1.** Cellular volatiles and media backgrounds. Volatiles from cellular headspace vs cellular headspace with media control deducted **(A, B)**. Media subtracted and protein normalised VOC flux for MCF10a (n = 9); MCF7 (n = 4); MDA-MB-231 cells (n = 6) **(C,D)**. Volatiles released from media alone. DMEM (n = 6), DMEM:F12 (n = 4), DMSO addition (n = 6) **(E, F, G)**. CHCl3 = Chloroform, DMS = Dimethyl sulfide, MeBr = Methyl bromide, MeCl = Methyl Chloride, MeI = methyl iodide, MeSH = Methanoethiol Boxplot whiskers show median ± Tukey distribution. ANOVA followed by Tukey or Bonferroni post hoc test was performed.




**Supplementary figure 2.** Doxorubicin treatment of MDA-MB-231 and MCF10a. **(A)** MTT assay of varying concentrations of doxorubicin for both MDA-MB-231 and MCF10a. Relative levels of vehicle (DMSO) are provided at each stage (mean ± SEM; n = 3). **(B, C)** Sulforhodamine B assay over time for with doxorubicin treatment (DOX) and vehicle (DMSO, 0.00008%) for MDA-MB-231 and MCF10a cells (mean ± SEM; n = 3). **(D, E)** Trypan blue exclusion assays following 24hr doxorubicin treatment and vehicle (DMSO, 0.00008%) for MDA-MB-231 and MCf10a. ANOVA followed by Bonferroni post hoc test was performed; ****p < 0.0001.

# Chapter 3:

## GC/MS analysis of hypoxic volatile metabolic markers in the MDA-MB-231 breast cancer cell line

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## Abstract

Hypoxia in disease describes persistent low oxygen conditions, observed in a range of pathologies, including cancer. In the discovery of biomarkers in biological models, pathophysiological traits present a source of translatable metabolic products for the diagnosis of disease in humans. Part of the metabolome is represented by its volatile, gaseous fraction; the volatilome. Human volatile profiles, such as those found in breath, are able to diagnose disease, however accurate volatile biomarker discovery is required to target reliable biomarkers to develop new diagnostic tools. Using custom chambers to control oxygen levels and facilitate headspace sampling, the MDA-MB-231 breast cancer cell line was exposed to hypoxia (1% oxygen) for 24 hours. The maintenance of hypoxic conditions in the system was successfully validated over this time period. Targeted and untargeted gas chromatography mass spectrometry approaches revealed four significantly altered volatile organic compounds when compared to control cells. Three compounds were actively consumed by cells: methyl chloride, acetone and n-Hexane. Cells under hypoxia also produced significant amounts of styrene. This work presents a novel methodology for identification of volatile metabolisms under controlled gas conditions with novel observations of volatile metabolisms by breast cancer cells.

## Introduction

The human 'volatilome' describes the production and metabolism by the human body of small, carbon-containing compounds called volatile organic compounds (VOCs) which are gaseous at room temperature and pressure (Amann et al. 2014; Drabinska et al. 2021). VOCs can be found in abundance in the breath and are reflective of processes within the body (Drabinska et al. 2021; Issitt et al. 2022b). Although fluctuations of VOCs vary between individuals and throughout the day, disease specific 'volatile fluxes', or biomarkers, could provide opportunities to non-invasively diagnose disease, monitor treatment and measure bodily functions (Issitt et al. 2022a; Issitt et al. 2022b).

The clinical potential of VOCs in diagnosis has been shown by a number of published breath studies (Issitt et al. 2022b). Diagnostic accuracy using breath VOC biomarkers has been achieved for a wide range of conditions, including various types of cancer (Jia et al. 2019; Issitt et al. 2022b), liver disease (De Vincentis et al. 2019), diabetes (Das et al. 2016), transplant rejection (Phillips et al. 2004), infections of the lung (Beccaria et al. 2018; Issitt et al. 2022b), liver function (using labelled VOCs) (Sangnes et al. 2019) and other conditions (Issitt et al. 2022b). Each study may independently achieve high sensitivity of disease detection (i.e. > 90%) but the reported compounds often do not translate between studies, slowing clinical application through conflicting and confounding results (Issitt et al. 2022b). However, our recent meta-analysis has shown underlying trends in chemical functional groups from published studies supporting potential clinical application (Issitt et al. 2022b). It is clear that in order to identify effective biomarkers more targeted methodological approaches are required to overcome variability (Hanna et al. 2019; Issitt et al. 2022b).

VOC profiles from cell types associated with pathological conditions have been identified, for example, differences between breast (Lavra et al. 2015; Issitt et al. 2022a), liver (Mochalski et al. 2013) and mesothelioma (Little et al. 2020) cancer cell lines. However, cellular VOC studies tend to be non-stressed cells in high (21%, atmospheric) oxygen conditions, which is not consistent with many disease or normal physiological states. To accelerate biomarker discovery, we propose models of pathophysiological stress. For example; stress from reactive oxygen species (ROS) induces alkane release in breast cancer cells (Liu et al. 2019), VOCs which have been observed in the breath of ROS associated conditions (Issitt et al. 2022b).

Hypoxia is a persistent reduction in oxygen from normal physiological conditions (normoxia). It is characteristic of a range of diseases, including, pulmonary hypertension (Young et al. 2019) and cancer (Samanta and Semenza 2018). It induces a range of metabolic alterations, including reduction in adenosine triphosphate generation and inhibition of fatty-acid desaturation through hypoxia inducible factor activity (Wheaton and Chandel 2011; Samanta

and Semenza 2018; Young et al. 2019), which can produce alterations in a range of associated breath volatiles (Harshman et al. 2015; Mazzatenta et al. 2021). Despite its relevance to pathophysiology, hypoxic volatiles have yet to be investigated *in vitro*. This is partially due to the challenges associated with development of a headspace sampling tool which can maintain an hypoxic environment. While volatile compounds in the available, limited, published studies associated with hypoxia show variation in breath (Harshman et al. 2015; Mazzatenta et al. 2021), translatable studies are required for target biomarker discovery.

Biomarker discovery in appropriate biological models can accelerate clinical delivery by identifying and allowing targeted analytical approaches, separating methodical challenges from pathology, and improving sensitivity. Multi-timepoint sampling and approaches considering local environment will also accelerate clinical application of breath diagnostics and consideration of methodological challenges around clinical application should drive experimental design. We have previously demonstrated a platform and method for both identification of VOC metabolisms in cellular headspace over time and VOC changes in response to cellular stress (Issitt et al. 2022a). However, models of pathological conditions require further investigation to ensure biomarker discovery is translatable from cell to human.

One of the primary sources of variance within the published literature revolves around methodology. Methods of breath VOC analysis can be split into 3 main sections where variability between studies can arise: initial collection, sample transfer and analytical approach. There are many effective breath collection methods for analysis of VOCs, such as simply breathing into a specialised bag or use of specialised technologies (Hanna et al. 2019; Di Gilio et al. 2020). Many studies use single time point collection (Issitt et al. 2022b), considering presence verses absence, which can miss valuable metabolic information, particularly volatile uptake, driven via chemical reactions reflective of cellular state or through cellular metabolism. Furthermore, variability in local environment influences and reduces reported outcome precision (Doran et al. 2017; Di Gilio et al. 2020; Issitt et al. 2022b) and approaches should consider sampling the environment (i.e. ambient air) along with breath (Hanna et al. 2019). A sample, once collected, is then transferred, either directly or indirectly (such as through chemical traps) to an analytical instrument. There are two main analytical approaches for discovery and accurate detection of VOCs: targeted and untargeted. Untargeted approaches, investigating the breath of patients, are capable of identifying relatively concentrated material (ppbv) whereas targeted approaches generally are capable of quantifying lower concentrations (pptv). Untargeted approaches therefore may miss changes in important, low concentration compounds, while targeted approaches can only look only for a limited number of known compounds of interest, reducing discovery potential.

Here, hypoxic stress is applied to a well studied breast cancer cell line with the intent of identifying process and disease linked physiological volatile metabolisms specifically linked to low oxygen conditions, so that more accurate diagnostic tools can be developed and applied in the clinic. Both targeted and untargeted analyses are applied after sampling with a static headspace method that accounts for the ambient air background and allows quantification of cellular uptake of VOCs. It was predicted that upon successful maintenance of a hypoxic environment, cellular VOC profiles from hypoxic versus hyperoxic cellular models would alter significantly.

## Methods

Methods for culture of MDA-MB-231 cells, headspace sampling from custom chambers and GC/MS analysis have been previously described in detail (Issitt et al. 2022a).

### Cell culture

MDA-MB-231 breast cancer cells (a gift from Professor Mustafa Djamgoz, Imperial College London) were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Scientific, Waltham, MA, USA), 25 mM glucose, supplemented with L-glutamine (4 mM) and 5 % foetal bovine serum (Thermo Scientific, Waltham, MA, USA). Cell culture medium was supplemented with 0.1 mM NaI and 1 mM NaBr (to model physiological availability of iodine and bromide). All cells were grown at 37 °C with 5 % $CO_2$.

Prior to volatile collection, cells were trypsinised, and 500,000 cells were seeded into 8 mL complete media in 10 cm polystyrene cell culture dishes. Cells were then allowed to attach for 3-4 h, washed with warm PBS and 6 mL treatment media was applied. Volatile headspace sampling was performed 24 h later.

### Induction of the hypoxic environment and VOC headspace sampling

Cells were placed in static headspace chambers as previously described (Issitt et al. 2022a) with new, clean silicon gaskets. Low oxygen, hypoxic gas (1 % $O_2$, 5 % $CO_2$, 94 % $N_2$; purchased from BOC Specialty Gases, Woking, UK) was flushed through the chambers at a rate of 4 L/min for 10 min (chamber volume = 25 L). Chambers were then closed and placed at 37˚C for 2 hours to allow residual oxygen in the media to equilibrate with chamber headspace. Chambers were then flushed again at a rate of 4 L/min for 10 min, sealed and returned to 37˚C.

After a further 24 hours, chambers were flushed again at a rate of 4 L/min for 10 min. 15 ml of gas standards (MeCl, 520 ppb (parts per billion); MeBr, 22 ppb; MeI, 26 ppb; DMS, 110 ppb; CFC-11, 400 ppb and $CHCl_3$, 110 ppb; BOC Specialty Gases, Woking, UK) were then injected into the chambers through a butyl seal and time zero sample taken. Injected compounds are either known metabolites for cancer cells, or internal standards (CFC-11) for the analysis and quantification of metabolism. Final chamber concentrations were similar to environmental concentrations, e.g MeCl, 1.2 ppb and MeBr 0.05 ppb, particularly more polluted urban spaces (Redeker et al. 2007). Injected gases are the same as those used for calibration. Compounds not injected but detected at first time point, due to residual presence from laboratory air,

(including isoprene, acetone, 2-MP, 3-MP and n-hexane) were quantified. Two time zero (T0) samples were taken using an evacuated 500 mL electropolished stainless steel canister (LabCommerce, San Jose, USA) through fine mesh Ascarite® traps (Archbold et al. 2005), after which the chamber was resealed and left on a platform rocker on its slowest setting for 120 min, at which point two further air samples (T1) were collected. Duplicate samples were analysed with targeted and untargeted MS approaches.

Cells were removed from the chamber, washed with PBS twice and lysed in 500 µL RIPA buffer (NaCl, 5 M; 5 mL Tris-HCl, 1 M, pH 8.0; 1 mL Nonidet P-40; 5 mL sodium deoxycholate, 10 %; 1 mL SDS, 10 %) with protease inhibitor (Sigma-Aldrich, Roche; Mannheim, Germany). Protein concentration of lysates were determined using BCA assay (Thermo Scientific, Waltham, MA, USA).

Media alone was taken through exactly the same process as cells. This has been visualised in supplementary Figure 1. Only acetone was shown to have any significant variability between conditions. These media blank outcome averages were subtracted from respective cellular samples prior to protein normalisation. Comparative controls include lab air blanks and those data available from the dataset and collection method published previously which created and quantified metabolic fluxes of volatile compounds from MDA-MB-231 under hyperoxic (lab air) conditions (Issitt et al. 2022a).

## Sample collection and GC/HID analysis

Ten mL headspace samples were taken from chambers using an airtight syringe (10 mL, SGE, Trajan, Milton Keynes, UK). 1 % $O_2$ (BOC Specialty Gases, Woking, UK) was flushed through sealed chambers containing 6 ml DMEM as described for cell treatments. Samples were taken at 5 and then 10 min post initial flush. In order to replicate cell treatments, the chamber was then closed for 2 hours, then flushed for 10 min, after which an air sample was taken. A further 20 min flush with 1 % $O_2$ air was employed and the chamber was closed, placed at 37 ˚C, and left to incubate for 24 hours, at which time the final sample was taken.

Air samples were immediately analyzed with a SRI 8610C Gas Chromatograph connected to a SRI 8690-0030 Helium Ionisation Detector (GC/HID (SRI Instruments Europe GmbH, Torrance, CA, USA). Peak separation was achieved using a Restek© PORAPAK Q porous polymer column (1.83 m x 2.1 mm ID x 3.175 mm OD), a solenoid switching valve (for backflushing CO2) and a Restek© MOLECULAR 5A sieve column (0.91 m x 2.1 mm ID x 3.175 OD) (Restek©, Bellefonte, PN, USA) connected in series. Helium was used as a carrier gas at 18 psi, and the flow rate and column temperatures (50 °C) were maintained during

separation. The valve was switched at 1.5 min to backflush the PORAPAK Q column. Measurement of compounds eluted from the MOLECULAR 5A sieve was achieved by using an SRI 8690-0030 Helium Ionisation Detector. SRI PeakSimple (version 453) software was used to generate a digital chromatograph for each sample and $O_2$ was quantified by comparing the peak area to known standards.

The standard curve was developed by flushing 120 mL Wheaton vials with butyl stoppers with pure nitrogen (BOC Gases, Woking, UK) for 30 mins. Ten mL of nitrogen only was injected to establish a background control. Because atmospheric air at sea level contains 21 % $O_2$, lab air was injected at 1 %, 2 %, 10 %, 20 % and 30 % within the $N_2$ filled vial to generate a standard curve consisting of 0 %, 0.21 %, 0.42 %, 2.1 %, 4.2 % and 6.3 % and 21 % (lab air only). Peak areas were integrated using Graphpad (Prism), and Padé (1,1). Linear regression demonstrated an R squared value of 0.96.

## GC/MS analysis of VOCs

Collected canister samples were transferred to a liquid nitrogen trap through pressure differential. Pressure change between beginning and end of “injection” was measured, allowing calculation of the moles of canister collected air injected  Sample in the trap was then transferred, via heated helium flow, to an Aglient/HP 5972 MSD system (Santa Clara, CA, USA) equipped with a PoraBond Q column (25 m x 0.32 mm x 0.5 µm film thickness) (Restek©, Bellefonte, PN, USA). Targeted samples were analyzed in selected ion monitoring (SIM) mode, and untargeted samples in full scan (SCAN) mode with the mass range of 45-200 amu. The mass spectrometer was operated in electron impact ionization mode with 70 eV ionization energy, and transfer line, ion source, and quadrupole temperatures of 250, 280 and 280, respectively. For details on SIM and significantly altered, identified SCAN compounds, see Table 1. All samples were analysed within 6 days of collection. The oven program for both SIM and SCAN analyses were identical and are as follows: 35 ˚C for 2 min, 10 ˚C/min to 155 ˚C, 1 ˚C/min to 131 ˚C and 25 ˚C/min to 250 with a 5 min 30 second hold.

| Compound | Retention time (min) | Mass charge ratio (m/z) |
|---|---|---|
| SIM | | |
| Methyl chloride (MeCl) | 7.6-7.9 | 50, 52 |
| Methyl bromide (MeBr) | 10.3-10.4 | 94,96 |
| Trichloroflouromethane (CFC-11) | 15.0-15.3 | 101,103 |
| Methyl iodide (MeI) | 15.4-15.7 | 127,142 |
| Dimethyl Sulfide (DMS) | 16.2-16.5 | 62 |
| Acetone | 18.2-18.4 | 58 |
| Isoprene | 18.4-18.6 | Total ion count |
| Trichloromethane ($CHCl_3$) | 25.4-25.7 | 83,85 |
| 2-Methyl pentane (2-MP) | 27.6-27.8 | 43, 57 |
| 3-Methyl pentane (3-MP) | 28.0-28.2 | 43, 57 |
| n-Hexane (n-Hex) | 28.5-28.7 | 43, 57 |
| SCAN | | |
| Styrene | 33.3-33.5 | 45-200 amu |

**Table 1.** Retention times, mass charge ratios and GC/MS modes used to characterise individual VOCs. SIM and SCAN refer to selected ion monitoring and full mass scanning (targeted and untargeted) GC/MS modes.

Calibration was performed using standard gases (BOC Specialty Gases, Woking, UK). Linear regression of calibration curves confirmed strong, positive linear relationships between observed compound peak areas and moles of gas injected for each VOC ($r^2 > 0.9$ in all cases). For compounds not purchased in gaseous state (BOC Specialty gases, as above), 1–2 mL of compound in liquid phase was injected neat into butyl sealed Wheaton style glass vials (100 mL) and allowed to equilibrate for 1 h. One mL of headspace air was then removed from neat vial headspace using a gas tight syringe (Trajan, SGE) and injected into the headspace of a second 100 mL butyl sealed Wheaton style glass vial. This was then repeated, and 1 mL of the 2nd serial dilution vial was injected into the GC/MS system with 29 mL of lab air to give ppb concentrations. This was performed for methanethiol (MeSH, SPEXorganics, St Neots, UK), isoprene (Alfa Aesar, Ward Hill, MA, USA), acetone (Sigma-Aldrich, Burlington, MA, USA), 2- & 3-methyl pentane and n-hexane (Thermo Scientific, Waltham, MA, USA). Reported compounds detected by the GC/MS were confirmed by matching retention times and mass–charge (*m*/*z*) ratios with known standards.

$$Equation\ 1\text{: } [VOC](ppt) = \frac{CF\ x\ 10^{12}\ x\ Peak\ area\ x\ Calibration\ slope}{n}$$

Equation 1 outlines the approach to calculating VOC concentrations in parts-per-trillion-by-volume, or pptv. Here *Peak area* refers to the combined peak areas for the mass-charge ratios identified in Table 1. Multiplying *Peak areas* by their associated calibration curves (*Calibration Slope*) generate molar amounts which, when divided by the number of moles of headspace air injected (*n*), generate a unitless (moles compound/moles of air) ratio. Pptv concentrations are then obtained by multiplying this unitless ratio by $1x10^{12}$. For clarity, part-per-billion-by-volume values would be obtained by multiplying the unitless ratios by $1x10^{9}$, or one billion. Sample VOC concentrations were then normalised to CFC-11 concentrations (240 parts-per-trillion-by-volume (pptv)) through multiplication by a "correction factor", or *CF*, Equation 1). CFC-11 was used as an internal standard, since atmospheric concentrations of CFC-11 are globally consistent and stable (Redeker et al. 2007). Quantification of Styrene was done as above but normalisation to CFC-11 was not possible under flushed, hypoxic conditions.

To account for differences in rates of cellular proliferation over 24 hours, cellular results from GC/MS analyses were normalised to protein content at time of sampling using a BCA assay. When comparing media blanks to cellular assays results are reported in grams compound per petri dish per hour.

Data has been made publicly available at the National Institute of Health Metabolomics workbench (project PR001638, DOI: http://dx.doi.org/10.21228/M8ZX4D) (Sud et al. 2016).

## Hydrogen peroxide (Amplex red) assay

Experiments were performed in phenol red free DMEM. DMEM containing 50 µM Amplex Red reagent (Thermo Scientific, Waltham, MA, USA ) and 0.1 U/mL horse radish peroxidase (HRP, Thermo Scientific, Waltham, MA, USA) was added to cells in 12 well dishes (500 µL per well) for 15 min following 24 hours in hypoxic or control conditions. Fluorescence at 590 nm was measured with a plate reader (Clariostar, BMG, Ortenberg, Germany) and compared against a $H_2O_2$ standard curve for quantification.

## Statistics

Figures were assembled and statically analysed in Graphpad Prism version 9.3. VOCs were separated based on their flux amount to allow visualisation on the y-axis and were analysed this way. Two-way ANOVA with Bonferroni post-hoc analysis was performed for graphs with multiple factors was performed (Figure 2A and B, Supplementary Figure 1A and B). One way

ANOVA with Tukey post hoc analysis was performed for acetone analysis (Figure 2B and supplementary Figure 1B). Student's T-test was performed for Styrene analysis against media only as none was detected for control cells, and these were presented on the graph for visual information. Amplex red data was analysed using Student's T-test.

# Results

## Chambers maintain low oxygen conditions over 24 hours

To confirm chambers maintained hypoxic conditions over 24 hours we sampled gas from chambers throughout our method, measuring $O_2$. When flushed with reduced oxygen air (1%) for 5 minutes, oxygen levels rapidly fell from atmospheric 21% to between 6% and 2% (Figure 1). After 10 min of reduced oxygen flushing, each chamber held less than 5%. Chambers left for 2 hours (120 mins) to allow media to equilibrate and flushed for 10 min revealed average $O_2$ levels of 1.15% ± 1.03 (Ch 1), 1.34% ± 0.93 (Ch 2) and 1.98% ± 4.07 (Ch 3) respectively. Sealed chambers maintained low oxygen levels over 24 hours with average $O_2$ levels of 1.31% ± 1.31 (Ch 1), 1.76% ± 1.02 (Ch 2) and 1.96% ± 0.28 (Ch 3) respectively.

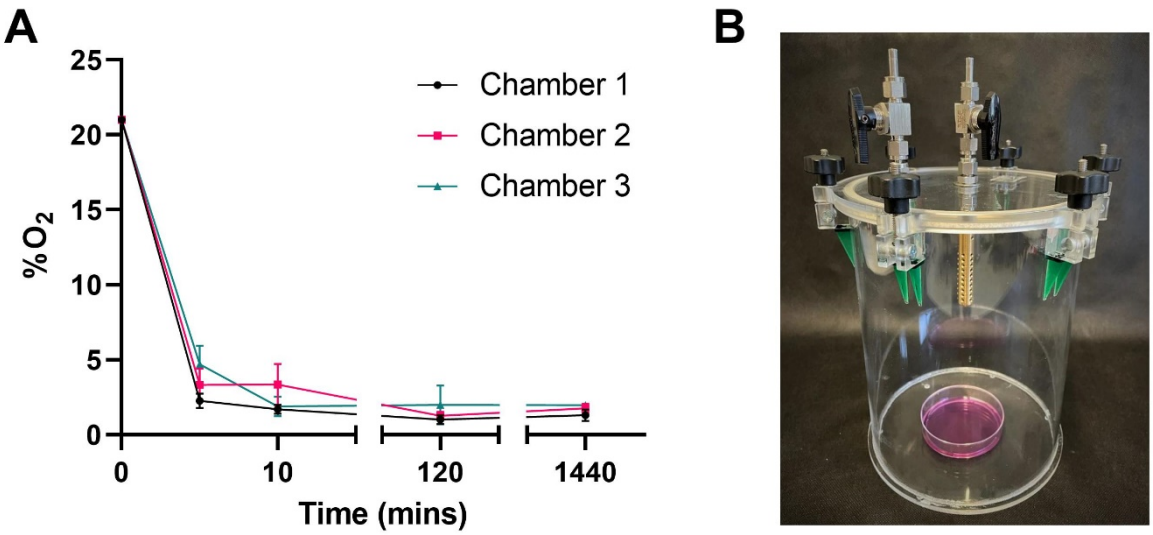


**Figure 1.** Chambers maintain hypoxic conditions over 24 hours. **(A)** Oxygen ($O_2$) content in 3 custom made chambers containing 6ml media was measured following a 10 min flush, 2 hour dwell and another 10 min flush (20 mins) with 1% $O_2$, 5% $CO_2$ gas mix. $O_2$% was then measured following chambers being sealed for 1440 mins (24 hours). Mean ± SEM; n=3. **(B)** Image of collection chamber.

## Hypoxia induces differing volatile fluxes in breast cancer cell line MDA-MB-231

Persistent hypoxia over 24 hours induced significant changes in flux for 3 targeted compounds (SIM analysis); MeCl, acetone and n-hexane (but not hexane isomers; 2-methyl pentane, or 3-methyl pentane), when compared to control (Figure 2A-C). MeCl was taken up by cells under hypoxia and released by cells under hyperoxic cell culture conditions. n-Hexane was produced by hyperoxic control cells while those under hypoxia consumed hexane.

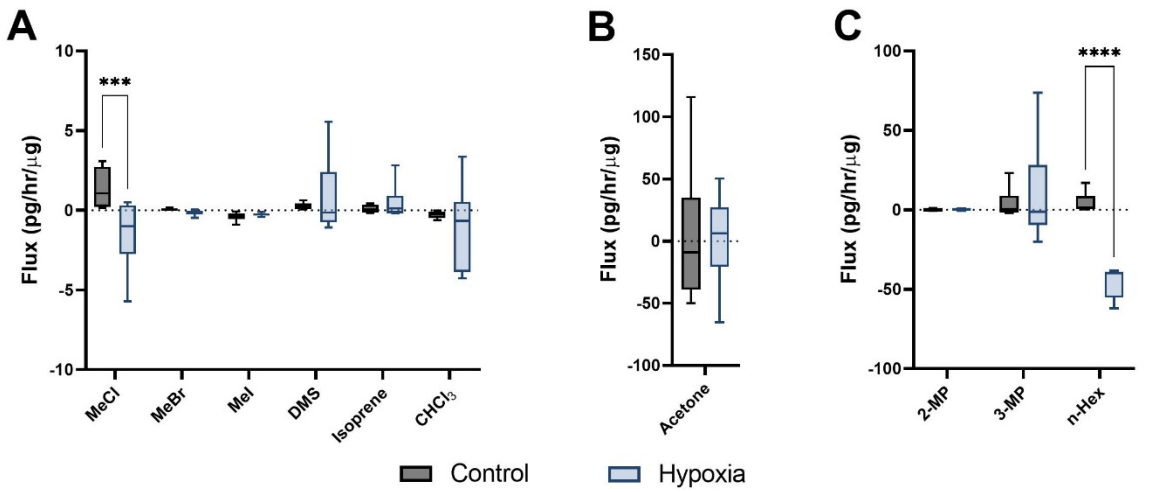


**FIGURE 2.** Cellular volatile response to hypoxia. Volatile flux (pg/hr/µg) for MDA-MB-231 cells in control conditions or hypoxia (24 h). Media subtracted and protein normalised VOC flux for MDA-MB-231 control cells (n = 6) and cells in hypoxia (n = 6). CHCl3, chloroform; OMS, dimethyl sulfide; MeBr, methyl bromide; MeCl, methyl chloride; MeI, methyl iodide; MeSH, methanoethiol; 2-MP, 2 methyl pentane; 3-MP, 3 methyl pentane; n-Hex, n-hexane. Boxplot whiskers show median ± Tukey distribution, n = 6. Two way ANOVA followed by Bonferroni post-hoc test was performed for **(A,B)**. One way ANOVA with Tukey post-hoc test performed for B; ***p < 0.001; ****p < 0.0001.

## Production of Styrene under hypoxic conditions

Cells maintained under hypoxic conditions significantly produced styrene as determined by untargeted GG/MS approaches (Figure 3A). Styrene was not found in the headspace of control cells (ND, or not detected) and styrene fluxes in media blanks were not significantly different from zero, while fluxes from hypoxic cells were significantly different from media blanks. Styrene was identified through spectral matching, followed by known standard injections. No other compounds were found to be significantly altered using the untargeted SCAN method.

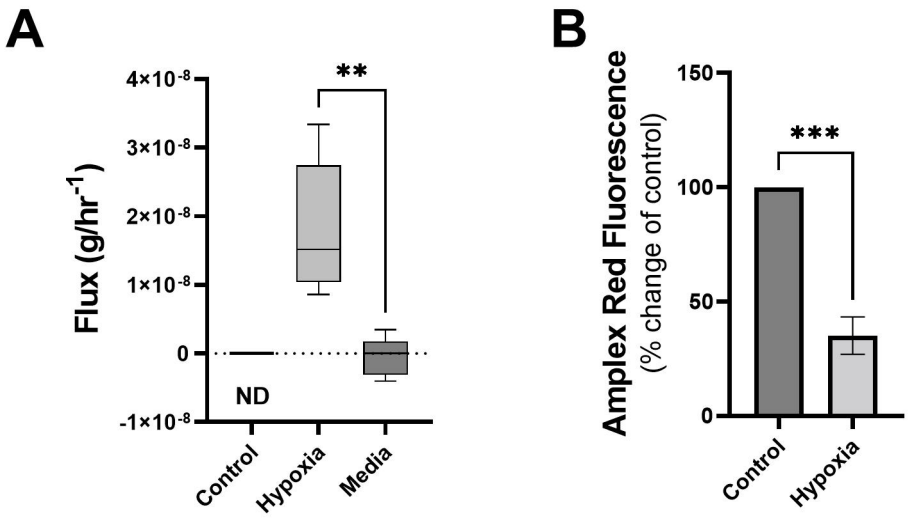


**FIGURE 3.** Cells under hypoxic conditions produce styrene and exhibit reduced ROS. Volatile flux (g/hr−1) for styrene from MDA-MB-231 cells in control conditions or hypoxia and media only (24 h). Non Detected (ND) for control cells. Amplex Red assay was performed following 24 h incubation as a measure of reactive oxygen species (ROS), H2O2. Shown as percentage change from relative control. Boxplot whiskers show median ± Tukey distribution, A; n = 6. Student's T-test was performed for **(A,B)**, ***p < 0.001.

## Reactive oxygen species are reduced under hypoxia

Changes in volatiles, including alkanes, have been linked to increases in ROS (Calenic et al. 2015). The observed uptake of n-Hexane in hypoxic MDA-MB-231 cells could therefore be correlated with alterations in ROS levels in these cells. Following 24 hours exposure to hypoxic conditions, ROS, as determined by Amplex Red assay, showed significant reduction compared to control (Figure 3B).

## Discussion

Static headspace sampling chamber was demonstrated to be capable of maintaining a low oxygen environment for >24 hours, as evidenced by chamber concentrations and cellular ROS response. Furthermore, VOCs from cells maintained under low oxygen conditions can be sampled, and that these cells produce a significantly different volatile profile than either media blanks or identical cells exposed to hyperoxic conditions.

Two out of 10 compounds targeted by SIM revealed quantifiable, differential metabolic responses in cells exposed to hypoxic conditions (1% $O_2$) relative to those maintained in normal laboratory conditions (21% $O_2$, physiological hyperoxia). Our previous results quantified alterations in MDA-MD-231 cells for these volatiles after treatment with the chemotherapeutic agent Doxorubicin. When placed under cellular stress through Doxorubicin treatment only MeCl showed a similar stress response (enhanced uptake). In contrast, hexane (or hexane isomers) were not consumed or degraded significantly (Issitt et al. 2022a).

Over 24 hours of doxorubicin treatment has been shown to increase ROS (Pilco-Ferreto and Calaf 2016) whereas the opposite has been shown in cells maintained in hypoxic conditions (Sgarbi et al. 2018). A significant reduction was demonstrated in ROS in MDA-MB-231 cells following 24hrs of hypoxia (Figure 3B). Cellular stress response mechanics and differences in cellular state could therefore be identified and quantified through volatile metabolic approaches. Alkanes have been positively correlated with ROS previously (Calenic et al. 2015), here a decrease was demonstrated in n-hexane within hypoxic cells (Figure 2C) with diminished ROS content while in cells treated with doxorubicin, non-significant increases were observed (Issitt et al. 2022a). Metabolic consumption n-hexane is through currently unidentified processes, however the demonstration of variable consumption of a compound demonstrates a potential biomarker dynamics missed by studies only focusing on production. Acetone, hexanes and other compounds shown here are commonly found in urban environments (Redeker et al. 2007) and so their expression in the breath is driven through a combination of equilibration in the bloodstream and chemical/biological uptake processes within the body.

The production of styrene by cells under hypoxia could be a defining VOC biomarker for cancer since hypoxia is characteristic of the tumour microenvironment (Samanta and Semenza 2018). Our recent review showed that, despite substantial variability in reported outcomes, aromatics are powerful descriptors of cancer (Issitt et al. 2022b). Five studies have previously reported styrene in the breath of lung cancer patients using untargeted approaches (Phillips et al. 1999; Chen et al. 2005a; Peng et al. 2009; Rudnicka et al. 2011; Corradi et al. 2015; Koureas et al. 2020). Styrene has also been reported as higher in the breath of lung

cancer patients in studies using other approaches (Chen et al. 2005b; Nardi-Agmon et al. 2016; Wang et al. 2022). However, styrene has been shown to be higher in the breath of smokers (Koureas et al. 2020) and so is often considered, along with other aromatics compounds, to be a confounding contaminant since high percentages of lung cancer patients have a history of smoking. (Issitt et al. 2022b). Styrene has also been reported in the breath of patients with ovarian (Amal et al. 2015), gastric (Amal et al. 2013; Amal et al. 2016) and liver (Qin et al. 2010) cancers.

Styrene utilisation as a breath based diagnostic biomarker may be challenging since environmental contamination would need to be considered (Hanna et al. 2019). The presented method accounts for environmental VOCs through a flux analysis that incorporates two temporal sampling points, a starting sample following equilibration with the local atmosphere and a second sample at a later time point. This allows us to determine when available environmental volatiles are being added to (metabolically produced) or consumed/degraded by cells. This is important where environmental VOCs may mask effects or differences, such as high traffic, urban environments or perfumed indoor spaces. It is worth stating however, that the observed degradation may be purely non-targeted chemical reactivity with available enzymes or active compounds. However, to some degree whether the process is substrate specific or nonspecific is unimportant. A different cell response under stress was observed, which points to different cellular states, inclusive of differing enzyme compositions, and points to new and novel potential biomarkers.

Environmental correction sampling approaches such as this chamber headspace method may present an opportunity to overcome challenges to applications within the clinic, particularly with breath samples taken from ambient air as well as exhalate from the patient. The two time point sampling approach is particularly important since production of compounds with large initial concentrations, or consumption/degradation of compounds are often challenging to detect using single time point sampling methods.

It was observed that cellular consumption of VOCs (MeCl, Acetone and n-Hexane) is descriptive of hypoxic stress and that chemotherapeutic stress also induces consumption of VOCs (Issitt et al. 2022a); notably MeCl. To our knowledge this is the first example of a controlled environment experiment performed under low oxygen conditions that both a) quantifies VOC fluxes from a cellular model and b) utilises a VOC injection of gases to monitor ongoing anaerobic metabolism of compounds. We have demonstrated a novel method for induction and maintenance of low oxygen for the study of volatile fluxes. This approach allows new dynamics to be explored for the discovery of cell to patient translational biomarkers. It is perhaps worthy of note that many of the published methods for breath research would not

have identified or quantified the methyl chloride or hexane results, due to the small changes (pptv) observed.

It was previously reported that cellular ‘volatile metabolic flux’ can separate cell type and response to chemotherapeutic stress (Issitt et al. 2022a). This chamber based method has also been successfully used with mice models, quantifying both mouse breath and faecal volatiles (Issitt et al. 2022a). Here, this chamber based approach was demonstrated to identify cells under hypoxic stress. A novel method is demonstrated to identify hypoxia induced VOCs, potential biomarkers of cancer. Importantly these biomarkers are both produced and consumed by cells under hypoxic stress. MeCl, n-hexane and styrene are clinically interesting compounds requiring further investigation. The compounds reported here have been reported as present in human breath (Shahi et al. 2022) and we have shown that these compounds vary in response to cellular stress, from previously published doxorubicin (Issitt et al. 2022a) and here, hypoxic stress. Together this suggests they are able to differentiate cellular response due to pathophysiological differences. These compounds are from diverse functional chemical groups and we have previously demonstrated the ability of functional chemical groups to separate disease groups with greater ability than individually considered compounds (Issitt et al. 2022b). A functionally diverse group of VOCs could give greater power when building a ‘breath print’ for diagnosis (Issitt et al. 2022b).

## Conclusion

The work presented here demonstrates a novel methodology investigating volatile metabolisms in a controlled environment for volatile biomarker discovery. Using this method we have shown distinct changes in VOCs, demonstrating the potential for VOCs in defining metabolic alterations to environmental changes.

## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

Conceptualization, T.I., W.J.B. and K.R.R.; Data curation, T.I.; Formal analysis, T.I.; Funding acquisition, S.T.S., W.J.B. and K.R.R.; Investigation, T.I.; Methodology, T.I., M.R. and K.R.R.; Project administration, T.I. and S.T.S.; Resources, M.R., W.J.B.; Visualization, T.I.; Writing—original draft, T.I.; Writing—review and editing, S.T.S., W.J.B., M.R. and K.R.R. All authors have read and agreed to the published version of the manuscript.

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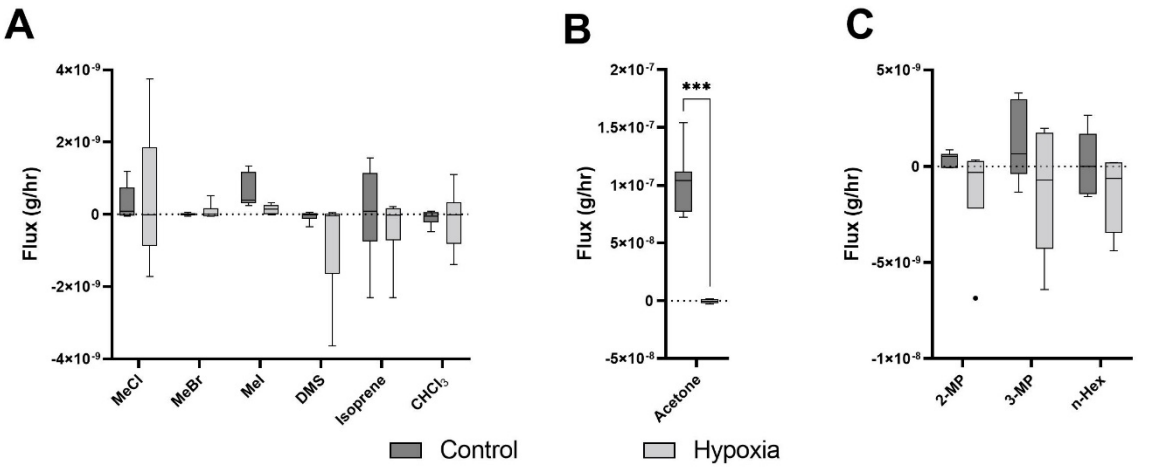
Supplementary Material




**Supplementary figure 1**. Volatile flux of media controls. Volatile flux in grams per hour (g/hr) for control media (n = 8) or media in hypoxia (n = 6) in 10cm dishes. CHCl3, chloroform; OMS, dimethyl sulfide; MeBr, methyl bromide; MeCl, methyl chloride; MeI, methyl iodide; MeSH, methanoethiol; 2-MP, 2 methyl pentane; 3-MP, 3 methyl pentane; n-Hex, n-hexane. Boxplot whiskers show median ± Tukey distribution, n = 6. Two way ANOVA followed by Bonferroni post hoc test was performed for **(A,B)**. One way ANOVA with Tukey post-hoc test performed for B; ***$p < 0.001$.

# Chapter 4:

## Cellular response to starvation provides biomarkers for breast cancer through volatile metabolites linked to methylation and methionine metabolism

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Key words

Methyl chloride, chloromethane, breath diagnostics, methylation, methionine, S-adenosylmethionine, SAM

## Highlights

- Glucose, serum and oxygen starvation induce significant changes in volatile flux
- Consumption of methyl chloride by cells translates to the breath of tumour bearing mice
- Methyl chloride production is linked to cellular methylation activity
- Methyl chloride consumption is linked to methionine synthesis

## Abstract

Volatile organic compounds (VOCs) demonstrate promise as non-invasive diagnostic tools, however, lack of mechanistically linked VOCs with biomarker discovery platforms limit delivery to the clinic. Previously identified putative VOC biomarkers were investigated in breast cancer cell lines under tumour pathophysiological conditions, in which they were deprived of serum, glucose or oxygen. VOCs in breast cancer cell cultures were significantly altered when placed under physiologically relevant cellular conditions. In particular, significant reduction of methyl chloride (MeCl) production, and in many cases consumption, is a consistently informative marker for cellular stress. The role of MeCl in cellular methylation activity was investigated and its production described in the context of cellular methylation potential and methyl-transferase activity. Blocking cellular methylation with 5-azacytidine effectively prevented MeCl production. A new "push-pull" model for cellular production and consumption of MeCl in human tissues is presented. Furthermore, clear separation by breath of MDA-MB-231 xenograft tumour bearing mice using the same suite of VOCs was observed where, consistent with this model, reduced MeCl production was a key biomarker. This work presents both a novel mechanism for cellular metabolism of available, ambient atmospheric VOCs, a new substrate for methionine synthesis and a potentially powerful translational breath biomarker for cancer.

## Introduction

Volatile metabolomics utilise volatile organic compounds (VOCs) released or consumed by biological processes to describe metabolic changes in organisms or systems. VOCs are small, carbon containing compounds which are gaseous at room temperature, often with a distinct odour, such ethanol or acetone. These characteristics present an attractive approach for non-invasive diagnostic approaches using VOCs, especially those present in human breath. While many studies have been conducted using human breath and are able to define disease in humans through changes in VOCs, understanding of the biological and mechanistic processes driving these changes remains limited (Issitt et al. 2022b).

To describe and monitor alterations in compounds linked to diseases, such as cancer, knowledge of volatile compounds linked to mechanistic process are required to improve diagnostic accuracy. VOC discovery through cellular models, such as cell type comparisons (Little et al. 2020; Issitt et al. 2022a), response to drugs(Issitt et al. 2022a), oxidative stress (Liu et al. 2019) and hypoxia (Taware et al. 2020; Issitt et al. 2023) provide focal points for pathologically linked VOCs in breath. There has been a range of cellular VOC studies for cells such as lung (Hanai et al. 2012; Thriumani et al. 2018), breast (Lavra et al. 2015; Issitt et al. 2022a) and liver (Mochalski et al. 2013) cancer, stem cells (Klemenz et al. 2019) and umbilical vein endothelial cells (Mochalski et al. 2015). The microbiome and infectious agents also drive VOC metabolisms (Sagar et al. 2015; Ahmed et al. 2017). Some metabolic processes have been linked to VOCs (Janfaza et al. 2019; Issitt et al. 2022b), such as ADH1 potentially driving *trans*-2-hexenol production in A539 cells (Furuhashi et al. 2020), CYP450 metabolism of limonene (Miyazawa et al. 2002) and alkane production linked to oxidative stress (Liu et al. 2019), however more studies into VOC metabolic mechanisms are required.

We have previously described a method for investigating volatile fluxes from cells and mice using a static headspace chamber with multiple time point sampling (Issitt et al. 2022a; Issitt et al. 2023). In these studies, variations in cell type (cancer vs non cancer) was clearly evident in the volatile profiles of these cells (Issitt et al. 2022a). However, it was also evident that cellular response to stress, from doxorubicin (Issitt et al. 2022a) or low oxygen (Issitt et al. 2023) produced clear changes in cellular VOC profile, markedly, metabolic transformation for some volatiles from production to consumption.

Cell type volatiles flux comparisons are warranted, useful and able to determine variability between cell type (Lavra et al. 2015; Issitt et al. 2022a), for example, biphasic and epithelioid sub-types in mesothelioma cells (Little et al. 2020). This highlights the power of volatile metabolomics, however, translation of these signals into more complex systems i.e. the human body introduces many confounding and conflicting signals (Issitt et al. 2022b). Cell

type comparisons are powerful tools for comparing variability inherent between cell types but may be lacking in modelling the environmental pathological conditions experienced by cells. Through-out our previous work, translational characteristics in the VOC profile, were more tightly determined by models of what cells might experience *In situ*. From this we hypothesise that pathophysiological models are more appropriate for VOC biomarker discovery.

In previous studies (Issitt et al. 2022a; Issitt et al. 2023) and throughout the presented research, methyl chloride (MeCl) is a consistently descriptive VOC biomarker. MeCl is exhaled in human breath (Keppler et al. 2017), produced from s-adensylmethionine dependent methylation activity in plants (Rhew et al. 2003) and consumed by bacteria for a range of processes (McAnulla et al. 2001). This VOC is therefore a potential target for mechanistically linked studies and we hypothesise that its observed production from cells in culture and in breath of mice (Issitt et al. 2022a) is linked to methylation activity.

In this study we set out to determine translation of the aggressive, double negative breast cancer cell line MDA-MB-231 VOC profile in culture to mice with MDA-MB-231 xenograft tumours. This study aims to model the substrate starvation experienced by a growing tumour (Osawa et al. 2009). Serum, glucose and hypoxic starvation are applied MDA-MB-231 breast cancer cells and non-transformed MCF10a breast derived cells to determine VOC response in a suite of volatiles previously described (Issitt et al. 2022a; Issitt et al. 2023). Methylation activity and methionine levels are investigated in the context of MeCl to determine potential mechanisms to explain observed variations in the same volatiles detected in the breath of MDA-MB-231 tumour bearing mice.

# Methods

## Cell Culture and treatments

MDA-MB-231 breast cancer cells (a gift from Professor Mustafa Djamgoz, Imperial College London) were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Scientific, Waltham, MA, USA), 25 mM glucose, supplemented with L-glutamine (4 mM) and 5 % foetal bovine serum (Thermo Scientific, Waltham, MA, USA). The nontransformed human epithelial mammary cell line MCF10A (a gift from Dr. Norman Maitland) was grown in DMEM/F12 (Thermo Scientific, Waltham, MA, USA) supplemented with 5% FBS, 4 mM L-glutamine (Thermo Scientific, Waltham, MA, USA), 20 ng/mL EGF (Sigma-Aldrich, Roche; Mannheim, Germany), 0.5 mg/mL hydrocortisone (Sigma-Aldrich, Burlington, MA, USA), 100 ng/mL cholera toxin (Sigma-Aldrich, Burlington, MA, USA) and 10 µg/mL insulin (Sigma-Aldrich, Burlington, MA, USA). Both of these formulations were considered control conditions. Cell culture medium was supplemented with 0.1 mM NaI and 1 mM NaBr (to model physiological availability of iodine and bromide). Serum free media and glucose free media were prepared in the same way but without those individual components. All cells were grown at 37 °C with 5 % $CO_2$.

Prior to volatile collection, cells were trypsinised, and 500,000 cells were seeded into 8 mL complete media in 10 cm polystyrene cell culture dishes. Cells were then allowed to attach for 3-4 h, washed with warm PBS and 6 mL treatment media was applied. Volatile headspace sampling was performed 24 h later.

Cells were treated with 10 µM (Thakur et al. 2012) 5-Azacytidine (5-AZA, prepared in sterile water, Sigma-Aldrich, Germany) to block methylation events; the MAT2A inhibitor to block production of MAT (the enzyme which primarily catalyses the synthesis of S-adenosylmethionine), FIDAS-5 (prepared in DMSO, Sigma-Aldrich, Germany) or sodium nitroprusside to block methionine synthase activity (SNP, prepared in water, Sigma-Aldrich, Germany) where stated. S-adenosylmethionine (SAM) treatment was performed at a concentration of 50µM, determined to produce significant effects upon MDA-MB-231 without considerable cell death or cell growth (Ilisso et al. 2015; Mahmood et al. 2018). Drug concentrations were determined as producing a significant effect without excessive cell death over 24 hours, from the literature and with alamar blue assay; 5-AZA treatment was performed at 10 µM (Thakur et al. 2012), FIDAS-5 at 10 µM(Zhang et al. 2021), SNP at 400 µM(Nicolaou et al. 1997).

## VOC headspace sampling

The method of headspace sampling has been described in detail (Issitt et al. 2022a; Issitt et al. 2023). Briefly, cells placed in static chambers were flushed with lab air (4 L/min for 10 mins) and time zero sample taken into evacuated electro polished steel canisters (LabCommerce, San Jose, USA). Samples were then left on a rocker for 120 mins at the slowest setting, at which point another sample was collected. Cells were removed from the chamber, washed with PBS twice and lysed in 500 µL RIPA buffer (NaCl (5 M), 5 mL Tris-HCl (1 M, pH 8.0), 1 mL Nonidet P-40, 5 mL sodium deoxycholate (10 %), 1 mL SDS (10%)) with protease inhibitor (Sigma-Aldrich, Roche; Mannheim, Germany). Protein concentration of lysates were determined using BCA Assay (Thermofisher, USA). Background (medium only) readings were taken for all medium types following 24 hours incubation 37 °C and 5% $CO_2$ (supplementary Figure 3A).

## GC/MS analysis of VOCs

Headspace samples were condensed in a liquid nitrogen trap before being transferred, via heated helium flow, to and Aglient/HP 5972 MSD system (Santa Clara, CA, United States) equipped with a PoraBond Q column (25m x 0.32mm x 0.5 µm film thickness) (Restek©, Bellefonte, PN, United States), as previously detailed (Issitt et al. 2022a; Issitt et al. 2023). Samples were analysed in selected ion monitoring (SIM) mode, specific details of which are available at Issitt et al. 2023.

Peak area/moles injected were calibrated from known standard injections. Sample moles injected were calculated using these peak area-based calibration curves, and concentration determined by moles of compound/moles of air injected. Sample VOC concentration were then normalised to CFC-11 concentrations (240 parts-per-trillion-by-volume (ppt)). CFC was used as an internal standard, per sample standard for normalisation as atmospheric concentrations of CFC-11 are globally consistent and stable (Redeker et al. 2007). Detailed equations are available (Issitt et al. 2022a; Issitt et al. 2023).

Media backgrounds were subtracted from cellular plus media volatile fluxes which were then normalised to protein concentration. No variation between media types was observed (Figure S1) and so these were pooled to create an average media blank value. As an extension of this, we have previously demonstrated no variation in DMSO containing media volatile flux for the compounds discussed in this research (Issitt et al. 2022a).

## MTT assay

MDA-MB-231 and MCF10A cells were seeded onto 96-well plates at a density of 8000 cells per well. Serial dilutions across the plate were performed once the cells had attached to the plate (4 h). Cells were then placed in cell culture incubation conditions. A total of 24 h later, 20 µL of MTT solution was added to each well and incubated for 3 h. Medium was removed, and precipitates solubilised in 100 µL DMSO. Absorbance was then measured at 570 nm using a Clariostar Plus microplate reader (BMG Labtech, Offenburg, Germany) across duplicate technical with triplicate experimental replicates.

## Sulphorhodamine B assay

To determine cell growth, SRB assays were performed. The SRB assay measures cell density based on protein content (Vichai and Kirtikara 2006). Following incubation, cell monolayers were fixed with 10% (wt/vol) trichloroacetic acid (TCA) and stained for 30 min, after which the excess dye was removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader (Vichai and Kirtikara 2006) using duplicate technical with triplicate experimental replicates.

## Trypan blue exclusion assay

Trypan blue exclusion assay was performed on MDA-MB-231 and MCF10A cells following treatment. Following a published protocol (Strober 2015), trypsinised cells were mixed with 0.4% Trypan blue solution and counted to determine the number of unstained (viable) and stained (nonviable) cells. Cells were counted at 20x magnification across 10 fields of view using duplicate technical and triplicate experimental repeats.

## Ion Chromatography

A previously published protocol was adapted to quantify chloride contents of cells and tissues (Chapp et al. 2018). Cells were grown to 80% confluency in a 10cm dish and treated for 24 hours, as appropriate. Cells were washed in 10 mL pH 7 phosphate buffer twice and lifted using a cell scraper in 5 mL phosphate buffer. Cells were then pelleted by centrifugation (200g 5 mins), supernatant aspirated and cells resuspended in 1 mL dd$H_2O$. Pellet was then snap frozen in liquid nitrogen, thawed and pulse sonicated (5 second pulse for 20 seconds), a

sample of 50 µL was removed at this point for protein quantification using BCA. The mixture was then added to 5ml $ddH_2O$ and syringe filtered through 0.2 µm prerinsed (with $ddH_2O$) PTFE filter. Tumour and mammary fat pad samples were weighed, washed in phosphate buffer, snap frozen, thawed and sonicated and treated as above.

Ion chromatography was performed using a Dionex ICS-2000 ion chromatograph fitted with an EGC III KOH Eluent generator cartridge, ADRS 600 2 mm suppressor, DS6 heated conductivity cell and AS40 autosampler. Dionex IonPac AS18 (2 mm i.d. x 250 mm length) analytical column fitted with an IonPac AG18 (2 mm i.d x 50 mm length) guard. Mobile phase gradient: 2 mM potassium hydroxide (hold 1 min) to 41 mM over 35 min (hold 4 min) at a flow rate of 0.25 ml/min column. A suppressor current of 26 mA, column temperature of 30 °C, detector temperature of 35 °C and a sample injection volume of 15 µL. Instrument control and data processing performed using Chromeleon software. Samples were quantified against calibration curves derived from known standard injections.

## Methylation ELISA

All samples were tested for global DNA methylation using the MethylFlash™ Methylated DNA Quantification Kit (Colorimetric) (Epigentek, Farmingdale, NY, USA). MethylFlash uses an enzyme-linked immunosorbent assay (ELISA) based method to quantify global DNA methylation. For each sample, 200 ng of DNA was used, in duplicate, as recommended by Epigentek. DNA was isolated from cell pellets or tissue using DNA extraction kit for tissue and blood (Qiagen). The ELISA was performed following manufacturers instructions. Absorbance readings from each plate were calculated at 450 nm using Clariostar (BMG) and Mars software (BMG) across duplicate technical with triplicate experimental replicates.

## HPLC

Cells were grown to 80% confluence in 6 well plates and treated. Cells were then washed with PBS (5mL x 2) and stored, sealed at -80 ˚C. Samples were prepared in ice cold 90% methanol with 0.1% triflouroacetic acid (Korinek et al. 2013). The following authentic standards were used: L-methionine (Met), L-homocysteine (Hom), cyanocob(III)alamin (B12), S-(5′-adenosyl)-L-homocysteine (SAH) and S-(5′-Adenosyl)-L-methionine chloride dihydrochloride (SAM), all supplied by Merck.

Standard stock solutions were prepared in water with 0.1% TFA at the following concentrations: Met 4 mg/mL, HCY 5 mg/mL, B12 6 mg/mL, SAH 4 mg/mL, SAM 2 mg/mL,

and stored at -20ºC for up to two weeks. Eight-level standard curves were constructed in 90% methanol at the following concentration ranges: Met 2.1 ng/mL-40 µg/mL, HCY 24 ng/mL-100 µg/mL, B12 38 ng/mL-2.4 µg/mL, SAH 9.8 ng/mL-40 µg/mL, SAM 0.31-20 µg/mL (the lower-end concentration was that at which a peak was clearly visible, and serves as an approximation of LOD).

A Waters Acquity IClass UPLC was interfaced to a Waters Synapt G2-Si, operated in positive ESI sensibility mode, scan rate 0.6 sec, mass range 50-1600 *m/z*. Lock mass leucin-enkephalin was used. Mobile phase A) was 10 mM ammonium formate, pH 3.0 (adjusted with ammonium hydroxide) and 1% acetonitrile, mobile phase B) acetonitrile. The gradient started at 90% B and decreased to 35% B over 19 min, where it remained until 24 min, to return to 90% B at 25 min and to re-equilibrate until 35 min; flow rate was 0.5 mL/min. A MilliporeSigma SeQuant® ZIC-HILIC column (100x4.6 mm, 200 Å, 5 µm) was used at 40ºC. The autosampler was kept at 7ºC, injection volume was 10µL. Analytes were identified according to accurate mass (all [M+H]+ adducts) and retention time, compared to authentic standards using Skyline software v22.2.0.351. Samples were stored at -80ºC and loaded into the autosampler 20 mins before each run as SAM degrades rapidly at room temperature.

## RNA sequencing analysis

Public RNA sequencing data was available for MCF10A and MDA-MB-231 cell lines (SRA:PRJNA302668; (Messier et al. 2016)), as well as MDA-MB-231 cells grown in normoxic and hypoxic conditions (1% O 2 ) from either total RNA sequencing after ribodepletion (SRA: PRJNA604033; (Chen et al. 2021)) or mRNA sequencing after polyA enrichment (SRA: PRJNA530760; (Wang et al. 2020)). Following standard quality control, including adapter removal and removal of low-quality reads using Trimmomatic v0.36 (Bolger et al. 2014), gene-level expression values in transcripts per million (TPM) were derived against the Gencode v41 human transcriptome using kallisto v0.46.1(Bray et al. 2016). TPMs were recalculated after exclusion of non-coding and mitochondrial genes. Differentially expressed genes in each experimental condition were identified using sleuth v0.30.0 (Pimentel et al. 2017) in R studio. MDA-MB-231 hypoxia datasets were also combined to provide a higher sample number, controlling for experimental design as a batch effect. Between experiment comparisons were drawn using π-values(Xiao et al. 2014), a product of fold change and significance value. Methyl transferases were selected for using identified methyl transferases (Petrossian and Clarke 2011), the gene list is available in supplementary information (supplementary table 1). π-values were used to direct a ‘prerank’ gene set enrichment analysis using a curated list of

198 methyltransferase genes (list provided in supplementary) using GSEApy v1.0.5 (Fang et al. 2023).

## Transfection

Cells were transfected using the transfection reagent TransIT-siQuest (Mirusbio reagents) in OPTIMEM (Gibco). Scrambled (control) siRNA and siDNMT (SMARTpool) were purchased from ThermoFischer and used at a concentration of 25nM with cells grown to 80% confluency, following manufacturers (Mirusbio) instructions. Knockdown was confirmed with western blot.

## Western blot

Cell were prepared for immunoblotting (Western blot) by lysing in RIPA buffer with phosphatase and protease inhibitor cocktails (Sigma-Aldrich, UK). 35 µg of protein lysate was resuspended in laemmli buffer, denatured for 5 minutes at 95˚C, separated by SDS PAGE and transferred to nitrocellulose membrane (Whatman, USA). Nitrocellulose membranes were immunoblotted with the following primary antibodies: rabbit polycolonal antihuman DNMT1 (Novus Biologicals, UK), mouse monclonal antihuman MAT I/II (F-12, Santa Cruz Biotechnology, USA), mouse monoclonal antihuman PRMT1 (B2, Santa Cruz Biotechnology, USA), mouse monoclonal antihuman α-tublulin (Cell signalling, USA). HRPconjugated secondary antibodies were used for chemiluminescence detection with Luminol Santa Cruz Biotechnology, USA) and protein levels were quantified by densitometry with ImageJ (NIH, Bethesda US).

## Hydrogen peroxide (amplex red) assay

Experiments were performed in phenol red free DMEM. DMEM containing 50 µM Amplex Red reagent (Thermo Scientific, Waltham, MA, United States) and 0.1 U/mL horse radish peroxidase (HRP, Thermo Scientific, Waltham, MA, United States) was added to cells in 12 well dishes (500 µL per well) for 15 min following 24 h in starvation, starvation plus SAM or control conditions. We have previously reported no change in ROS using this assay following 24 hours of hypoxia (Issitt et al. 2023). Fluorescence at 590 nm was measured with a plate reader (Clariostar, BMG, Ortenberg, Germany) and compared against a $H_2O_2$ standard curve for quantification.

## Orthotopic xenograft breast tumour model

Tumour model has previously been reported by our group (James et al. 2022). *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ (bred in-house) mice were housed in individually ventilated cages with enrichment (3-5 mice per cage) in temperature-controlled rooms with access to water and food ad libitum. At 6 weeks of age, female mice were anaesthetised (2% isoflurane in oxygen (2 L/min)) and $5 \times 10^5$ MDA-MB-231 cells (suspended in Matrigel, 50% v/v in saline, 50 µL of volume) were injected into the left inguinal mammary fat pad. Animal weight, condition and tumour growth (calliper measurement) were monitored daily. Mice were euthanized at 5 weeks after cell implantation or at 15-mm tumour diameter and tumours and isolated. Mammary fat pad, without tumour was also collected from the same animals (contralateral side).

We have previously published our method for sampling mouse headspace (Issitt et al. 2022a). Nine-week-old female Rag2−/− Il2rg−/− mice were selected for sampling. Experimental replicates were 2 mice from a cage across 3 separate litters/cages: 6 mice in total for each experimental group (control or with tumour as littermates). Mice were chosen randomly for experimental groups in each litter, blinding was not possible with animal care, sampling and analysis performed by TI. Experiments have been reported in-line with the ARRIVE guidelines (Percie du Sert et al. 2020).

Using tube handling methods, mice were gently placed with a cardboard tube and blue paper into the custom chambers. Flushing the chamber for 10 min using a Yamitsu air pump with a flow rate of 750 mL per min in undisturbed conditions, mice were allowed to acclimatise. T0 samples were then taken, and as with cellular headspace, the chambers were sealed for 20 min and T1 samples were then taken. This was performed for all mice, once, 4 weeks post implantation, in chambers, first with mice and then again without the mice present but with their faecal material. Number of faecal pellets recorded once mice were removed from chamber, this was used to normalise faecal VOCs for comparison in supplementary Figure S7.

## Database searching and alignment

The methyl chloride transferase (*Batis maritima,* UNIPROT ID: Q9ZSZ7) and cmuA methyltransferase (UNIPROT ID: F8J7J8 (Goulding et al. 1997)) were aligned against the human proteome using Clustal (1.2.4) multiple sequence alignment by UNIPROT BLAST.

## Data analysis

Graphs and statistics were generated/performed in Graphpad (Prism). Details of specific tests are provided in figure legends. RNA seq data was arranged in R studio following analysis (described above).

## Ethical approval

Approval for all animal procedures was granted by the University of York Animal Welfare and Ethical Review Body. All procedures were carried out under authority of a UK Home Office Project Licence and associated Personal Licences.

## Results

The work presented attempts to model pathophysiological environments experienced by cells in a growing tumour. Starving cells of serum, glucose and oxygen (hypoxia) models one part of tumour pathophysiology as growing tumours experience poor availability of substrate delivery (Li et al. 2021). The dynamics of this cellular metabolic response to resource starvation was explored through volatile profiles and associated metabolites investigated which are linked to the most informative VOCs. These changes were then compared to the breath of mice bearing tumours of the same cells investigated under stress conditions.

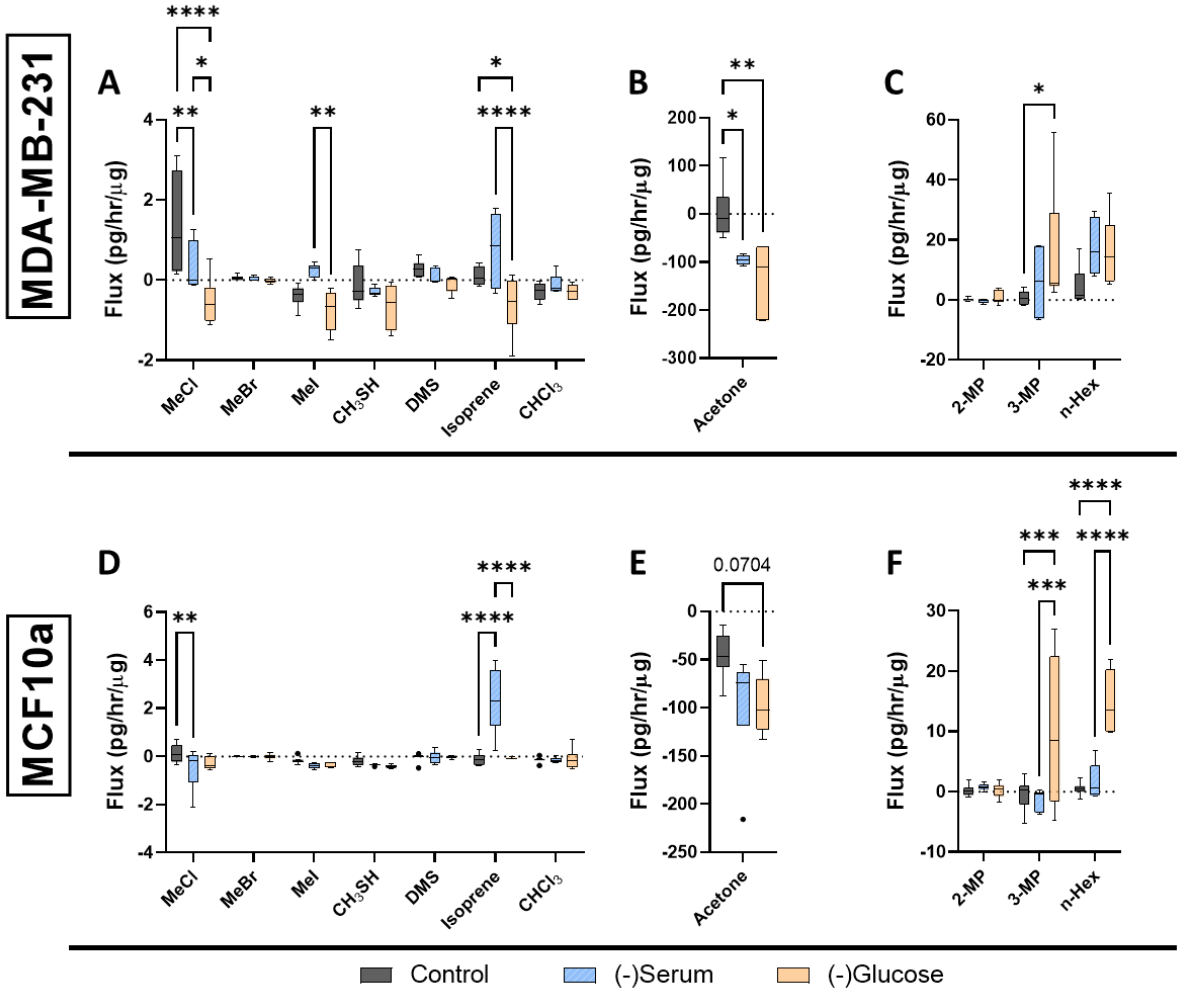


Figure 1. **Starvation of breast derived cells produces detectable changes in select volatile organic compounds.** Volatile flux (pg/hr/µg) for the cancerous MDA-MB-231 (**A-C**) and noncancerous MCF10a **(D-F).** Media subtracted and protein-normalised. (-)Serum = media without serum, (-)Glucose = media without glucose. $CHCl_3$ = Chloroform, DMS = Dimethyl sulphide, MeBr = Methyl bromide, MeCl = Methyl chloride, MeI = Methyl iodide,

MeSH = Methanoethiol. Boxplot whiskers show median ± Tukey distribution (n=6). Two-way ANOVA with Bonferroni post hoc test was performed for **A, C, D** and **F**. One-way ANOVA with Tukey post hoc test was performed for **B** and **E**; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

## Starvation produces detectable changes in volatile flux for select volatile compounds

MDA-MB-231 and MCF10a cells were exposed to starvation medium to compare VOC responses to starvation stress. Cells in starvation medium (without serum or without glucose) over 24 hours exhibit significant changes in flux for 5 compounds.

MDA-MB-231 cells under serum starvation exhibited a significant reduction in fluxes of MeCl (Figure 1A) and acetone (Figure 1B) flux compared to control. Serum starvation also produced an increase in MeI, and these fluxes were significantly different from glucose starvation (Figure 1A). Further to this, a non-significant increase in n-Hex was observed (Figure 1C). Glucose starvation of MDA-MB-231 cells produced a significant metabolic transformation, from production to consumption, for MeCl (Figure 1A). Consumption of isoprene and acetone was also observed in glucose starved cells (Figure 1A and B). Glucose starved cells had an increased 3-MP flux and a non-significant increase in n-Hex (Figure 1C).

Glucose and serum starved MCF10a cells exhibited significantly greater uptake of MeCl compared with control (Figure 1D). Uptake of acetone was also observed in MCF10a serum starved cells (Figure 1E). Serum starved cells also generated significantly more isoprene when compared to control (Figure 1D). Glucose starvation in MCF10a led to greater consumption of acetone (Figure 1E) and a non-significant, but greater, uptake of MeCl. Glucose starved cells n also generated substantially more 3-MP and n-Hex when compared to both control and serum starvation (Figure 1F).

Growth and metabolic activity of both cell lines under starvation was investigated to determine cellular responses to these stresses over time. At 24 hours, there was a considerable reduction in cells following serum starvation (Figure S1B and C). Glucose starvation produced no changes in growth for MDA-MB-231 cells and an increase in MCF10a over 24 hours, with relatively less growth after 48 hours compared to control (Figure S1B and C). Metabolic activity of cells was measured using MTT assay (Figure S1D and E). A reduction in metabolic activity was observed in all starvation conditions for both cell lines with the only significant difference recorded for glucose starved MCF10a (Figure S1D). Starvation of MDA-MB-231 cells showed reduction in viability by MTT, without significance (Figure S1E). Cell death in these conditions was investigated using trypan blue assay. Both cell lines showed significant reduction in

percentage viable cells in both serum and glucose free medium of between 70 and 50% (Figure S1F and G).

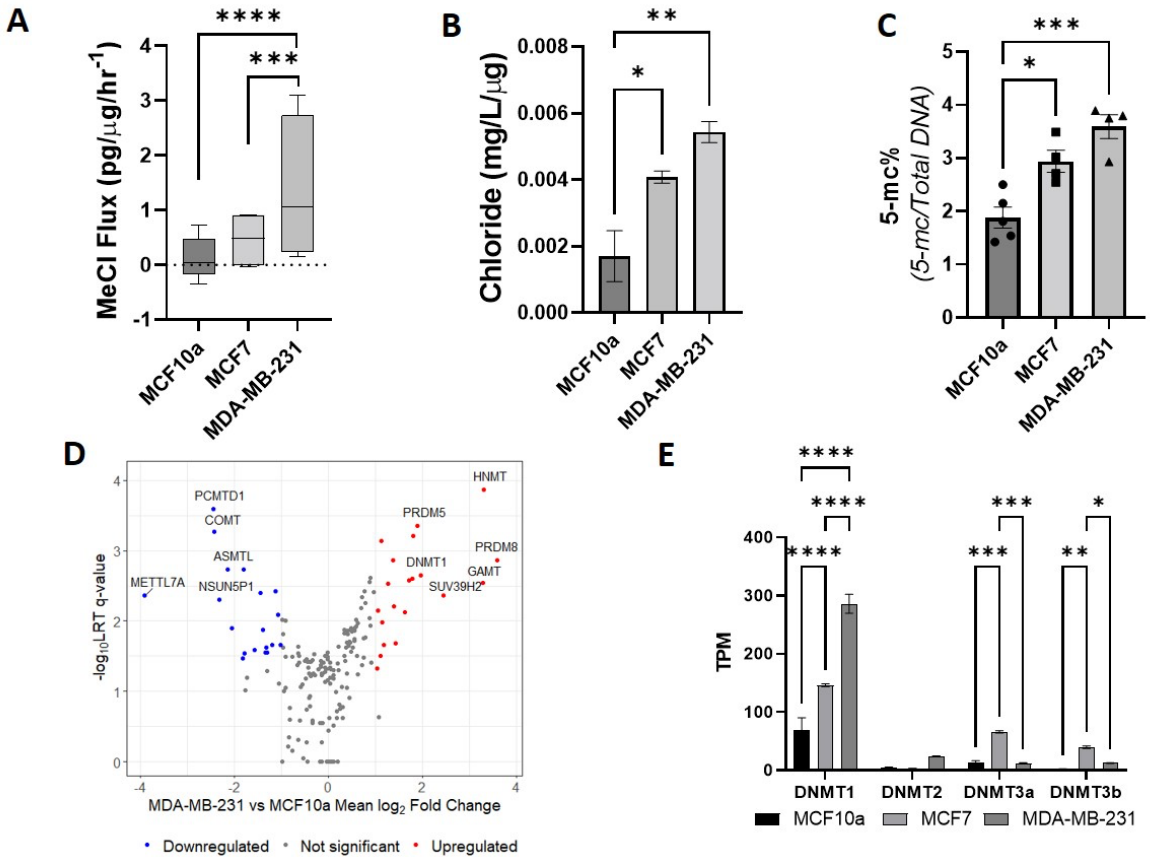


Figure 2. **Methyl chloride flux corresponds with chloride content and DNA methylation in breast derived cells. (A)** Methyl chloride (MeCl) flux only in pg/μg/hr$^{-1}$, statistical analysis performed within expanded data set; two-way ANOVA with bonferonni post hoc test represented here independently (n=6). **(B)** Cellular chloride content in parts per million (ppm) for different cell types. **(C)** Percentage of 5-methylcytosine (5-mC) of total DNA loaded for each cell type. **(D)** Volcano plot of RNA seq data for -log$_{10}$ LRT (likelihood ratio test) q values (corrected p values using Benjamini-Hochberg) of methyl transferase genes shown vs mean log$_2$ fold change of MDA-MB-231 vs MCF10a **(E)** Transcripts per million (TPM) of RNA seq data for DNA methyl transferases (DNMT) for different cell lines. Bar plots shown with mean ± SEM for **A-C** and mean ± SD for **E**. One-way ANOVA with Tukey post hoc test was performed for **B, C** and **E**; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

## Methyl chloride flux correlates with methylation in breast cell types

MeCl flux is increased significantly in MDA-MB-231 compared with MCF10a and MCF7 (Figure 2A and Issitt et al. 2022). Because of this, intracellular chloride was investigated with

ion chromatography, which showed a similar trend to MeCl flux between these cell lines, with MCF7 and MDA-MB-231 intracellular chloride significantly increased compared with MCF10a (Figure 2B).

There are no defined pathways for methyl chloride metabolism in humans, despite reports of its elevated concentration in human breath and interaction with the human gut microbiome (Shahi et al., 2022). MeCl production has been linked to s-adenosyl methionine dependent methylation in plants (Rhew et al. 2003) and Maritime seawort (Ni and Hager 1998; Ni and Hager 1999), therefore a protein:protein alignment was performed with BLAST from UNIPROT (Coudert et al. 2023) for methyl chloride transferase (*Batis maritima,* UNIPROT ID: Q9ZSZ7) against the human database. The top result was thiopurine methyltransferase (TPMT, UNIPROT ID P51580), which was subsequently aligned, producing 24.8% similiarity (Figure S2A). This alignment indicates some similarity between proteins, with some conservation in the binding domain of TPMT (Figure S2A) suggesting a potential avenue for MeCl interaction.

DNA methylation content of the three cell lines, MCF10a, MCF7 and MDA-MB-231 cells was investigated to determine any correlation with MeCl levels. 5-methyl cytosine residue percentage, determined by ELISA, of total DNA content within cells was seen to significantly increase from 1.8% for MCF10a, to 3% for MCF7 and 3.6% for MDA-MB-231 cells in control conditions (Figure 2C).

As human methyl chloride metabolism is unclear and plant studies suggest multiple methyl transferases may be involved in methyl chloride production, RNA sequencing data was investigated to identify variation in the TPMT gene and methyl transferases generally. Methyl transferase RNA variation is shown as a volcano plot (Figure S2B). Differential expression analysis (Figure 2D) revealed 20 (red) and 19 (blue) methyl-transferase (from a report list in Petrossian & Clarke, 2011) genes which were relatively and respectively up- and down-regulated in the MDA-MB-231s. Of these the most 6 significantly upregulated were *HNMT, PRDM8, SUV39H2, DNMT1, PRDM8*. The 6 significantly upregulated in MCF10a were *METTL7A, PCMTD1, COMT, NSUN5P1, ASMTL and COMTD1.* Whilst individual methyl-transferase gene expression differed between these divergent cell lines, gene seat enrichment analysis (GSEA) found no significant shift across the entire gene family (NES (normalised enrichment score): 0.971; FDR (false discovery rate): 0.503). To further describe observable increases in DNA methylation seen between cell types, DNA methylation specific genes were investigated as transcript per million (TPM) from the same public dataset. Increases in DNMT1 for MCF7 and MDA-MB-231 were seen compared to MCF10a (Figure 2E) which was comparable to changes seen in DNA methylation (Figure 2E). DNMT2 was slightly increased

for MDA-MB-231 compared to other cells and MCF7 shows increases in DNMT3a/b (Figure 2E).

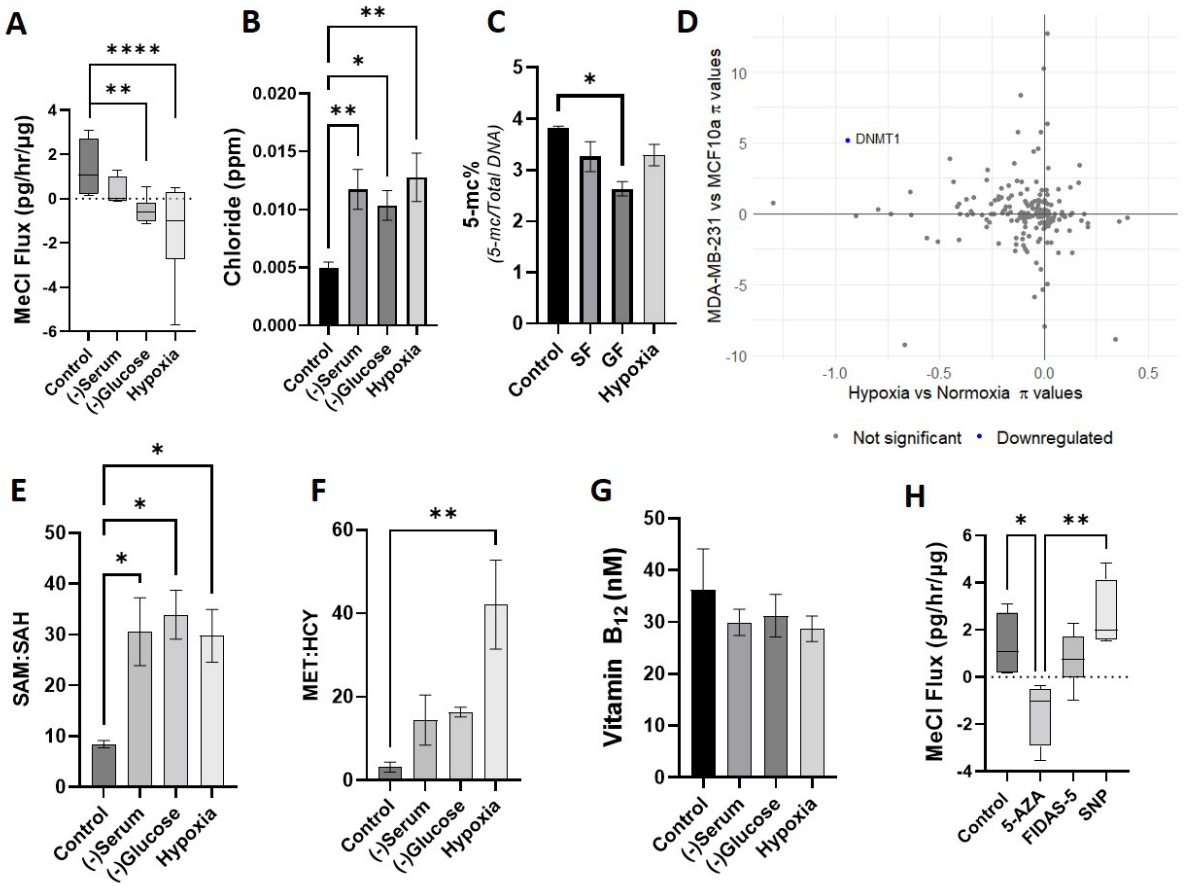


Figure 3. **Methyl chloride (MeCl) flux correlates with starvation and methylation levels. (A)** MeCl flux (pg/hr/µg) of MDA-MB-231 cells without (-) serum, glucose or hypoxia. Data presented from figure 1 and hypoxia data from previously published data for comparative purposes, statistics for **A** are from Two-way ANOVA in the presence of the whole dataset (n=6). **(B)** Intracellular chloride content of cells under starvation conditions in part per million (ppm) (n=4). **(C)** DNA methylation of cells under starvation conditions given as percentage 5-methylcytosine of total DNA (n=4). **(D)** Pi-chart of RNA data, comparing MDA-MB-231 (upwards on y-axis), MCF10a (down on y-axis), normoxia (right, x-axis) and hypoxia (left, x-axis), pi-values. **(E)** Ratio of S-adenosylmethionine (SAM) to s-adenosyl-homocysteine (SAH) for cells under starvation conditions (n=4). **(F)** Ratio of methionine (MET) to homocysteine (HCY) for cells under starvation conditions (n=4). **(G)** Vitamin B12 (nM) for cells under starvation conditions (n=4). **(H)** MeCl flux (pg/hr/µg) of cells treated with 10 µM 5-azacytadine (5-AZA), 10 µM FIDAS-5 or 400 µM sodium nitroprusside (SNP) (n=6). Bar plots shown with

mean ± SEM. One-way ANOVA with Tukey post hoc test was performed for **B, C, E-H**; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

## Methyl chloride flux correlates with methylation response in MDA-MB-231

We have demonstrated MeCl reduction in response to serum starvation and uptake in glucose starvation (Figure 1A) and previously reported a MeCl uptake in response to oxygen starvation in MDA-MB-231 (Issitt et al. 2023), outlined in Figure 3A. This reduction/consumption correlated with accumulation of chloride for all conditions Figure 3B.

Analysis of global DNA methylation revealed a reduction in all conditions with significance found in the glucose starvation condition alone (Figure 3C), correlating with results seen in Figure 2. Interestingly, differential analysis between MDA-MB-231 cells grown in hypoxic rather than normoxic conditions found only one significantly different gene: downregulation of *NOP2* (log2FC= -1.30; q=1.81) in hypoxia. Whilst no other genes met our significance thresholds (Figure S3A), including *DNMT1* (log2FC= -0.92; q=1.73), the data suggested an overall downregulation of methyl-transferase genes in hypoxia, supported by GSEA (NES: -1.701; FDR q: 0.0001, Figure S3B) Under normoxia, MCF10a MeCl levels are lower than MDA-MB-231 in normoxia (Figure 1), we investigated the similarities between MT gene expression in MCF10a with hypoxic MDA-MB0231s using a π-plot: a method for comparing multiple differential expression analyses by combining fold change and significance value (Figure 3C). The only significantly changed gene was DNMT1, which was significantly down-regulated in both hypoxic conditions and in MCF10a cells when compared with MDA-MB-231 in normoxic conditions.

HPLC analysis of SAM and SAH can be expressed as a ratio to show methylation potential in cells (Caudill et al. 2001; Castro et al. 2003). SAM:SAH ratio increased our cells in starvation conditions (Figure 3E) SAH is described as a potent inhibitor of methylation (Castro et al. 2003) and decreases relative to SAM show potential for methylation increasing and a lack of consumption of SAM. SAM increased slightly, without significance following starvation (Figure S3C) and SAH decreased (Figure S3D).

## Active consumption of MeCl linked to methionine synthesis

The observed reduction/consumption of MeCl suggests an entirely different metabolism in use by cells, potentially using MeCl as a metabolic substrate rather than a metabolic output. As with methyl chloride production, methyl chloride consumption has not been described or

detailed in humans. Use of MeCl as a substrate has, however, been demonstrated and described in methylotrophic bacteria, via the *cmuA* methyltransferase (Goulding et al. 1997; McAnulla et al. 2001; Studer et al. 2001). The chloromethane methyltransferase protein (UNIPROT ID: F8J7J8 (Goulding et al. 1997)) was therefore compared to the human proteasome via UNIPROT BLAST. The top result with 31.08% alignment was methionine synthase (MTR, UNIPROT ID: Q99707), alignment for these two proteins was then performed (Figure S4). High conservation was observed, with some conserved regions lying in the binding domains of the MTR structure, providing confidence that similar substrates are used for both proteins.

MTR generates methionine from homocysteine, using vitamin b12 to add a methyl group. To investigate if there was potential MeCl consumption relating to MTR, methionine, homocysteine and vitamin B12, the molecule which facilitates MTR's consumption of homocysteine to methionine, were investigated. A similar trend was see in methionine (Figure S3E) and homocysteine (Figure S3F), as SAM and SAH changes. The ratio of methionine to homocysteine (MTH:HCY) increased for all starvation conditions with significance seen for cells in hypoxic environments (Figure 3F). Vitamin B12 levels were not shown to change under starvation conditions (Figure 3G).

## Preventing methylation with 5-Azacytadine blocks MeCl production

Because results indicated a role for methylation in MeCl production and alterations to SAM:SAH and MTH:HCY, MDA-MB-231 cells were treated with 5-AZA (to block methylation (Muller and Florek 2010)), FIDAS-5 (to block cellular generation of SAM (Zhang et al. 2013)) and SNP (which has been shown to block methionine synthase (Nicolaou et al. 1997)). 5-AZA treatment clearly generated a MeCl uptake response (Figure 3H), similar to effects of glucose or hypoxic starvation (Figure 1 and 2A). FIDAS-5 produced no change in MeCl flux and SNP treatment generated a slight increase in MeCl flux (Figure 3X). The viability of cells under these conditions was investigated using trypan blue assay, 5-AZA and SNP treatment produced a significant reduction in viability of 10-20% (Figure S3G).

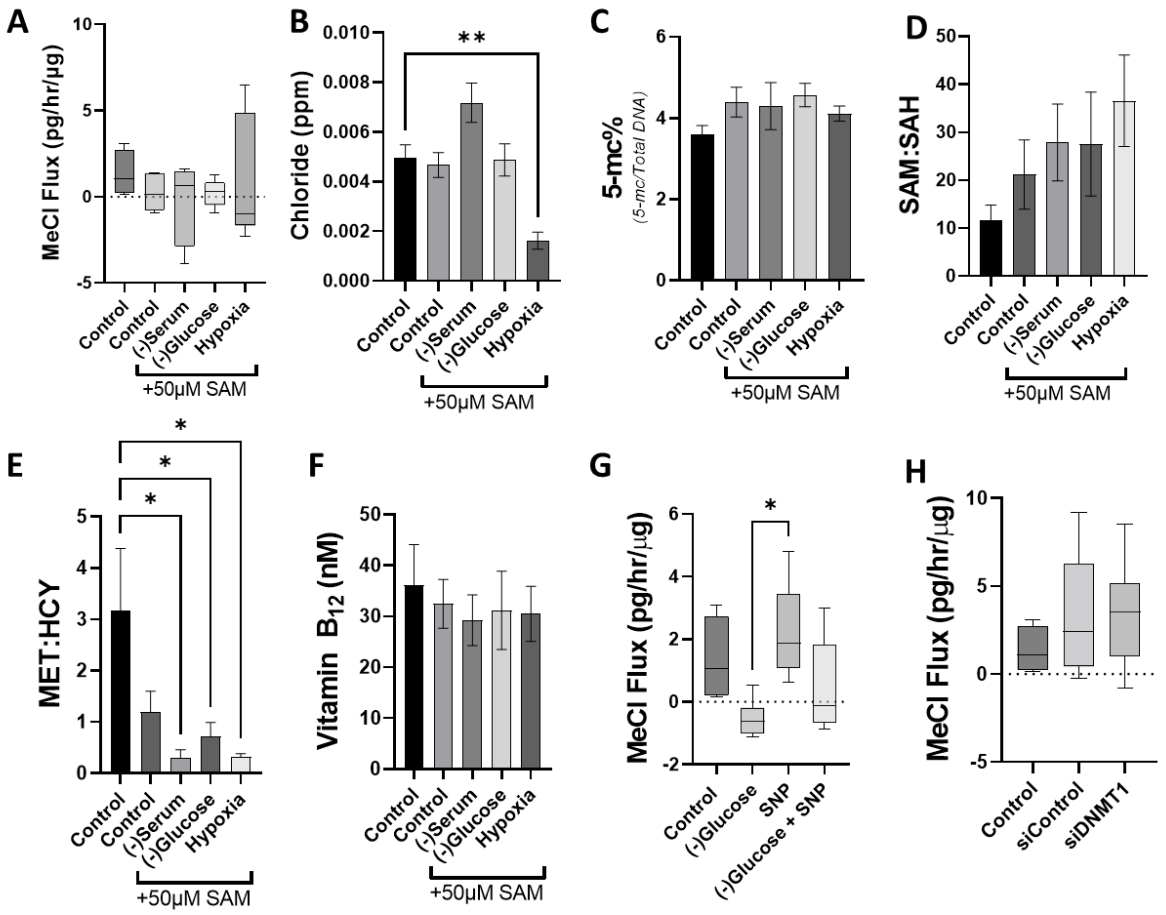


Figure 4. **SAM recovers MeCl flux in starvation conditions. (A)** MeCl flux (pg/hr/µg) of MDA-MB-231 cells treated S-adensylmethionine (SAM) under control conditions or without (-) serum, glucose, hypoxia. (n=6). **(B)** Intracellular chloride content of cells under starvation conditions or control, treated with SAM, in part per million (ppm) (n=4). **(C)** DNA methylation of cells under starvation conditions or control, treated with SAM, given as percentage 5-methylcytosine of total DNA (n=4). **(D)** Ratio of SAM to s-adenosyl-homocysteine (SAH) for cells under starvation conditions or control, treated with SAM (n=4). **(E)** Ratio of methionine (MET) to homocysteine (HCY) for cells under starvation conditions or control, treated with SAM (n=4). **(F)** Vitamin B12 (nM) for cells under starvation conditions or control, treated with SAM (n=4). ). **(G)** MeCl flux (pg/hr/µg) of glucose starved cells treated with 400 µM sodium nitroprusside (SNP) (n=6). **(H)** MeCl flux (pg/hr/µg) of untreated cells or those treated si-Control or si-DNMT1 (n=6). Bar plots shown with mean ± SEM. One-way ANOVA with Tukey post hoc test was performed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

## S-adenosylmethionine treatment recovers methyl chloride flux response under starvation

Because MeCl release by cells was suspected of being a product of methyl transferase activity, treatment with the primary substrate for methyl transferases, SAM, was performed for cells under starvation and MeCl levels quantified. In contrast to untreated cells, treatment of MDA-MB-231 (starved and control) with SAM resulted in no significant differences in MeCl fluxes between starved and unstarved cells (Figure 4A). While there was an observable decrease in MeCl within all treatments compared to control, glucose starved cells which, when untreated, actively consumed MeCl, no longer consumed MeCl under SAM treatment (Figure 4A).

Control and glucose starved cells show no change in chloride content following SAM treatment (Figure 4B). Serum starvation with SAM treatment produced a slight accumulation of chloride (Figure 4B), but to a lesser extent than serum starvation alone (Figure 3B). Cells in hypoxia with SAM supplementation, showed a significant reduction in chloride accumulation (Figure 4B), far reduced compared to cells in hypoxia alone (Figure 3B). SAM supplementation, in all cases reduced the accumulation of chloride in starved MDA-MB-231. SAM supplementation in serum and glucose starvation media produced no significant changes in media background volatile profiles (Figure S5A-C).

SAM is synthesised by methionine adenosyltransferase (MAT) (Lu 2000), and levels were investigated in starvation and SAM supplemented MDA-MB-231. Starvation of serum and glucose revealed a lower level of expression, shown by western blot (Figure S5D), which was significant when quantified (Figure S5E). SAM supplementation alone revealed little change compared to control and starvation with SAM revealed a similar trend, however slightly reduced (Figure S5D and E).

## S-adenosylmethionine treatment recovers methylation response under starvation

Protein arginine methyl transferase 1 (PRMT1) was investigated, to see if this methyl transferase also altered under starvation. Western blot revealed PRMT1 reduced considerably under starvation conditions (w/o serum or glucose) compared with control (Figure S5D), however this was not statically significant with 3 replicates (Figure S5F). SAM supplementation appeared to increase PRMT1 in control conditions and recover this effect in starvation conditions (Figure S5D and F). Further to this, DNMT1 mRNA clearly shows a decrease in hypoxic conditions (Figure S5G).

Cells under starvation showed no significant changes in DNA methylation when treated with SAM (Figure 4C). Methylation potential (SAM:SAH ratio) increased in cells treated with SAM and was further increased in cells under starvation (Figure 4D), however, this increase was not as pronounced as that seen in cells under starvation without SAM (Figure 3E). Intracellular SAM, increased to a lesser extent in cells under starvation with SAM supplementation (Figure S5H) than cells under starvation alone (Figure S3C). Effects upon intracellular SAH were also reduced in cells under starvation with SAM supplementation (Figure S5I) than those under starvation alone (Sigure S3D).

## Methionine and homocysteine under starvation conditions with s-adensylmethionine

Methionine to homocysteine ratio in MDA-MB-231 cells was drastically reduced under starvation conditions with SAM treatment (Figure 4E) in stark contrast to the increases seen in cells under starvation alone, where this ratio increased (Figure 3F). Methionine levels increased, but not significantly in all SAM treated cells (Figure S5J), as with cells under starvation alone but homocysteine levels drastically increased (Figure S5K), whereas they decreased under starvation alone (Figure S3F). Further to this, methionine synthase (MTR) levels from public RNA data, show no change under hypoxia compared to normoxia (Figure S6A). Vitamin B12 levels did not change under treatment with SAM (Figure 4F).

To determine if consumption of MeCl, which is pronounced in glucose starved cells is a result of MTR activity, cells were treated with sodium nitroprusside (SNP) in control and glucose starvation conditions (Figure 4G). SNP treatment produced a slight increase in MeCl flux, a similar amount to that consumed under glucose starvation (Figure 4G and 3A). Treatment of glucose starved cells with SNP effectively removed consumption of MeCl by these cells (Figure 4G).

This data shows that SAM is linked to MeCl production but not methionine metabolism.

## Knockdown of DNMT1 does not alter MeCl flux in MDA-MB-231

To determine if DNMT1 was driving MeCl production, cells were treated with siRNA for DNMT1 or a si-scramble as control. Knockdown was successful, shown by western blot (Figure S6B), however no change was observed in MeCl flux in these conditions (Figure 4H).

## Glutathione and reactive oxygen species are not linked to methyl chloride flux under starvation

Glutathione synthesis is driven by methionine and it is linked to epigenetic changes and methylation activity (Garcia-Gimenez and Pallardo 2014; Sedillo and Cryns 2022). Glutathione levels were investigated to see if their levels corresponded with MeCl flux. In glucose starvation, glutathione reduced compared to serum and control conditions (Figure S6C). Treatment with SAM did not alter glutathione levels in control cells and produced no changes under serum or glucose starvation (Figure S6C). Therefore, alterations in MeCl were not associated with glutathione

Reactive oxygen species (ROS) have been linked to VOCs in the breath and in cellular headspace (Issitt et al. 2022b). To check for changes in ROS, as increases could be responsible for observed increases and decreases in VOCs an amplex red assay was performed for cells under starvation conditions and with SAM treatment. Previously we have published a significant reduction in ROS for MDA-MB-231 in hypoxic conditions for 24 hours (Issitt et al. 2023). Under serum and glucose starvation following 24 hours, ROS was slightly elevated compared to control (Figure S6D). Treatment with SAM produced a slight increase in ROS in control cells, a significant increase in serum starved and no change in glucose starvation (Figure S6D). Together with previously published results (Issitt et al. 2023), these results suggest the VOCs studied here are not associated with variations in cellular ROS.

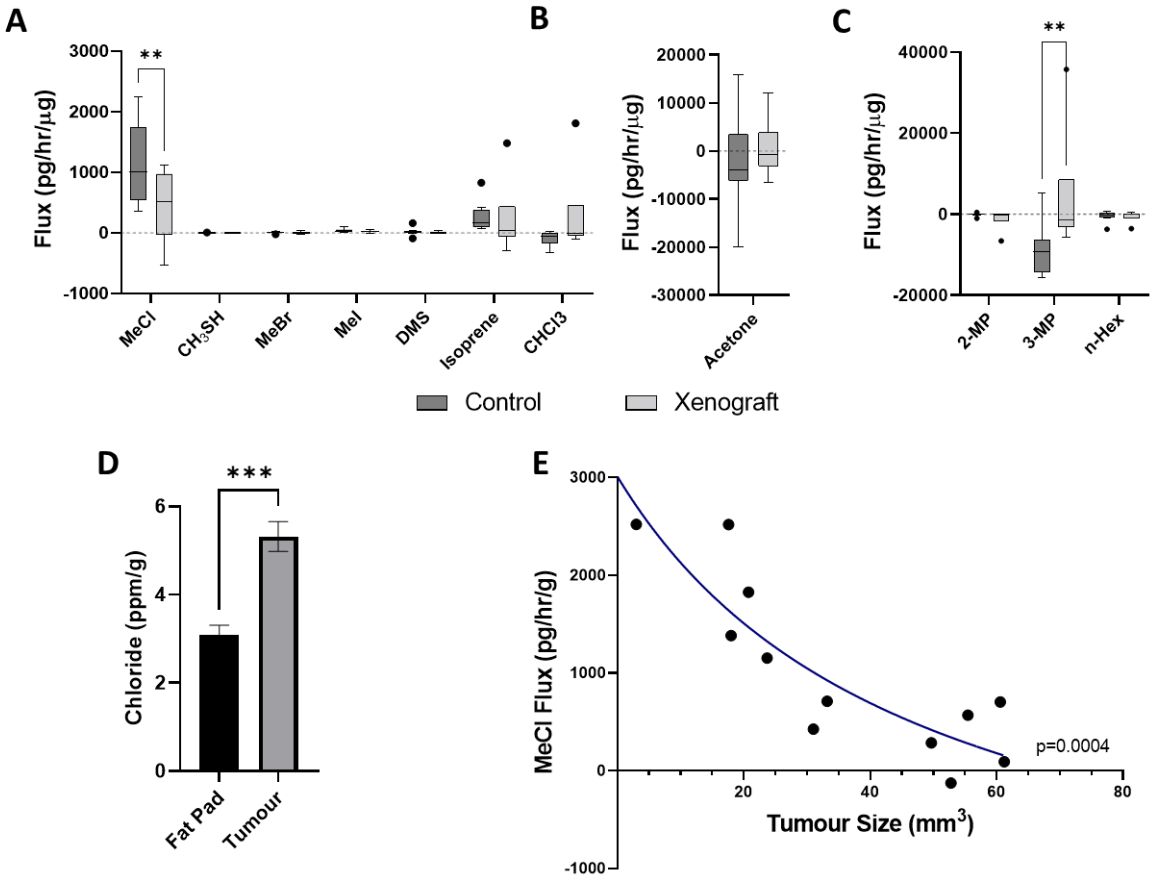


Figure 5. **Volatile flux (g/hr/g) of mice breath in control and MDA-MB-231 xenograft tumour bearing mice. (A-C)**. Faecal VOCs subtracted and normalised to mouse weight. **(D)** Intra-tumour and contralateral mammary fat chloride content in ppm. **(E)** Methyl chloride (MeCl) flux (pg/hr/g) of mouse breath (faecal MeCl flux subtracted and mouse weight normalised) for tumours of varying size ($mm^3$). $CHCl_3$ = Chloroform, DMS = Dimethyl sulphide, MeBr = Methyl bromide, MeI = Methyl iodide, MeSH = Methanoethiol. Boxplot whiskers show median ± Tukey distribution (n=6). Bar plot **(D)** shown with mean ± SEM. Two-way ANOVA with Bonferroni post hoc test was performed for **A** and **C**. Students T-test performed for **D**. Pade (1,1) approximant, least squares fit with non-linear fit test performed, shown by line of fit in **E**.

## Volatile flux is altered in the breath of MDA-MB-231 tumour xenograft mice

The breath of mice with and without tumour xenografts was investigated to determine if VOC profiles of MDA-MB-231 would transfer to the breath of mice. These mice are age and sex matched, with consistent sampling time point sampling at 4 weeks post protocol. Two VOCs were altered (Figure 5), MeCl was significantly reduced (Figure 5A) and 3MP was significantly

increased (Figure 5C) compared with control mice. This method requires the subtraction of faecal headspace from the chamber without the mice in, and no variations were seen in the VOCs from faecal material (Figure S7A).

Because we have shown consumption or reduced levels of MeCl correlating with accumulation of intracellular chloride, chloride was investigated in tumours compared to the mammary fat pad without xenograft. Using this comparator, chloride was significantly increased in tumours compared with the contralateral mammary fat pad (from 3 ppm to 5 ppm, Figure 5D).

Tumour size was compared to MeCl flux, which revealed MeCl flux reducing as tumour size increased (Figure 5E). A non-linear regression with padé fit, showed this trend to be significant ($p=0.0004$). Chloride and DNA methylation content of these same tumours was investigated, however no significant trends were observed (Figure S7D and E).

## Overview of results

Briefly, cellular volatiles are significantly altered under serum and glucose starvation. Consistently, methyl chloride (MeCl), which is produced by cells, is taken up under stress. This uptake is matched by chloride accumulation. Variations in baseline cell type MeCl release correlates with DNA methylation, but not activity of the methyl-transfersome as a whole. Starvation induced MeCl uptake, with reduction in global DNA methylation, increases in methylation potential and changes in associated metabolites. Publicly available RNA sequencing data supports these findings by revealing DNMT1 as a commonly reduced gene between starvation conditions and cell types where MeCl is reduced. Blocking methylation activity in cells produces a switch from production to uptake of MeCl but knockdown of DNMT1 produced no change. RNA data reveals significantly reduced enrichment of the methytransfersome under starvation conditions, suggesting MeCl production is a result of global methyl transferase activity and not DNMT1 alone. This is further supported by supplementation with SAM in starvation conditions lessening the effect of starvation upon MeCl. Further to this, active consumption of MeCl is linked to methionine synthase, which is supported by methionine synthase blocking agents removing starvation effects upon MeCl. The usefulness of these volatile biomarkers is further validated in the breath of cancer bearing mice, which show significantly reduced MeCl.

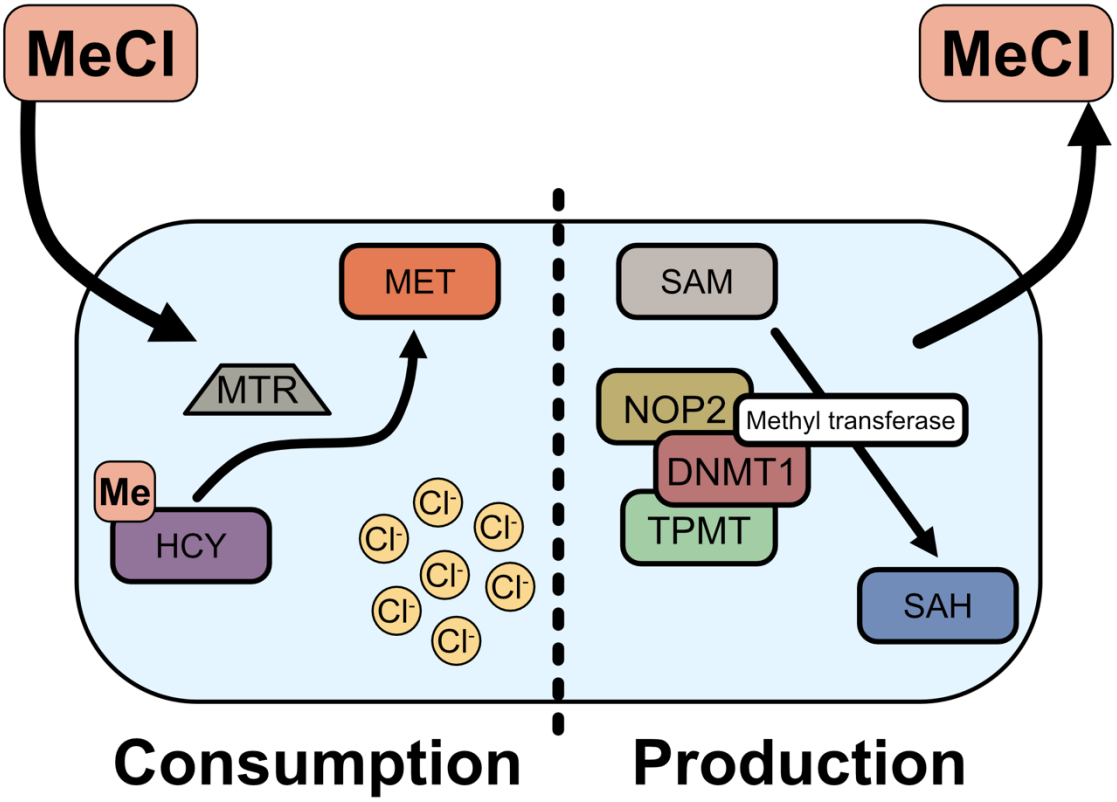


Figure 6. **Proposed mechanisms of methyl chloride (MeCl) consumption and production.** MeCl consumption by cells is through the action of methionine synthase (MTR), generating methionine (MET) through the addition of a methyl group from MeCl to homocysteine (HCY), resulting in an accumulation of chloride ions. Production of MeCl by cells through the activity of S-adenosylmethionine (SAM) dependent methyl transferases which use SAM as a methyl donor, producing S-adenosyl-L-homocysteine.

# Discussion

This research has utilised a methodology capable of measuring cellular metabolism of VOCs in models of tumour pathological environment, namely serum, glucose and oxygen starvation, to discover biomarkers capable of distinguishing cancer in the breath of tumour bearing mice.

DNA methylation profiles within cancer, manifesting as hypomethylation across the genome and hypermethylation for specific loci, present a current emerging technology for cancer diagnostics (Papanicolau-Sengos and Aldape 2022; Li et al. 2023), with large scale ongoing human trials (Neal et al. 2022; Pons-Belda et al. 2022). We present, through this work, methyl chloride (MeCl) production as a novel biomarker of methylation, present in human breath.

## Cellular processing of VOCs – biomarkers of methylation

Volatile flux linked to mechanistic process has been demonstrated in this research, primarily MeCl both as a product of methyl transferase activity and as a potential substrate for methionine synthesis. To highlight this process, a schematic is presented in Figure 6. Here, the activity of methyl transferases, generates MeCl and the consumption of MeCl is driven by methionine synthase, in place of vitamin b12, resulting in the observed accumulation of chloride ions. We have demonstrated that MeCl levels rise and fall alongside DNA methylation levels, however, knockdown of DNMT1, the primary enzyme for DNA methylation, did not alter MeCl flux. Protein alignment of MeCl producing enzymes in plants with the human database revealed a methyl transferase (TPMT) as a likely candidate. We determined that DNA methylation alone may not drive MeCl production as publicly available data shows a down regulation of methyl transferases under hypoxic conditions and treatment with 5-AZA, which blocks a range of methylation events, prevented release of MeCl. Furthermore, treatment with SAM, prevented the significant reduction of MeCl production under starvation conditions. Additional SAM, in this instance may promote methyl transferase activity, increasing MeCl production. Which is supported by evidence that cells under hypoxic conditions are hypo methylated (Shahrzad et al. 2007), potentially through the impairment of the production of SAM in hypoxic conditions (Chawla et al. 1996; Avila et al. 1998).

MeCl, is a potent methylating agent and is exhaled in human breath in the range of 2.5 to 33 parts per billion by volume (ppbv), 60 times that of inhaled air (Keppler et al. 2017). Isoprene is the most studied VOCs in human breath (Mochalski et al. 2023), with reported concentrations of 100ppbv (at rest) and much higher concentrations following exercise (Mochalski et al. 2023). Compared to isoprene and other compounds such as acetone (1.2-1880 ppbv), methanol (160-2000 ppbv) and ethanol (13-1000 ppbv) (Fenske and Paulson

1999), MeCl, is found in lower amounts but is still a considerable component of human breath. MeCl described in the breath of healthy humans (Statheropoulos et al. 2005; Keppler et al. 2017; Drabinska et al. 2021), appears to be altered little in the breath of smokers (Filipiak et al. 2012) but we have yet to find evidence of it altered in the breath of cancer patients.

Because starvation of glucose, hypoxia and treatment with 5-AZA not only prevented production of MeCl but generated an active consumption by cells, we propose that MeCl is both used and produced by cells. This 'push-pull' model presents a novel method for methionine generation, an essential amino acid for protein synthesis and other biochemical reactions required for cell viability and growth (Sedillo and Cryns 2022). Methionine is used heavily by cancer cells in growth and response to stress (Sedillo and Cryns 2022) and the stress responses shown here could be linked to this dependence. MDA-MB-231 cells under hypoxic conditions have been shown to have increased levels of methionine (Tsai et al. 2013), in line with our findings. Furthermore, we have shown cells under variable stress consuming MeCl, including chemotherapeutic stress with Doxorubicin (Issitt et al. 2022a), which has been shown to increase intra and extra cellular methionine in skeletal muscle cells (Fabris and MacLean 2018).

The balance between production and consumption of MeCl is observed through the breath of mice in this study, where by larger tumours have increased levels of cells persisting in conditions with low nutrient delivery. This is observed in the breath of mice with larger tumours having reduced levels of MeCl compared to smaller tumours (Figure 5E). Increased methionine levels and decreased methylation in cells under stress, may explain reduced MeCl in the breath of tumour bearing mice. MeCl consumption through methionine synthesis coupled with a decrease in release through reduced methylation may explain the observed trends, supporting the mechanism presented in figure 6. Furthermore, intra-cellular methionine levels have been shown to increase with tumour size (Kawaguchi et al. 2018).

## Limitations of the study

More research is required to describe these mechanisms in detail and here we present a hypothesis upon which to build. We have shown across multiple models, VOC flux in response to stress and generated evidence to describe the consumption and production of methyl chloride as a cellular mechanism. However, we have presented a consistent and translational biomarker of cellular stress and pathology both in this work and in previously published work (Keppler et al. 2017; Issitt et al. 2022a; Shahi et al. 2022; Issitt et al. 2023), and provided evidence to begin to describe the mechanism of metabolism.

## Conclusion

The methodology described here for both mouse breath (static headspace) and cellular headspace, as previously described (Issitt et al. 2022a; Issitt et al. 2023), takes two headspace samples to determine flux of VOC over time. This methodology varies from the majority of breath and cellular VOC approaches which often concentrate VOCs onto materials for thermal desorption of a single time point (Issitt et al. 2022b). While the presented method is less automated, it allows description of active consumption, which we have shown here to be important, descriptive characteristics of stress. In mice breath for example, while levels of 3-MP in the breath of tumour bearing mice is no different from zero, it is significantly different from control, where mice are consuming this VOC. How these results will translate to a clinical application remains to be seen but it highlights the need for alternate approaches for volatile biomarker discovery, while considering international benchmarking (Wilkinson et al. 2021).

By attempting to model tumour environment in cell models, measuring select VOCs and then testing the breath of tumour bearing mice for those same targets, we identified two VOCs (MeCl and 3-MP) out of seven which were significantly altered in the cellular model (Figure 1), which translated to the mouse model (Figure 5). We have then attempted to describe likely mechanisms for MeCl flux (Figure 6), within the context of methylation and methionine synthesis, both of which are altered in cancer (Klutstein et al. 2016; Xie et al. 2019; Sedillo and Cryns 2022).

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## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

Conceptualization, T.I., W.J.B. and K.R.R.; Data curation, T.I.; Formal analysis, T.I., A.M.; Funding acquisition, S.T.S., W.J.B. and K.R.R.; Investigation, T.I.; Methodology, T.I., A.M. and K.R.R.; Project administration, T.I. and S.T.S.; Resources, , W.J.B.; Visualization, T.I., A.M.; Writing—original draft, T.I.; Writing—review and editing, S.T.S., W.J.B., M.R. and K.R.R.

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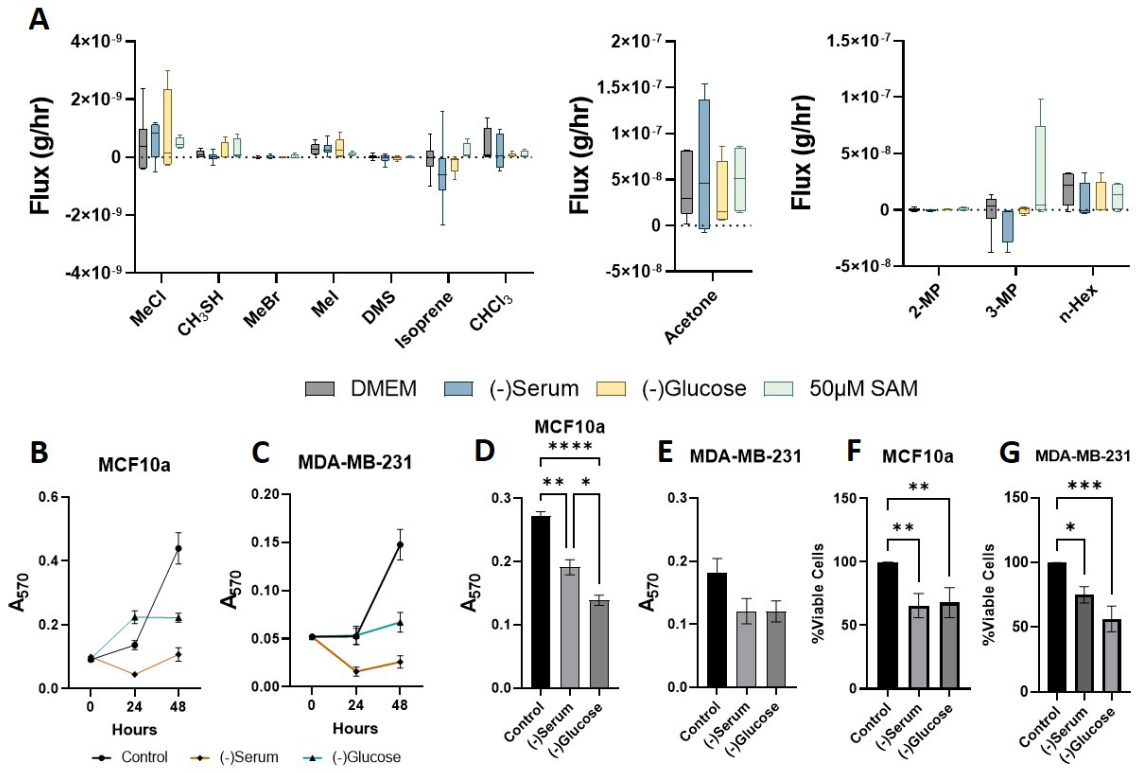
Supplementary Material




Supplementary Figure 1**. Volatile flux for media backgrounds and cell response to starvation.** Select volatile compound flux (g/hr$^{-1}$) for media control conditions **(A).** SAM, S-adensolymethionine; $CHCl_3$, chloroform; DMS, dimethyl sulfide; MeBr, methyl bromide; MeCl, methyl chloride; MeI, methyl iodide; MeSH, methanoethiol; 2-MP, 2 methyl pentane; 3-MP, 3 methyl pentane; n-Hex, n-hexane. Boxplot whiskers show median ± Tukey distribution (n=6). **(B,C)** Cell growth curves measured by sulphorhodamine B assay for MCF10a **(B)** and MDA-MB-231 **(C)** (n=3). **(D)** MTT assay for cells following 24 hour starvation (n=3). **(D)** Cell viability/death measured by trypan blue assay for MCF10a **(F)** and MDA-MB-231 **(G)** (n=3). Mean One-way ANOVA with tukey post hoc analysis performed for **D-G,** error bars are mean ± SEM, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

**A**

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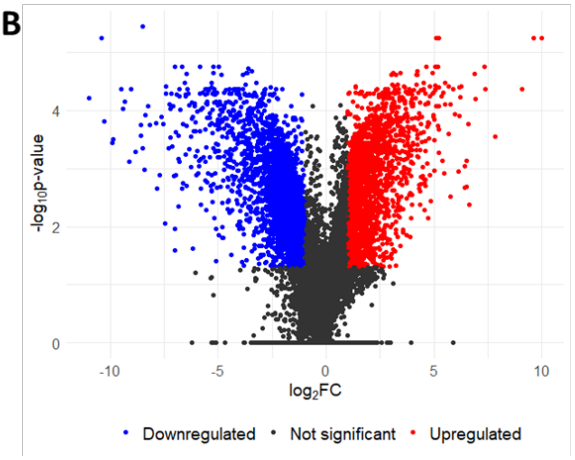
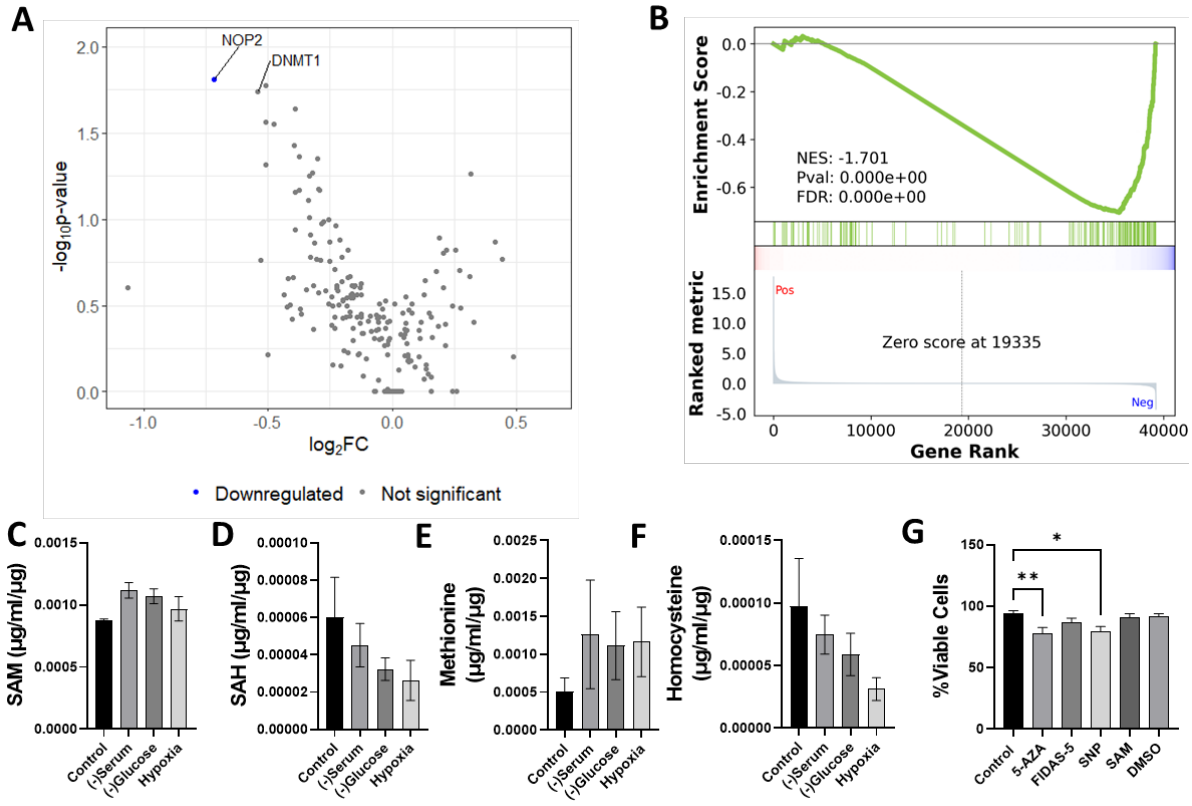
tr|Q9ZSZ7|Q9ZSZ7_BATMA      MSTVANIAPVFTGDCKTIPTP--EECATFLYKVVNSGGWEKCWVEEVIPWDLGVPTPLVL    58
sp|P51580|TPMT_HUMAN        ----------MDGTRTSLDIEEYSDTEVQKNQVLTLEEWQDKWVNGKTAFHQEQGHQLLK    50
                                     : *  .::     .:  .   :*:.   *:. **:    :.      *:
tr|Q9ZSZ7|Q9ZSZ7_BATMA      HLVKN--NALPNGKGLVPGCGGGYDVVAMANPERFMVGLDISENALKK---------ARE    107
sp|P51580|TPMT_HUMAN        KHLDTFLKGKSGLRVFFPLCGKAVEMKWFADRGHSVVGVEISELGIQEFFTEQNLSYSEE    110
                            : :..  :.  . : :.* ** . ::  :*:  : :**::*** .:::         :.*
tr|Q9ZSZ7|Q9ZSZ7_BATMA      TFSTMPNSSCFSFVKE-------DVF--TWRPEQPFDFIFDYVFFCAIDPKMRPAWGKAM    158
sp|P51580|TPMT_HUMAN        PITEIPGTKVFKSSSGNISLYCCSIFDLPRTNIGKFDMIWDRGALVAINPGDRKCYADTM    170
                             :: :*.:. *.  .        .:*         **:*:*   : **:*  * .:..:*
tr|Q9ZSZ7|Q9ZSZ7_BATMA      YELLKPDGELI--TLMYPITNHEGGPPFSVSESEYEKVLVPLGFKQLSLEDYSDLAVEPR    216
sp|P51580|TPMT_HUMAN        FSLLGKKFQYLLCVLSYDP-TKHPGPPFYVPHAEIERLFGKICNIR-CLEKVDA------    222
                            :.**  . : :  .* *   .:. **** * .:* *::: :   : .**. .
tr|Q9ZSZ7|Q9ZSZ7_BATMA      KGKEKLARWKKMNN----------230
sp|P51580|TPMT_HUMAN        -FEERHKSWGIDCLFEKLYLLTEK 245
                              :*:   *
```

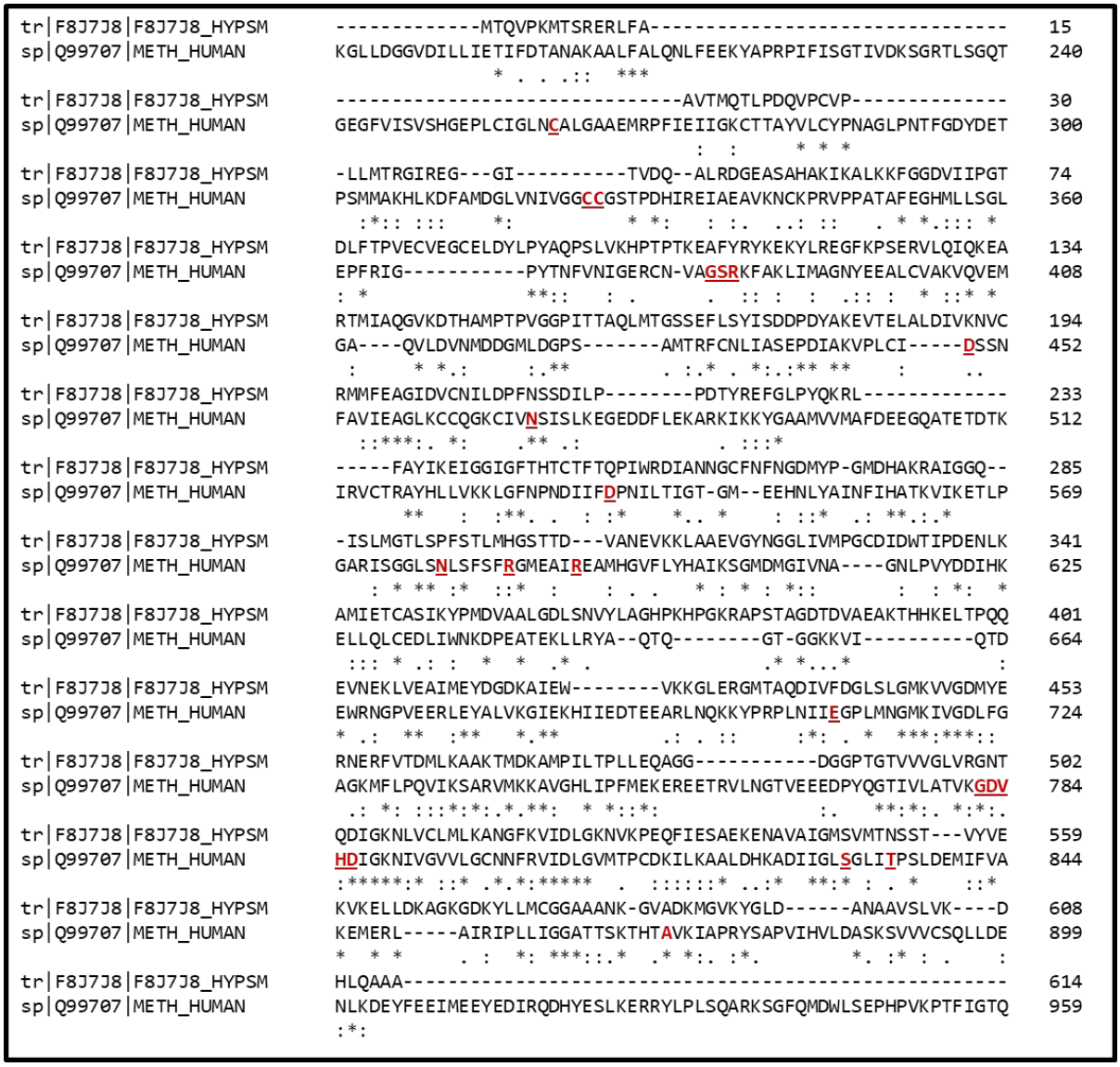
**B**



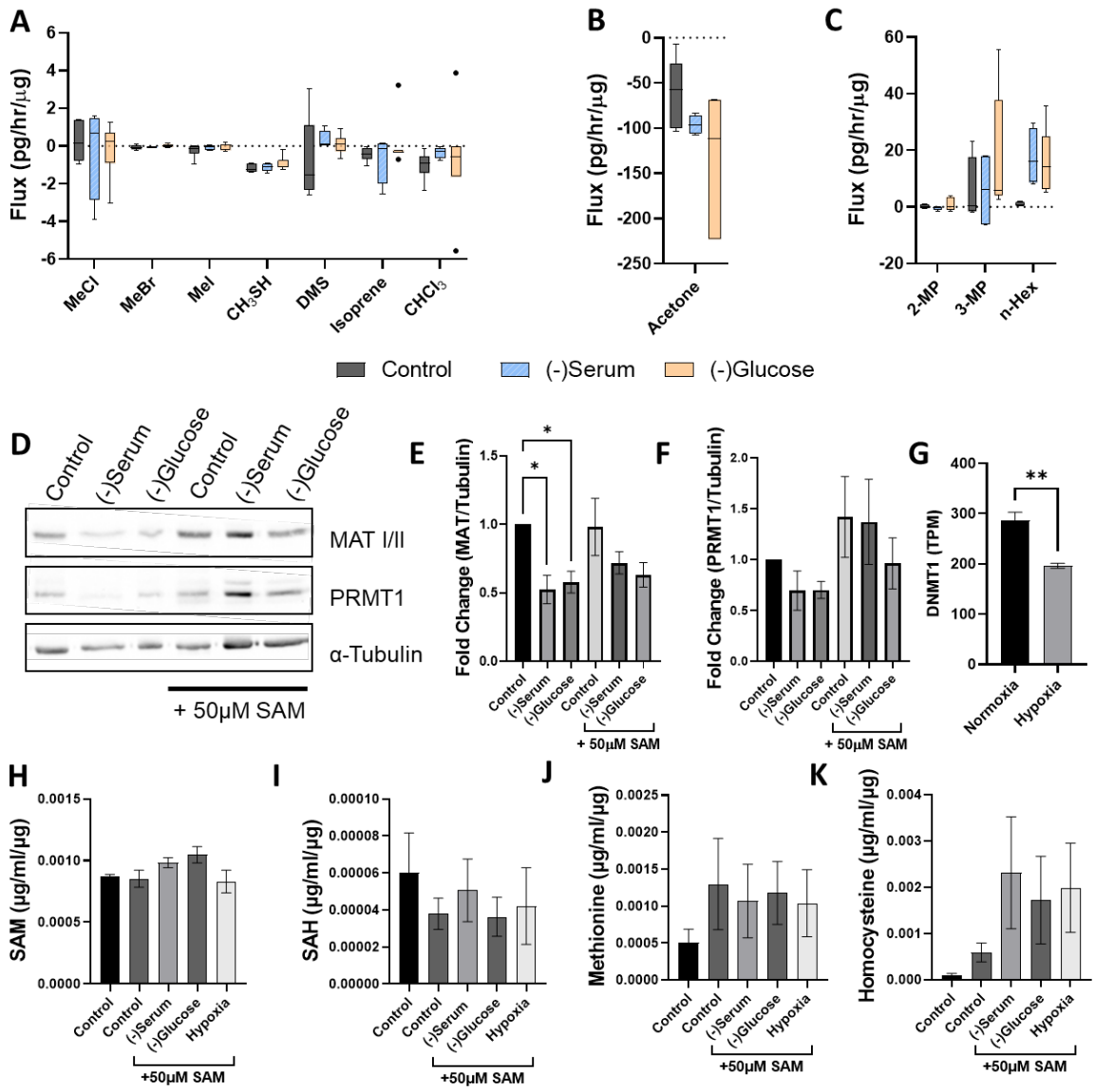

Supplementary Figure 2**. Protein alignment for TPMT and methyl chloride transferase and volcano plot. (A)** Alignment results from UNIPROT for human TPMT (thiopurine methyltransferase, UNIPROT ID: P51580) and plant methyl chloride transferase (*Batis maritima,* UNIPROT ID: Q9ZSZ7*).* Alignment results revealed 24.8% similiarity, the most similar protein in the UNIPROT human proteasome against methyl chloride transferase. ***** = same residue **:** , **.** =closely linked residues. Underlined and red sections show binding domains. **(B)** Volcano plot of RNA seq data for -log10 LRT (likelihood ratio test) q values (corrected p values using Benjamini-Hochberg) vs mean log2 fold change of MDA-MB-231 vs MCF10a.




Supplementary Figure 3**. Effects of starvation upon mRNA and methylation metabolites in MDA-MB-231. (A)** Volcano plot of RNA seq data for -log10 LRT (likelihood ratio test) q values (corrected p values using Benjamini-Hochberg) vs mean log2 fold change of hypoxia vs normoxia. **(B)** Gene set enrichment analysis results of RNA data from **(A)**. S-adenosylmethioine (SAM) **(C)**, S-adenosyl-L-homocysteine (SAH) **(D)**, Methioine **(E)** and homocysteine **(F)** intracellular MDA-MB-231 content in µg/ml/ug, for cells under starvation conditions or control. **(G)** Percentage viable cells for MDA-MB-231 cells following 24 hours treatment with 10 µM 5-azacytadine (5-AZA), 10 µM FIDAS-5 or 400 µM sodium nitroprusside (SNP), 50 µM SAM or 0.00005% DMSO measured by Trypan blue assay. Bar plots shown with mean ± SEM, n=4 **.** One-way ANOVA with Tukey post hoc analysis performed for all bar charts. *$p < 0.05$; **$p < 0.01$.

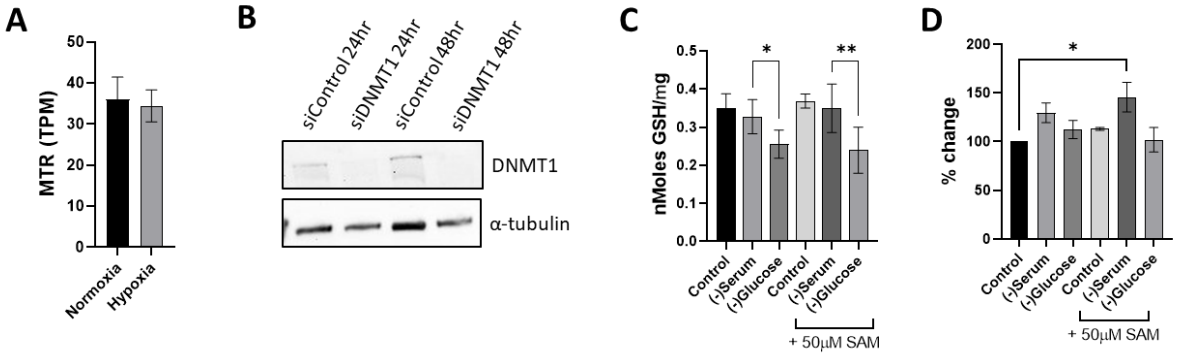



Supplementary Figure 4**. Protein alignment of the methylotroph cmuA protein and human methionine synthase.** Alignment results from UNIPROT for human MTR (methionine synthase, UNIPROT ID: Q99707) and bacterial chloromethane (methyl chloride) methyltransferase (UNIPROT ID: F8J7J8*).* Alignment results revealed 31.08% similarity, the most similar protein in the UNIPROT human proteasome against chloromethane methyl transferase. *= same residue : , . =closely linked residues. Underlined and red sections show binding domains.

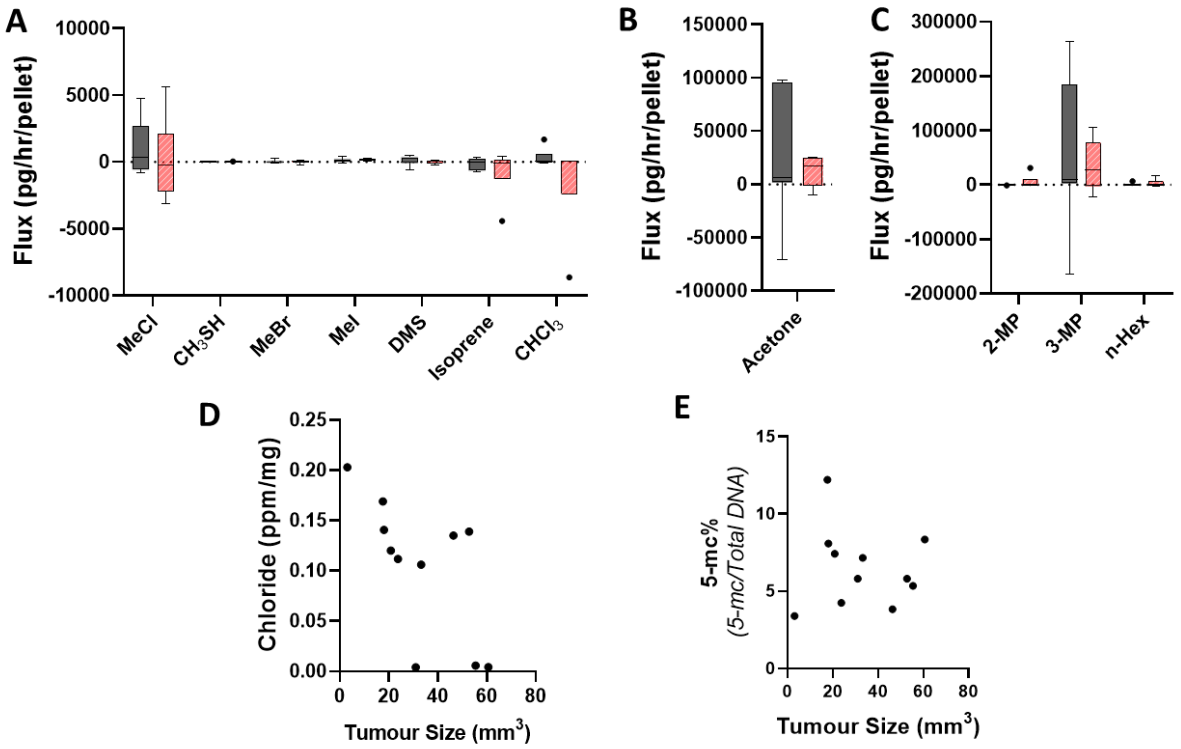



Supplementary Figure 5**. Effects of S-adenosylmethionine (SAM) treatment upon MDA-MB-231 cells. (A-C)** Volatile flux (pg/hr/µg) for MDA-MB-231 in control media, media without serum or media without glucose, all supplemented with 50 µM SAM. Media subtracted and protein-normalised. **(D)** Representative western blot of MDA-MB-231 cell lysates probed for MATI/II, PRMT1 and α-Tubulin in starvation conditions with or without SAM. **(E)** Quantification of MATI/II western blots by densitometry analysis of conditions in **D** normalised to α-tubulin and expressed as fold change compared to control **(F) )** Quantification of PRMT1 western blots by densitometry analysis of conditions in **D** normalised to α-tubulin and expressed as fold change compared to control. **(G)** DNMT1 RNA levels in transcripts per million (TPM) of MDA-MB-231 cells in normoxic or hypoxic conditions from publicly available data. S-adenosylmethioine (SAM) **(H)**, S-adenosyl-L-homocysteine (SAH) **(I)**, Methioine **(J)** and homocysteine **(K)** intracellular MDA-MB-231 content in µg/ml/ug, for cells under starvation conditions or control supplemented with SAM. (-)Serum = media without serum, (-)Glucose =

media without glucose. $CHCl_3$ = Chloroform, DMS = Dimethyl sulphide, MeBr = Methyl bromide, MeCl = Methyl chloride, MeI = Methyl iodide, MeSH = Methanoethiol. Boxplot whiskers show median ± Tukey distribution (n=6). Two-way ANOVA with Bonferroni post hoc test was performed for **A-C**. Bar plots shown with mean ± SEM. One way ANOVA with Tukey posthoc analysis performed for bar charts **E, F, H-K**. Students T-test performed for **G**. *$p$ < 0.05; ***$p$ < 0.001.




Supplementary Figure 6**. Glutathione and reactive oxygen species investigation and DNMT1 knockdown in MDA-MB-231. (A)** MTR (methionine synthase) RNA levels in transcripts per million (TPM) of MDA-MB-231 cells in normoxic or hypoxic conditions from publicly available data. (n=3) . **(B)** Representative western blot of MDA-MB-231 cell lysates probed for DNMT1 and α-Tubulin in treated with si-RNA targeting DNMT1 or scrambled control at 24 hours or 48 hours post treatment. **(C)** Glutathione content of MDA-MB-231 cells, normalised to protein content (GSH/mg) in nMoles, for cells in control, serum or glucose starvation with our with out S-adenslymethioine (SAM) treatment (n=3). **(D)** Amplex red assay for reactive oxygen species. Treatment conditions as with **C**, expressed as percentage change compared to control (n=3). Bar plots shown with mean ± SEM. One way ANOVA with Tukey posthoc analysis performed for bar charts **C** and **D**. *$p$ < 0.05; **$p$ < 0.01.




Supplementary Figure 7**. Volatile flux and tumour chloride and DNA methylation content from MDA-MB-231 tumour xenograft bearing mice. (A-C)** . Volatile flux (g/hr/pellet) of mouse faecal pellets from control and MDA-MB-231 xenograft tumour bearing mice. Normalised to number of pellets (n=6). **(D)** Chloride content in parts per million (ppm) normalised to weight of sample in mg compared to total tumour size in $mm^3$. **(E)** 5-methyl cytosine (5-mc) content as percentage of total DNA for tumours compared to total tumour size in $mm^3$.

| ENSEMBL ID | Gene ID | Approved symbol | Approved name | Location |
|---|---|---|---|---|
| ENSG00000188573.8 | FBLL1 | FBLL1 | fibrillarin like 1 | 5q34 |
| ENSG00000169519.21 | METT5D1 | METTL15 | methyltransferase like 15 | 11p14.1 |
| ENSG00000244026 | FAM86D | FAM86DP | family with sequence similarity 86 member D, pseudogene | 3p12.3 |
| ENSG00000214756.8 | METTL12 | CSKMT | citrate synthase lysine methyltransferase | 11q12.3 |
| ENSG00000227835 | CARM1L | CARM1P1 | coactivator associated arginine methyltransferase 1 pseudogene 1 | 9p24.2 |
| ENSG00000107614.22 | TRDMT1 | TRDMT1 | tRNA aspartic acid methyltransferase 1 | 10p13 |
| ENSG00000100462.16 | PRMT5 | PRMT5 | protein arginine methyltransferase 5 | 14q11.2 |
| ENSG00000101654.18 | RNMT | RNMT | RNA guanine-7 methyltransferase | 18p11.21 |
| ENSG00000132275.11 | RRP8 | RRP8 | ribosomal RNA processing 8 | 11p15.4 |
| ENSG00000071462.12 | WBSCR22 | BUD23 | BUD23 rRNA methyltransferase and ribosome maturation factor | 7q11.23 |
| ENSG00000168806.8 | LCMT2 | LCMT2 | leucine carboxyl methyltransferase 2 | 15q15.3 |
| ENSG00000145194 | ECE2 | ECE2 | endothelin converting enzyme 2 | 3q27.1 |
| ENSG00000185238.13 | PRMT3 | PRMT3 | protein arginine methyltransferase 3 | 11p15.1 |
| ENSG00000241644.2 | INMT | INMT | indolethylamine N-methyltransferase | 7p14.3 |
| ENSG00000171806.12 | C1orf156 | METTL18 | methyltransferase like 18 | 1q24.2 |
| ENSG00000169093.16 | ASMTL | ASMTL | acetylserotonin O-methyltransferase like | Xp22.3_Yp11.3 |
| ENSG00000145002.13 | FAM86B2 | FAM86B2 | family with sequence similarity 86 member B2 | 8p23.1 |
| ENSG00000174912 | METT5D2 | METTL15P1 | methyltransferase like 15 pseudogene 1 | 3q25.31 |
| ENSG00000141744.4 | PNMT | PNMT | phenylethanolamine N-methyltransferase | 17q12 |
| ENSG00000093010.15 | COMT | COMT | catechol-O-methyltransferase | 22q11.21 |
| ENSG00000120265.19 | PCMT1 | PCMT1 | protein-L-isoaspartate (D-aspartate) O-methyltransferase | 6q25.1 |
| ENSG00000105202.9 | FBL | FBL | fibrillarin | 19q13.2 |
| ENSG00000130816.17 | DNMT1 | DNMT1 | DNA methyltransferase 1 | 19p13.2 |
| ENSG00000166741.8 | NNMT | NNMT | nicotinamide N-methyltransferase | 11q23.2 |
| ENSG00000111641.12 | NOP2 | NOP2 | NOP2 nucleolar protein | 12p13.31 |
| ENSG00000196433.13 | ASMT | ASMT | acetylserotonin O-methyltransferase | Xp22.3_Yp11.3 |
| ENSG00000169710.9 | FASN | FASN | fatty acid synthase | 17q25.3 |

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|---|---|---|---|---|
| ENSG00000150540.14 | HNMT | HNMT | histamine N-methyltransferase | 2q22.1 |
| ENSG00000160310.19 | PRMT2 | PRMT2 | protein arginine methyltransferase 2 | 21q22.3 |
| ENSG00000037474.15 | NSUN2 | NSUN2 | NOP2/Sun RNA methyltransferase 2 | 5p15.31 |
| ENSG00000130005.13 | GAMT | GAMT | guanidinoacetate N-methyltransferase | 19p13.3 |
| ENSG00000124713.6 | GNMT | GNMT | glycine N-methyltransferase | 6p21.1 |
| ENSG00000164603.12 | C7orf60 | BMT2 | base methyltransferase of 25S rRNA 2 homolog | 7q31.1 |
| ENSG00000126814.7 | TRMT5 | TRMT5 | tRNA methyltransferase 5 | 14q23.1 |
| ENSG00000291151 | NSUN5P1 | NSUN5P1 | NSUN5 pseudogene 1 | 7q11.23 |
| ENSG00000110871.15 | COQ5 | COQ5 | coenzyme Q5, methyltransferase | 12q24.31 |
| ENSG00000203791.15 | METTL10 | EEF1AKMT2 | EEF1A lysine methyltransferase 2 | 10q26.13 |
| ENSG00000162639.16 | C1orf59 | HENMT1 | HEN methyltransferase 1 | 1p13.3 |
| ENSG00000101247.18 | C20orf7 | NDUFAF5 | NADH:ubiquinone oxidoreductase complex assembly factor 5 | 20p12.1 |
| ENSG00000203740.4 | METTL11B | NTMT2 | N-terminal Xaa-Pro-Lys N-methyltransferase 2 | 1q24.2 |
| ENSG00000139780.8 | C13orf39 | METTL21C | methyltransferase 21C, AARS1 lysine | 13q33.1 |
| ENSG00000005194.15 | NSUN5C | NSUN5P2 | NSUN5 pseudogene 2 | 7q11.23 |
| ENSG00000005194 | CIAPIN1 | CIAPIN1 | cytokine induced apoptosis inhibitor 1 | 16q21 |
| ENSG00000165055.16 | METTL2B | METTL2B | methyltransferase 2B, methylcytidine | 7q32.1 |
| ENSG00000164169.13 | PRMT10 | PRMT9 | protein arginine methyltransferase 9 | 4q31.23 |
| ENSG00000150756.14 | FAM173B | ATPSCKMT | ATP synthase c subunit lysine N-methyltransferase | 5p15.2 |
| ENSG00000170439.8 | METTL7B | TMT1B | thiol methyltransferase 1B | 12q13.2 |
| ENSG00000146834.15 | MEPCE | MEPCE | methylphosphate capping enzyme | 7q22.1 |
| ENSG00000003509.16 | C2orf56 | NDUFAF7 | NADH:ubiquinone oxidoreductase complex assembly factor 7 | 2p22.2 |
| ENSG00000121486.12 | TRM1L | TRMT1L | tRNA methyltransferase 1 like | 1q25.3 |
| ENSG00000066651.20 | TRMT11 | TRMT11 | tRNA methyltransferase 11 homolog | 6q22.32 |
| ENSG00000186666.6 | BCDIN3D | BCDIN3D | BCDIN3 domain containing RNA methyltransferase | 12q13.12 |
| ENSG00000143919.15 | C2orf34 | CAMKMT | calmodulin-lysine N-methyltransferase | 2p21 |
| ENSG00000165644.11 | COMTD1 | COMTD1 | catechol-O-methyltransferase domain containing 1 | 10q22.2 |
| ENSG00000127804.13 | METT10D | METTL16 | methyltransferase 16, N6-methyladenosine | 17p13.3 |

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|---|---|---|---|---|
| ENSG00000142453.12 | CARM1 | CARM1 | coactivator associated arginine methyltransferase 1 | 19p13.2 |
| ENSG00000181038.14 | C17orf95 | METTL23 | methyltransferase like 23 | 17q25.2 |
| ENSG00000139160.13 | C12orf72 | ETFBKMT | electron transfer flavoprotein subunit beta lysine methyltransferase | 12p11.21 |
| ENSG00000108592.17 | FTSJ3 | FTSJ3 | FtsJ RNA 2'-O-methyltransferase 3 | 17q23.3 |
| ENSG00000155275.19 | C4orf23 | TRMT44 | tRNA methyltransferase 44 homolog | 4p16.1 |
| ENSG00000180917.18 | FTSJD1 | CMTR2 | cap methyltransferase 2 | 16q22.2 |
| ENSG00000099899.15 | TRMT2A | TRMT2A | tRNA methyltransferase 2 homolog A | 22q11.21 |
| ENSG00000137200.13 | FTSJD2 | CMTR1 | cap methyltransferase 1 | 6p21.2 |
| ENSG00000156017.13 | C9orf41 | CARNMT1 | carnosine N-methyltransferase 1 | 9q21.13 |
| ENSG00000165171.11 | WBSCR27 | METTL27 | methyltransferase like 27 | 7q11.23 |
| ENSG00000127720.8 | C12orf26 | METTL25 | methyltransferase like 25 | 12q21.31 |
| ENSG00000010165.20 | METTL13 | METTL13 | methyltransferase 13, eEF1A lysine and N-terminal methyltransferase | 1q24.3 |
| ENSG00000186523.15 | FAM86B1 | FAM86B1 | family with sequence similarity 86 member B1 | 8p23.1 |
| ENSG00000179299.17 | NSUN7 | NSUN7 | NOP2/Sun RNA methyltransferase family member 7 | 4p14 |
| ENSG00000138780.15 | GSTCD | GSTCD | glutathione S-transferase C-terminal domain containing | 4q24 |
| ENSG00000206562.12 | METTL6 | METTL6 | methyltransferase 6, methylcytidine | 3p25.1 |
| ENSG00000241058.4 | NSUN6 | NSUN6 | NOP2/Sun RNA methyltransferase 6 | 10p12.31 |
| ENSG00000104885.19 | DOT1L | DOT1L | DOT1 like histone lysine methyltransferase | 19p13.3 |
| ENSG00000029639.11 | TFB1M | TFB1M | transcription factor B1, mitochondrial | 6q25.3 |
| ENSG00000144401.14 | FAM119A | METTL21A | methyltransferase 21A, HSPA lysine | 2q33.3 |
| ENSG00000123427.17 | FAM119B | EEF1AKMT3 | EEF1A lysine methyltransferase 3 | 12q14.1 |
| ENSG00000137760.15 | ALKBH8 | ALKBH8 | alkB homolog 8, tRNA methyltransferase | 11q22.3 |
| ENSG00000117481.11 | NSUN4 | NSUN4 | NOP2/Sun RNA methyltransferase 4 | 1p33 |
| ENSG00000143303.12 | C1orf66 | METTL25B | methyltransferase like 25B | 1q23.1 |
| ENSG00000166166.13 | TRMT61A | TRMT61A | tRNA methyltransferase 61A | 14q32.33 |
| ENSG00000118894.15 | FAM86A | EEF2KMT | eukaryotic elongation factor 2 lysine methyltransferase | 16p13.3 |
| ENSG00000188917.15 | TRMT2B | TRMT2B | tRNA methyltransferase 2 homolog B | Xq22.1 |
| ENSG00000087995.16 | METTL2A | METTL2A | methyltransferase 2A, methylcytidine | 17q23.2 |

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|---|---|---|---|---|
| ENSG00000198890.9 | PRMT6 | PRMT6 | protein arginine methyltransferase 6 | 1p13.3 |
| ENSG00000168300.14 | PCMTD1 | PCMTD1 | protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1 | 8q11.23 |
| ENSG00000130305.17 | NSUN5 | NSUN5 | NOP2/Sun RNA methyltransferase 5 | 7q11.23 |
| ENSG00000137574.11 | TGS1 | TGS1 | trimethylguanosine synthase 1 | 8q12.1 |
| ENSG00000130731.16 | C16orf13 | METTL26 | methyltransferase like 26 | 16p13.3 |
| ENSG00000126457.22 | PRMT1 | PRMT1 | protein arginine methyltransferase 1 | 19q13.33 |
| ENSG00000103254.10 | FAM173A | ANTKMT | adenine nucleotide translocase lysine methyltransferase | 16p13.3 |
| ENSG00000137364 | TPMT | TPMT | thiopurine S-methyltransferase | 6p22.3 |
| ENSG00000138050.15 | THUMPD2 | THUMPD2 | THUMP domain containing 2 | 2p22.1 |
| ENSG00000067365.15 | C16orf68 | METTL22 | methyltransferase 22, Kin17 lysine | 16p13.2 |
| ENSG00000134077.16 | THUMPD3 | THUMPD3 | THUMP domain containing 3 | 3p25.3 |
| ENSG00000148335.15 | METTL11A | NTMT1 | N-terminal Xaa-Pro-Lys N-methyltransferase 1 | 9q34.11 |
| ENSG00000171103.11 | TRMT61B | TRMT61B | tRNA methyltransferase 61B | 2p23.2 |
| ENSG00000197006.15 | METTL9 | METTL9 | methyltransferase like 9 | 16p12.2 |
| ENSG00000162851.8 | TFB2M | TFB2M | transcription factor B2, mitochondrial | 1q44 |
| ENSG00000178694.10 | NSUN3 | NSUN3 | NOP2/Sun RNA methyltransferase 3 | 3q11.2 |
| ENSG00000165792.18 | METT11D1 | METTL17 | methyltransferase like 17 | 14q11.2 |
| ENSG00000123600.21 | METTL8 | METTL8 | methyltransferase 8, methylcytidine | 2q31.1 |
| ENSG00000100483.14 | C14orf138 | VCPKMT | valosin containing protein lysine methyltransferase | 14q21.3 |
| ENSG00000185432.12 | METTL7A | TMT1A | thiol methyltransferase 1A | 12q13.12 |
| ENSG00000214435.9 | AS3MT | AS3MT | arsenite methyltransferase | 10q24.32 |
| ENSG00000111218.12 | PRMT8 | PRMT8 | protein arginine methyltransferase 8 | 12p13.32 |
| ENSG00000138382.15 | METTL5 | METTL5 | methyltransferase 5, N6-adenosine | 2q31.1 |
| ENSG00000122435.11 | CCDC76 | TRMT13 | tRNA methyltransferase 13 homolog | 1p21.2 |
| ENSG00000203880.12 | PCMTD2 | PCMTD2 | protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2 | 20q13.33 |
| ENSG00000132600.18 | PRMT7 | PRMT7 | protein arginine methyltransferase 7 | 16q22.1 |
| ENSG00000104907.13 | TRMT1 | TRMT1 | tRNA methyltransferase 1 | 19p13.13 |
| ENSG00000132423.12 | COQ3 | COQ3 | coenzyme Q3, methyltransferase | 6q16.2 |

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|---|---|---|---|---|
| ENSG00000250305.9 | C8orf79 | TRMT9B | tRNA methyltransferase 9B (putative) | 8p22 |
| ENSG00000037897.17 | METTL1 | METTL1 | methyltransferase 1, tRNA methylguanosine | 12q14.1 |
| ENSG00000068438.15 | FTSJ1 | FTSJ1 | FtsJ RNA 2'-O-methyltransferase 1 | Xp11.23 |
| ENSG00000122687.19 | FTSJ2 | MRM2 | mitochondrial rRNA methyltransferase 2 | 7p22.3 |
| ENSG00000205629.12 | LCMT1 | LCMT1 | leucine carboxyl methyltransferase 1 | 16p12.1 |
| ENSG00000086189.11 | DIMT1L | DIMT1 | DIM1 rRNA methyltransferase and ribosome maturation factor | 5q12.1 |
| ENSG00000156239.12 | N6AMT1 | N6AMT1 | N-6 adenine-specific DNA methyltransferase 1 | 21q21.3 |
| ENSG00000114735.10 | HEMK1 | HEMK1 | HemK methyltransferase family member 1 | 3p21.31 |
| ENSG00000150456.11 | N6AMT2 | EEF1AKMT1 | EEF1A lysine methyltransferase 1 | 13q12.11 |
| ENSG00000168228.16 | ZCCHC4 | ZCCHC4 | zinc finger CCHC-type containing 4 | 4p15.2 |
| ENSG00000165819.12 | METTL3 | METTL3 | methyltransferase 3, N6-adenosine-methyltransferase complex catalytic subunit | 14q11.2 |
| ENSG00000101574.15 | METTL4 | METTL4 | methyltransferase 4, N6-adenosine | 18p11.32 |
| ENSG00000145388.15 | METTL14 | METTL14 | methyltransferase 14, N6-adenosine-methyltransferase subunit | 4q26 |
| ENSG00000085276.19 | MECOM | MECOM | MDS1 and EVI1 complex locus | 3q26.2 |
| ENSG00000167548.18 | MLL2 | KMT2D | lysine methyltransferase 2D | 12q13.12 |
| ENSG00000099381.19 | SETD1A | SETD1A | SET domain containing 1A, histone lysine methyltransferase | 16p11.2 |
| ENSG00000101945.17 | SUV39H1 | SUV39H1 | SUV39H1 histone lysine methyltransferase | Xp11.23 |
| ENSG00000057657.17 | PRDM1 | PRDM1 | PR/SET domain 1 | 6q21 |
| ENSG00000109685.19 | WHSC1 | NSD2 | nuclear receptor binding SET domain protein 2 | 4p16.3 |
| ENSG00000141956.14 | PRDM15 | PRDM15 | PR/SET domain 15 | 21q22.3 |
| ENSG00000118058.24 | MLL | KMT2A | lysine methyltransferase 2A | 11q23.3 |
| ENSG00000116731.23 | PRDM2 | PRDM2 | PR/SET domain 2 | 1p36.21 |
| ENSG00000085276.19 | MECOM | TTLL12 | tubulin tyrosine ligase like 12 | 22q13.2 |
| ENSG00000100304.13 | TTLL12 | SETDB1 | SET domain bifurcated histone lysine methyltransferase 1 | 1q21.3 |
| ENSG00000143379.13 | SETDB1 | EZH2 | enhancer of zeste 2 polycomb repressive complex 2 subunit | 7q36.1 |
| ENSG00000106462.12 | EZH2 | KMT5B | lysine methyltransferase 5B | 11q13.2 |
| ENSG00000110066.15 | SUV420H1 | SETMAR | SET domain and mariner transposase fusion gene | 3p26.1 |
| ENSG00000170364.13 | SETMAR | SMYD5 | SMYD family member 5 | 2p13.2 |

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|---|---|---|---|---|
| ENSG00000135632.12 | SMYD5 | SETD3 | SET domain containing 3, actin histidine methyltransferase | 14q32.2 |
| ENSG00000183576.13 | SETD3 | KMT5C | lysine methyltransferase 5C | 19q13.42 |
| ENSG00000133247.14 | SUV420H2 | ZFPM1 | zinc finger protein, FOG family member 1 | 16q24.2 |
| ENSG00000179588.9 | ZFPM1 | SMYD4 | SET and MYND domain containing 4 | 17p13.3 |
| ENSG00000186532.12 | SMYD4 | KMT2E | lysine methyltransferase 2E (inactive) | 7q22.3 |
| ENSG00000005483.22 | MLL5 | SMYD1 | SET and MYND domain containing 1 | 2p11.2 |
| ENSG00000115593.15 | SMYD1 | SETD9 | SET domain containing 9 | 5q11.2 |
| ENSG00000155542.12 | C5orf35 | KMT2C | lysine methyltransferase 2C | 7q36.1 |
| ENSG00000055609.21 | MLL3 | SETD6 | SET domain containing 6, protein lysine methyltransferase | 16q21 |
| ENSG00000103037.12 | SETD6 | SETD7 | SET domain containing 7, histone lysine methyltransferase | 4q31.1 |
| ENSG00000145391.14 | SETD7 | ZFPM2 | zinc finger protein, FOG family member 2 | 8q23 |
| ENSG00000169946.14 | ZFPM2 | EZH1 | enhancer of zeste 1 polycomb repressive complex 2 subunit | 17q21.2 |
| ENSG00000108799.13 | EZH1 | EZH2 | enhancer of zeste 2 polycomb repressive complex 2 subunit | 7q36.1 |
| ENSG00000204371 | EHMT2 | EHMT2 | euchromatic histone lysine methyltransferase 2 | 6p21.33 |
| ENSG00000165671.22 | NSD1 | NSD1 | nuclear receptor binding SET domain protein 1 | 5q35.3 |
| ENSG00000136169.17 | SETDB2 | SETDB2 | SET domain bifurcated histone lysine methyltransferase 2 | 13q14.2 |
| ENSG00000181555.22 | SETD2 | SETD2 | SET domain containing 2, histone lysine methyltransferase | 3p21.31 |
| ENSG00000147548.17 | WHSC1L1 | NSD3 | nuclear receptor binding SET domain protein 3 | 8p11.23 |
| ENSG00000168137.20 | SETD5 | SETD5 | SET domain containing 5 | 3p25.3 |
| ENSG00000147596.4 | PRDM14 | PRDM14 | PR/SET domain 14 | 8q13.3 |
| ENSG00000112238.12 | PRDM13 | PRDM13 | PR/SET domain 13 | 6q16.2 |
| ENSG00000130711.5 | PRDM12 | PRDM12 | PR/SET domain 12 | 9q34.12 |
| ENSG00000152455.16 | SUV39H2 | SUV39H2 | SUV39H2 histone lysine methyltransferase | 10p13 |
| ENSG00000185420.19 | SMYD3 | SMYD3 | SET and MYND domain containing 3 | 1q44 |
| ENSG00000181090.21 | EHMT1 | EHMT1 | euchromatic histone lysine methyltransferase 1 | 9q34.3 |
| ENSG00000175213.3 | ZNF408 | ZNF408 | zinc finger protein 408 | 11p11.2 |
| ENSG00000142611.17 | PRDM16 | PRDM16 | PR/SET domain 16 | 1p36.32 |
| ENSG00000183955.14 | SETD8 | KMT5A | lysine methyltransferase 5A | 12q24.31 |

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|---|---|---|---|---|
| ENSG00000019485.14 | PRDM11 | PRDM11 | PR/SET domain 11 | 11p11.2 |
| ENSG00000170325.16 | PRDM10 | PRDM10 | PR/SET domain 10 | 11q24.3 |
| ENSG00000164256.11 | PRDM9 | PRDM9 | PR/SET domain 9 | 5p14.2 |
| ENSG00000152784.16 | PRDM8 | PRDM8 | PR/SET domain 8 | 4q21.21 |
| ENSG00000126856.16 | PRDM7 | PRDM7 | PR/SET domain 7 | 16q24.3 |
| ENSG00000061455.11 | PRDM6 | PRDM6 | PR/SET domain 6 | 5q23.2 |
| ENSG00000138738.11 | PRDM5 | PRDM5 | PR/SET domain 5 | 4q27 |
| ENSG00000116539.14 | ASH1L | ASH1L | ASH1 like histone lysine methyltransferase | 1q22 |
| ENSG00000143499.14 | SMYD2 | SMYD2 | SET and MYND domain containing 2 | 1q32.3 |
| ENSG00000185917.14 | SETD4 | SETD4 | SET domain containing 4 | 21q22.12 |
| ENSG00000110851.12 | PRDM4 | PRDM4 | PR/SET domain 4 | 12q23.3 |
| ENSG00000272333.8 | WBP7 | KMT2B | lysine methyltransferase 2B | 19q13.12 |
| ENSG00000139718.12 | SETD1B | SETD1B | SET domain containing 1B, histone lysine methyltransferase | 12q24.31 |
| ENSG00000059588.10 | TARBP1 | TARBP1 | TAR (HIV-1) RNA binding protein 1 | 1q42.2 |
| ENSG00000198917.13 | C9orf114 | SPOUT1 | SPOUT domain containing methyltransferase 1 | 9q34.11 |
| ENSG00000278619 | MRM1 | MRM1 | mitochondrial rRNA methyltransferase 1 | 17q12 |
| ENSG00000165275.10 | RG9MTD3 | TRMT10B | tRNA methyltransferase 10B | 9p13.2 |
| ENSG00000174173.7 | RG9MTD1 | TRMT10C | tRNA methyltransferase 10C, mitochondrial RNase P subunit | 3q12.3 |
| ENSG00000145331.14 | RG9MTD2 | TRMT10A | tRNA methyltransferase 10A | 4q23 |
| ENSG00000126749.16 | EMG1 | EMG1 | EMG1 N1-specific pseudouridine methyltransferase | 12p13.31 |
| ENSG00000171861.11 | RNMTL1 | MRM3 | mitochondrial rRNA methyltransferase 3 | 17p13.3 |
| ENSG00000162623.16 | TYW3 | TYW3 | tRNA-yW synthesizing protein 3 homolog | 1p31.1 |
| ENSG00000145692.15 | BHMT | BHMT | betaine--homocysteine S-methyltransferase | 5q14.1 |
| ENSG00000132840.10 | BHMT2 | BHMT2 | betaine--homocysteine S-methyltransferase 2 | 5q14.1 |
| ENSG00000116237.16 | ICMT | ICMT | isoprenylcysteine carboxyl methyltransferase | 1p36.31 |
| ENSG00000137404 | NRM | NRM | nurim | 6p21.33 |
| ENSG00000133027.18 | PEMT | PEMT | phosphatidylethanolamine N-methyltransferase | 17p11.2 |
| ENSG00000116984.15 | MTR | MTR | 5-methyltetrahydrofolate-homocysteine methyltransferase | 1q43 |

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|---|---|---|---|---|
| ENSG00000117543 | DPH5 | DPH5 | diphthamide biosynthesis 5 | 1p21.2 |
| ENSG00000145996.11 | CDKAL1 | CDKAL1 | CDK5 regulatory subunit associated protein 1 like 1 | 6p22.3 |
| ENSG00000101391.21 | CDK5RAP1 | CDK5RAP1 | CDK5 regulatory subunit associated protein 1 | 20q11.21 |
| ENSG00000134014.17 | ELP3 | ELP3 | elongator acetyltransferase complex subunit 3 | 8p21.1 |
| ENSG00000136444.10 | RSAD1 | RSAD1 | radical S-adenosyl methionine domain containing 1 | 17q21.33 |

Supplementary Table 1. Identified methyl transferase genes used for analysis of RNA sequencing data.

# Thesis Conclusion

This research has explored the presence of volatile organic compounds (VOCs) linked to diseases in humans with a focus on cancer biomarkers in breath. Research aims were progressively fulfilled through the thesis's four chapters, three of which are published papers and the fourth is in publication format, awaiting additional clinical data not included in this thesis.

In brief, chapter 1 identified trends in VOC chemistry in the breath of cancer patients compared with patients with other diseases. Using the results of chapter 1, chapters 2 and 3 applied novel methods to sampling VOCs in cellular and animal models, successfully identifying VOC flux which are not only descriptive of cell type, but also cellular state. In the final chapter and using the results and insights of chapters 1, 2 and 3 a model is presented to describe the mechanism of VOC metabolism for methyl chloride (MeCl), which offers the potential to act as a powerful biomarker of cellular malfunction and disease.

In the first chapter, the research landscape was contextualised and investigated to reveal trends and methodologies which compromise and limit the accuracy of biomarker discovery. This meta-analysis increased success of biomarker targeting and discovery and informed the approaches used throughout the research particularly by targeting biomarker discovery in cellular models of starvation. Chapter 1 recommends inclusion of as many functional groups as possible and during the research inclusion of a suite of alkanes, ketones and aromatics has evolved to represent these findings, evident in the subsequent chapters.

The range of functional groups considered as potential biomarkers is likely to change with ongoing research but my current study of the breath of breast cancer patients at York hospital has applied the results of results detailed in chapters 1-4 to search for an updated suite of compounds as potential biomarkers. The study removed methyl bromide, methane thiol and isoprene, molecules which have not shown to be of interest, and replaced them with dichloromethane, butanone and styrene This method also now includes, halogenated compounds, sulphur containing compounds, ketones, alkanes and aromatics. The inclusion of aldehydes would have been ideal, however the column used in the method, which allows good identification of halogenated compounds, did not easily allow aldehyde identification.

Chapter 2 presented successful methodology and demonstrated how volatile profiles described response to stress in the form of chemotherapeutic, doxorubicin. This highlighted that simple cell:cell comparisons may not provide all the biomarkers required for clinical applications. As such this drove the research in chapter 3, where stress is applied to the cells in the form of low oxygen, to mirror stress which cells in a growing tumour might experience. Chapter 4 expands on this research by inducing stress through serum and glucose starvation

which revealed specific stress responses in the volatile profile for comparison with results from chapters 2 and 3.

## Future directions

The most successful conclusion of this research has been the award from the Elsie May Sykes fund to conduct the pilot study in the breath of breast cancer patients at York District Hospital using the methodologies developed in the research detailed above – this research is currently in progress.

My research has highlighted MeCl as a mechanism of VOC processing integral to cellular metabolism and epigenetic response. The first next step is to identify the function of MeCl, which can then lead to further investigations of MeCl flux. The determination of mechanisms linked to, in particular, MeCl consumption and production are potentially useful in the identification of cancer and offer the diagnostic potential.

To further investigate MeCl production and the methyl transferase activity there are several experiments to conduct. Treatment with 5-AZA and SAM to test if recovery of MeCl flux seen in other conditions treated with SAM still persists (if methyl transferases are responsible for MeCl production then we would expect no change in MeCl levels in 5-AZA and SAM treated cells). An expanded range of knockdowns would also be ideal in order to target those methyl transferases most changed under hypoxic conditions, such as NOP2. The next experiment, could be to determine if Carbon-13 labelled SAM integrates to produce MeCl with a carbon-13.

Experiments to validate targets of MeCl consumption, namely methionine synthase and integration of the methyl group of MeCl to homocysteine, generating methionine could include a knockdown of methionine synthase, first in control cells, which may generate a slight increase in MeCl production. This could then be examined in cells under conditions of starvation.

In time, the peppermint breath test should be conducted to assess our gas chromatography mass spectrometry (GC/MS) methods[1,2]. This test seeks to inform the standardisation of breath and VOC analysis methods. Peppermint oil capsules are consumed and breath samples taken over time to determine sensitivity to metabolic products. The products, including menthol and $\alpha$-pinene, may not be seen with the GCMS equipment we are currently using, however this method of standardisation for breath VOC testing, could be applied to our condensation trap to better understand variations between our approach and thermal desorption approaches in breath collection.

Of the most interesting results presented in this thesis is the finding that deviation from MeCl baseline metabolism, to lesser production or even flipping to consumption, increases with tumour size. To expand on this finding and for future directions in human work, blood work investigating chloride content would have been ideal, as I found no intracellular tumour changes in chloride content compared to tumour size. This is unsurprising as cells in a tumour may work to remove their chloride content, therefore an investigation into blood chloride content would be warranted.

## Final Reflection

This research has added new knowledge to the rapidly expanding field of metabolomics and breath science. Through my research and interactions with the wider research community, I have recognised the need for the precise identification of more mechanisms under pinning VOC metabolisms in order to accurately target breath diagnostics. My first chapter shows that the vast majority of previous research into VOCs associated with disease have been through quantification of the production of VOCs released in the breath of patients. By quantifying flux and investigating starvation as a model of tumour microenvironment, this PhD demonstrates that more accurate models are possible and are required for biomarker discovery and that time point based methods of collection allow biomarker discovery in the context of both metabolic consumption and production.

# References


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