Journal of Breath Research

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To cite this article before publication: Edoardo Gaude *et al* 2019 *J. Breath Res.* in press https://doi.org/10.1088/1752-7163/ab1789

Targeted breath analysis: exogenous volatile organic compounds (EVOC) as metabolic pathway-specific probes

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Keywords

Breath Biopsy, Breathomics, Targeted Metabolomics, Metabolic Phenotyping, Pharmacobreathomics, Translational Metabolomics

Abstract

Breath research has almost invariably focussed on the identification of endogenous volatile organic compounds (VOCs) as disease biomarkers. After five decades, a very limited number of breath tests measuring endogenous VOCs is applied to the clinic. In this perspective article, we explore some of the factors that may have contributed to the current lack of clinical applications of breath endogenous VOCs. We discuss potential pitfalls of experimental design, analytical challenges, as well as considerations regarding the biochemical pathways that may impinge on the application of endogenous VOCs as specific disease biomarkers. We point towards several lines of evidence showing that breath analysis based on administration of exogenous compounds has been a more successful strategy, with several tests currently applied to the clinic, compared to measurement of endogenous VOCs. Finally, we propose a novel approach, based on the use of exogenous VOC (EVOC) probes as potential strategy to measure the activity of metabolic enzymes *in vivo*, as well as the function of organs, through breath analysis. We present longitudinal data showing the potential of EVOC probe strategies in breath analysis. We also gathered important data showing that administration of EVOC probes induces significant changes compared to previous exposures to the same compounds. EVOC strategies could herald a new wave of substrate-based breath tests, potentially bridging the gap between research tools and clinical applications.

Introduction

Exhaled human breath contains hundreds of volatile organic compounds (VOCs) [1] - low molecular weight metabolites that are excreted in breath as the result of metabolic processes in the body - that can be of endogenous and/or exogenous origin [2]. Endogenous VOCs are produced from internally available metabolic substrates and are part of physiological and pathological mechanisms. Exogenous VOCs are introduced into the body from an external source such as diet, environmental exposure, medication, etc, and are expressed in exhaled breath after circulation and/or internal metabolism. etc [3].

Several endogenous and exogenous breath VOCs have been investigated, in the last decades, as potentially useful and non-invasive biomarkers for various diseases with applications ranging as wide as lung cancer, cardiovascular diseases, asthma, cystic fibrosis, infectious diseases, and chronic inflammatory diseases [4–6]. The general approach that has been applied to the discovery of mainly

endogenous disease-related biomarkers entails the comparison of breath VOC profiles between groups of supposedly healthy subjects, and/or cohorts of patients with a documented disease. Application of statistical techniques helps with discovery of VOCs that are differentially expressed in control and diseased groups, leading to identification of candidate biomarker(s). Although this approach has been trialled and tested in several hundreds of experiments, none of these candidate endogenous or exogenous exhaled VOCs has paved its way into routine diagnostic use. In this article we will explore potential reasons for the current lack of VOC tests in the clinic, and present some data pointing towards a different experimental strategy which could complement current unsupervised discovery research.

The historic 'omics' approach to breath research

Historically, breath research has deployed the standard model of 'omics' research described in the previous paragraph: (small-size) cross-sectional case-control studies, screening as many potential biomarkers as possible, using complex mathematical algorithms to distil signal from noise, and to assess their statistical power when compared to control groups [6,7]. Upon observation of potentially discriminating repeatable spectral features, these non-targeted approaches rely on identification of compounds through comparison with database of chemical suspects, for which an exhaustive framework has been proposed [8]. Although many technical and methodological aspects are constantly being addressed and improved, two main challenges are however fundamentally linked to the scientific method applied in untargeted biomarker discovery studies and are shared with other 'omics' approaches such as transcriptomics and proteomics.

1: The challenges of "finding the needle in the haystack"

Untargeted study designs typically have a very small ratio of subjects to tested variables. This creates a considerable challenge to find relevant biomarkers in a very complex highly dimensional dataset. Especially, if a biomarker is expected to only be relevant in a minority of patients there are two 'evils' which need to be carefully balanced; the risk of overfitting a model resulting in falsely identified 'voodoo' correlations and the risk of obscuring true biomarkers [8][9]. Currently, early stage omics research frequently results in unvalidated and overfitted models, breath VOC research being no exception [6,9–11].

2: Balancing resolution and selectivity

The statistical challenges described above are in many ways aggravated by fundamental technological challenges with omics platforms: an untargeted 'measure-all' approach requires analytical platforms that can cover the widest possible range of molecular species. Although breath collection approaches and analytical techniques such as sorbent tubes and gas chromatography mass spectrometry (GC-MS) can be optimised to detect hundreds or even thousands of different compounds, the more biomarker you measure automatically compromises the ability to reliably identify and quantitate specific compounds. In untargeted discovery experiments this can result in considerable challenges to validate the chemical identity of candidate biomarkers in a background of chemically similar compounds [12]. This challenge is aggravated with most non-specific chemical sensors (referred to as eNoses), that perform collective/pattern loosely defined ranges of chemical classes with limited ability to identify or quantify the actual biomarkers [13]. To overcome these challenges, a chemical validation step should be taken. Comparison of the measured ion spectrum to universal libraries and available databases could provide information regarding molecular structure of the compound of interest, in the presence of good match with spectral library. Subsequent analysis of pure standards

should then be performed to confirm compound identity, based on the quality of the data [14]. Complementary analysis, in these cases, using for example, GC-GC, Orbitrap, or High Rresolution - Mass Spectormetry (HR-MS), would result with a less convoluted data with higher signal to noise ratio, that would facilitate the identification of unknown compounds [8,15–18][19]. Other techniques, such as Proton-transfer-reaction mass spectrometry (PTR-MS)/ Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) [20] ion mobility spectrometers (IMS)[21]are used for real time analysis. Although highly sensitive and accurate for exhaled VOCs analysis, it might be challenging to perform untargeted biomarker discovery studies. However, once a given targeted compound is known and/or the optimal sampling time-points are established, these techniques can have a great benefit over other off-line sampling methods, that could increase pre-analytical variation [22].

Fundamentally, this results in a trade-off which should be carefully considered when designing any VOC based experiment. Irrespective of the choices made, upon conducting the study, appropriate pre-analytical and analytical validation should be performed to obtain insight into the accuracy and precision of a method. This informs parameters such as the lower limit of detection and linear dynamic range which serve as important benchmarks to interpret candidate biomarkers against.

These two fundamental challenges have resulted in a situation where research results tend to gravitate to those exhaled VOCs occurring in the widest range of subjects and at the highest concentrations as these are easiest to detect. Consequently, there is an overrepresentation of these biomarkers in VOC literature where they are associated with a very wide range of health states. Acetone and isoprene are important examples in this context as they are present in virtually every breath sample and occur at a concentrations 2-4 orders of magnitude higher than other VOCs making them much easier to identify and quantify. Yet, breath acetone and isoprene have been associated to a plethora of diseases, impinging on the application of these compounds as specific disease biomarkers.
Although breath acetone has been suggested as a biomarker for monitoring ketosis in patients with epilepsy [23], acetone has also been associated to lung cancer [24–26], cystic fibrosis [27], asthma [28], malaria [29], and pneumonia [30], as well as to other factors such as age, gender, current medication, drugs of abuse, and race [31].

Similarly, breath isoprene has been used as a marker of endogenous biosynthesis of cholesterol [32–34] but it has been associated to influenza [35], renal disease [36], muscle activity [37] lung cancer [24–26] and advanced liver disease [38] as well as to other factors such as age, and circadian rhythms, peaking early in the morning and decreasing during the day [39,40]. The association of breath acetone and isoprene with such a diverse array of different disorders, physiological states and exposures, severely complicates the use of these compounds as specific disease biomarker [31,41].

This limited specificity of acetone and isoprene indicates they are unlikely to be valuable diagnostic disease biomarkers for the general population. Both compounds could still hold value, but their utility is probably limited to use cases where the biomarker is highly specific for the disease state of interest or is used for monitoring of within subject changes.

Where are we now in breath research?

Unfortunately, almost fifty years after the first studies in breath research [42], there are no endogenous VOC-based tests routinely applied in the clinic. Generally speaking, the success of research translating endogenous VOCs biomarkers beyond proof of concept, has been very limited.

To our knowledge, the only approved application based on endogenous VOCs is the detection of breath alkanes for diagnosing Grade 3 heart allograft rejection in patients who received heart transplant [43,44]. This method showed similar diagnostic performance to endomyocardial biopsy [43] Yet, breath alkanes have been associated with lung cancer [45], breast cancer [46], pneumonia [47], COPD [48], and asthma [49], suggesting that increased breath alkanes might be general markers of inflammation and lipid peroxidation, rather than specific markers of transplant rejection. Perhaps this aspect contributes to the low specificity observed for breath alkanes in the detection of heart allograft rejection [43].

Various factors have contributed to the lack of clinical applications of endogenous VOCs in breath analysis. For instance, several breath tests reported in the literature could never be reproduced. This is likely due to the lack of standardised equipment for breath collection [50], standardised collection methodologies [51], as well as appropriate controls for potential VOC contaminants present in the environment at the time of experiment [52].
Research studies have been conducted to develop tests without a clear clinical application, for instance in cases where an effective diagnostic test was already available [53,54]. In addition, patients with late-stage disease are often considered in biomarker discovery studies, posing a clear challenge for the use of those biomarkers for early disease detection. Finally, most of breath research studies published to date have not been validated in their intended use to diagnose population with realistic disease prevalence [55].

This clearly points at the need to investigate complementary alternative strategies. Firstly, building a detailed understanding of the biological pathways which underpin the production of VOCs allow targeted investigation of candidate biomarkers in a target population. Testing such a small set of biomarkers bypasses the risk of overfitting and allows adopting a targeted analytical method. Such a method could more reliably identify and quantitate target biomarkers as a fully non-invasive method to measure specific pathways through breath analysis. An obvious challenge will be to conduct the translational research that builds the understanding of the community with respect to the origins of VOCs. When done adequately this can provide a powerful route to progress a biomarker into clinical use. Although it has several limitations and is not a VOC, fractional exhaled nitric oxide (FeNO) is an interesting model of how such an approach could work [56]. A second strategy could be the collection of multiple phases of exhaled breath, including exhaled breath condensate (EBC) and aerosol (EBA). This might help to associate breath biomarkers with hydrophilic compounds usually present in other phases (e.g. cytokines, oxidative stress markers, etc) and improve our understanding of the origin of VOCs.

A commonly overlooked potential approach is to explore the utility of exogenous VOCs. These are usually seen as something to be removed as much as possible or otherwise corrected for. This however ignores the fact that the vast majority of successful breath tests to date measure exogenous VOCs.

Exogenous VOCs in breath analysis for health and disease

Use of exposure VOCs as probes for disease

Exogenous compounds are continuously introduced into the body through diet, skin, exposure to chemicals, medications, drugs, etc, and follow kinetics of absorption, distribution, metabolism and excretion. This can result in breath secretion of the exogenous compound itself (if volatile), or of volatile downstream products that directly originate from the exogenous compound. Several examples can be drawn on the use of exogenous volatile compounds in the development of viable breath tests. These may provide valuable insights into how VOC research can be approached in a radically different way.

Cigarette smoke has been long associated with increased levels of breath benzene [57], as well as other products originating from metabolism of tobacco compounds such as 2-methylfuran [58] or acetonitrile due its prolonged retention in the body.[59] Although exposure to benzene is common, especially in urban areas, smoking has been shown to increase breath benzene irrespective of background benzene exposure [60], suggesting that introduction of high levels of exogenous compounds can help differentiate population subgroups beyond interindividual variation originating from the environment. Similarly, detection of breath ethanol is used to assess alcohol consumption. Absorption of ethanol contained in alcoholic beverages leads to increased blood levels of ethanol, which is metabolised by the liver.[61] Liver metabolism can take minutes to hours to biotransform ethanol to acetaldehyde and acetone, and during this time ethanol is distributed via the bloodstream throughout the body and secreted via breath. This allows the use of breathalyzers as tools for assessing recent alcohol consumption. However, it is important to understand the kinetics of each metabolite, in terms of absorption, metabolism, half-life, diffusion, blood:breath concentrations and other important factors, prior to design of such an approach.[61]

Interestingly, the ethanol breath test also indirectly assesses a metabolic heterogeneity. As an example, following alcohol ingestion, breath ethanol levels were associated with different rates of alcohol absorption and metabolism in men and women [62]. Ethanol metabolite acetaldehyde has also been shown to diagnose genetic defects of ALDH2, a major enzyme implicated in ethanol metabolism [63]. This evidence indicates that exogenous ethanol, coupled with breath analysis, can be a useful tool for investigating phenotypic differences of alcohol metabolism.

In addition, several exposure VOCs that are known to be toxic to humans, such as benzene and naphthalene [64,65], trichloroethylene and tetrachloroethylene [66], chloroform and haloketones [67], toluene, ethylbenzene, and m-xylene [68], have been the subject of dedicated studies to elucidate their ADME kinetics by the human body. Elucidation of toxicokinetic properties of these compounds via administration of compound probes is a valid strategy to evaluate the potential risks of developing adverse reactions to the toxicants [69].

Taking this concept one step further, an exogenous substrate can be provided to investigate metabolic activity of the microbiome in relation to health and disease. As an example, fructose is currently applied in the clinic as metabolic probe to assess malabsorption through the gastrointestinal tract [70,71]. This application relies on the ability of bacteria to produce molecular hydrogen (H_2) when coming in contact with carbohydrates, and detection of breath H_2 is used to measure the extent of bacterial metabolism. In normal conditions carbohydrates such as glucose and fructose are fully absorbed in the small intestine and levels of breath H_2 are <20 parts per million (ppm) [70]. In case of malabsorption, higher levels of fructose are passed in the lower intestine and colon, where metabolism by gut microbiota can increase production and breath secretion of H_2 [71]. Importantly, fructose shows

high diagnostic performance for the detection of malabsorption, with a sensitivity of 98% and specificity of 86% [72] and it correlates with symptoms of malabsorption, despite not predicting change in diet [73]. Applying the same concept, administration of glucose or lactulose, together with detection of breath H_2, is used for the detection of small intestinal bacterial overgrowth (SIBO) [74].

Fernández del Río et al. have recently offered promising results supporting the link between exogenous VOCs and organ function. By analysing the breath of patients with liver cirrhosis before and after liver transplant, they found that breath limonene was associated with dysfunctional liver [75]. This finding is supported by other studies reporting association of breath limonene with liver cirrhosis [76], as well as hepatic encephalopathy [77]. Limonene is a monoterpene contained in most plant-based foods, especially citrus fruits. High limonene secretion in the breath of cirrhotic patients is the result of reduced limonene clearance from the bloodstream due to dysfunctional biotransformation in the liver [75,77]. These results suggest that exogenous limonene introduced through diet could reveal differences in liver function via measurement of limonene secretion in breath. Finally, the group of Joachim Pleil suggested the use of gas-phase probe molecules to assess the effect of potentially toxic compounds on known metabolic pathways [78]. The authors proposed an *in vitro* adverse outcome pathway (AOP) approach, where cell lines are exposed to a gas-phase probe, such as methyl tert-butyl ether (MTBE), and the toxic effects of exposure molecules are measured via monitoring of MTBE metabolism through specific enzymes [78].

Together, these studies indicate that exogenous VOCs, introduced as an experimental tool or through daily exposure, can be exploited to identify population subgroups and to assess specific disease-associated processes. In line with this concept, monitoring breath excretion of volatile anaesthetics, and corresponding metabolic products, has been suggested as a strategy to assess organ function [79,80].

Use of stable isotope labelled probes in breath analysis

The evidence reported in the previous section highlights that use of targeted exogenous probes can help investigating specific enzyme activities or organ functions. The concept of administering exogenous probes to assess metabolic functions *in vivo* has been applied in the past, but it has been limited to the administration of stable isotope-labelled probes. Research on the utilisation of labelled compounds as metabolic probes in breath analysis has led to several approved clinical applications.

Probably the most well-established of these applications is the use of ^{13}C-labelled urea for the diagnosis of *Helicobacter pylori* (*H. pylori*) [81–83]. The test comprises collection of a baseline breath sample before ingestion of a pill containing ^{13}C-urea, and collection of a further breath sample 20 minutes after ingestion [82]. The ^{13}C-urea breath test (UBT) is based on the concept that human cells are incapable of metabolising urea, whilst presence of *H. pylori* in the stomach will quickly lead to breakdown of urea, with production and secretion of $^{13}CO_2$ in breath. UBT has shown strong diagnostic accuracy for detection of *H. pylori* infection, providing 100% specificity and 92% sensitivity [83].

A further clinical application that uses an isotope-labelled compound is the administration of ^{13}C-labelled *Spirulina platensis* (*S. platensis*), that has been recently approved for the assessment of gastric emptying [84]. This product is composed of ^{13}C-enriched (99%) *S. platensis*, an algal food supplement that is mixed with a solid food meal. Absorption and metabolism of ^{13}C-*S. platensis* leads to excretion of $^{13}CO_2$ in breath. Delayed gastric emptying and absorption of the labelled probe leads to different kinetics of metabolism and breath $^{13}CO_2$ secretion [85]. Measurement of breath $^{13}CO_2$ over time (1-3 hours) allows the identification of patients with delayed gastric emptying with high diagnostic accuracy (sensitivity 93%, specificity 80%) [86].

Finally, ^{13}C-methacetin breath test (MBT) was approved in the UK at the end of 2017 for the assessment of maximal liver function [87]. In this test, 4mg/mL solution of ^{13}C-methacetin is injected intravenously and converted by the liver enzyme cytochrome P450 1A2 (CYP1A2) [88] into

acetaminophen and $^{13}CO_2$, with the latter being secreted in breath. Secretion of $^{13}CO_2$ occurs within 1-2 minutes after injection and is measured through infrared laser spectroscopy (LiMAx® system). CYP1A2 constitutes between 4 and 16% of the total hepatic CYP450 pool [89], and liver dysfunction that can be induced by different diseases is likely to result in alterations of CYP1A2 activity. The MBT has been extensively applied and validated for the detection of liver cirrhosis induced by infection of hepatitis C virus (HCV) [90–92], displaying high diagnostic accuracy (sensitivity 96%, specificity 92%) [90]. MBT has also been successfully applied for the detection of non-alcoholic fatty liver disease (NAFLD) [93,94], and primary biliary cirrhosis [95], as well as breath secretion of $^{13}CO_2$ after MBT has been shown to recover after liver transplantation [96]. This evidence indicates that exogenous stable isotope probes constitute a very powerful strategy for assessing metabolic phenotypes and organ functions *in vivo*. Nevertheless, if intravenous administration of a probe is required this means a clinical environment is needed for the test to be administered, and the test is no longer non-invasive. This will also impact on the regulatory approval for such tests. These factors could perhaps explain why among the several patented strategies involving stable isotope probes, only a handful of products are currently approved for commercialisation [96,97].

In conclusion, through understanding biological pathways exogenous (unlabelled or labelled) compounds can be used to assess specific enzymatic activities or organ functions. This can provide accurate disease biomarkers which have made it into the clinic for specific applications. In clear contrast with the untargeted approach applied for the discovery of endogenous VOCs, design of strategies that deploy exogenous VOC probes relies on *a priori* understanding of the molecular mechanisms of disease. A targeted approach presents an opportunity for much more rigorous method development allowing high performance of a target analyte. This is not possible for an untargeted approach, where the method must perform generally well for many different compounds with a wide range of concentrations. Targeted approaches based on exogenous VOCs allow optimisation of every aspect from sampling through to data analysis and ensure any trends in the data can be attributed to biology rather than technical variability. This approach has proven to enable the construction of highly accurate tests. (Figure 1)

Exogenous VOC (EVOC) probes for *in vivo* metabolic phenotyping

The evidence mentioned above about the association between breath levels of limonene and liver dysfunction [75,77] as well as other studies that clearly indicates that washout curves of administered exogenous VOCs can be used for assessing metabolic function and pharmacokinetics *in vivo*.[98] [99] Yet, the inability to control limonene intake in the general population complicates the definition of threshold values of breath limonene for the identification of liver dysfunction in this application. However, administering an exogenous VOC as a probe constitutes a conceptually different strategy from those applied to date in breath analysis. Whilst ^{13}C-labelled applications are based on biotransformation of the substrate to generate $^{13}CO_2$, followed by isotope enrichment analysis in breath, exogenous VOCs can be directly used as probes by monitoring the breath clearance (or washout) of the substrate itself, as well as by detecting multiple products that can derive from metabolism of the substrate (Figure 1). This novel application in breath analysis can make use of virtually any exogenous VOC that, metabolised by the human body, can offer a readout of metabolic enzymes/organs.

Here, we propose the use of exogenous volatile organic compound (EVOC) probes as tracers of specific *in vivo* metabolic activities. EVOC probes can be volatile compounds that, administered to a subject through various routes, undergo metabolism and distribution in the body and are excreted via breath. Additionally, metabolism of EVOC probes by specific enzymes can lead to production of other

volatile compounds that could be detected in breath. The kinetics of metabolism and subsequent breath excretion of the EVOC probe, or of its products, can be used as an indication of the metabolic activity of specific enzymes or organs/tissues. In case breath levels of the EVOC probe itself are to be monitored, clearance or washout of the EVOC probe will be a function of the metabolic activity of the enzyme(s) under investigation. On the contrary, if breath secretion of the product(s) originating from the EVOC probe are to be determined, the rate of product generation will be associated to the enzymatic activity of interest. This approach is essentially different from previous strategies involving the use of non-volatile probes (e.g. fructose, glucose) coupled to detection of non-VOC breath compounds (H_2) [72,74].

A fundamental requirement during the design of EVOC probe strategies is a detailed understanding of the enzymes or organs/tissues that are affected by the disease or condition of interest. Understanding the pathophysiological mechanisms of a specific disease can help identify specific metabolic targets that can be exploited to reveal the presence of disease. The design of EVOC probe strategies can then be directed based on *a priori* knowledge of a target metabolic (dys)function. Administration of an EVOC probe that is metabolised by a disease-specific mechanism, could result in differential breath secretion of the EVOC probe itself, or of its metabolites, in diseased subjects compared to healthy people.

Several factors have to be considered when developing an EVOC probe strategy for disease diagnosis:

1. Optimise pairing of substrate to enzyme(s): different enzymes have different substrate-specificity, and this might also be affected by disease conditions. Screening of different EVOC probes, and analysis of the specificity of different enzymes for the same probe, can finally lead to an optimised match between EVOC probe and enzyme of interest.
2. Identify viable route of administration: several routes of administration (oral, intravenous, sublingual, inhalation, transdermal, etc) can be envisioned based on the enzyme activity of interest. Organ/tissue distribution of the target enzyme will dictate the choice of the route of administration.
3. Distribution kinetics: distribution of different compounds in the body is affected by route of administration, metabolism kinetics, as well as by physicochemical properties of the EVOC probe itself. For instance, lipophilic compounds will be retained in fat deposits, and excreted via breath, for longer time than hydrophilic compounds. These considerations have to be taken into account when selecting EVOC probes, as they might introduce a variability which would induce heterogeneity in washout curves that might lead to incorrect data interpretation. for example, different absorption kinetics, would lead to variance in the timing of C-max, an important time point.
4. Biological variation assessment: As with any biomarker it is relevant to understand the biological variability that can be expected to be found when conducting repeated measurement under (seemingly) identical situations. Key-factors, such as, health status and pre-existing conditions, diet, medication intake, exercise, smoking habits, breath patterns (while sampling) and other, could affect the mass of compounds diffusing to exhaled breath and eventually captured on the tubes. Understanding biological variability in a population of healthy individuals allows researchers to relate the effect size of their candidate biomarker to the variability in a general population. An important caveat here is that the most important assessment of population variability is that done within the intention to diagnose population as it is this variability which ultimately impacts the diagnostic accuracy. The index of individuality [100] is a useful concept in this context allowing the researcher to assess whether changes within or differences between subjects are the most appropriate use of a biomarker. In addition, taking into account that EVOC probes consist mainly of compounds that the population might

be exposed to, that could induce variability in baseline measurements, which could negatively affect the washout study. Therefore, it would be recommended to use concentrations that are several folds higher than expected background levels, to minimize its effect. Finally, it is important to realise that the intra- and inter- instrument variability of the assay is crucial to understand in detail when analysing such data.

5. Likelihood of secretion in breath: this aspect will depend on the EVOC probes, or derived metabolites, and is based on volatility of the compounds of interest. This depends on physical properties of the compounds, such as boiling point and water/air partition coefficient [101]. Selection of EVOC probes that, not only are metabolised by the enzyme of interest, but also are secreted in breath at high proportions, is fundamental for the development of EVOC probe strategies.
6. Dosage of EVOC probes: the amounts of EVOC probe that reach the enzyme of interest will determine the ability of the assay to reveal differences in enzymatic activity. Indeed, evaluation of enzymatic activity is usually measured as a function of substrate concentration [102]. Defined ranges of substrate concentrations are needed to assess differences in enzyme Vmax (the maximal catalytic rate with saturating concentrations of substrate) or Km (enzyme affinity for the substrate). Appropriate dosage of EVOC probe will change according to the enzyme of interest.
7. Kinetics of metabolism and breath excretion of the EVOC probe itself, and/or of product metabolites, in healthy subjects have to be determined in order to measure intra- and inter-individual variability, as well as to assess contribution of potential confounding factors such as diet, lifestyle, age, gender, current medication, etc. Breath values from healthy subjects can then be determined, in order to establish reference values of the healthy population.

Importantly, experimental groups (disease vs controls) might respond differently to administration of EVOC probes, through mechanisms that are independent from the disease under investigation. This is a potential hurdle of the proposed strategies, as it can confound the interpretation of results. A possible strategy to circumvent this approach could be to analyse the correlation of EVOC probe effect with disease severity (to ascertain the link with disease within the same experimental group), as well as to validate findings with different control groups. Regardless, final assessment of assay performance in the intended use case is required to confirm validity of the EVOC strategy, where possible using a randomised control trial incorporating a clinically relevant interventions.

Terpenes as an example of EVOC probes

To offer an example of EVOC probes we investigated changes in breath composition upon administration of terpenes, a class of VOCs that are found in a variety of plants. We administered peppermint oil capsules to one healthy subject at rest, in the morning, without prior fasting, and measured the composition of exhaled terpenes in breath over time while the subject sitting in an upright position, snacks/drinks were allowed during the experiment. (Figure 2A),

Breath samples were collected with the ReCIVA breath sampler, continues 4 minutes of end tidal exhaled breath collected onto Tenax TA/Carbograph 5TD sorbent tubes (Markes International). All tubes were analyzed upon sampling avoiding any storage, through thermal-desorption gas chromatography linked with mass spectrometry (TD-GC-MS). (for detailed methods please refer sup. materials) to Ingestion of the EVOC probe resulted in a marked increase in breath concentrations of *alpha*-pinene, *beta*-pinene, limonene, eucalyptol and p-menthan-3-one, within 30 minutes of ingestion compared to baseline levels. Importantly, this increase was not observed for endogenous compounds that are not contained in the EVOC probe, such as acetone and isoprene (Figure 2A). The washout kinetics that we observed after administration of the EVOC probe suggest involvement of first-pass

intestinal and hepatic metabolism. For most of the terpenes analysed, the peak breath excretion was observed at 0.5 hours, indicating relatively fast gastrointestinal absorption into the bloodstream. Subsequent decrease of breath levels of all terpenes is due to biotransformation and clearance from the bloodstream via liver metabolism, kidney filtration, and probably a small fraction in exhaled breath. As VOCs are metabolised and gradually cleared, blood concentrations decrease over time, with progressively lower secretion in breath. Importantly, different terpenes followed different washout kinetics, possibly indicating metabolism and/or clearance of different compounds by different liver enzymes.

Breath composition is known to change over the course of days and weeks, and even in the same sampling session based on the breath patterns of the subject[103], leading to high intra-individual variation [104]. To assess the stability of our approach in determining reproducible changes in breath, we administered the EVOC probes to one healthy subject and collected longitudinal breath samples over the course of five weeks (Figure 2B). We compared the fold changes of several terpenes at baseline (before EVOC probe), peak (45 minutes after EVOC probe) and 3 hours after EVOC, across the repeated breath collections. Administration of the EVOC probe induced significant changes in the peak breath secretion of several terpenes compared to baseline levels, and fold changes were highly reproducible (Figure 2B).

An important factor to consider when administering EVOC probes is the background level of breath VOCs present at baseline. Background terpene levels are expected in the normal population due to dietary consumption. Different background levels of VOCs can result from different dietary intake, as well as from different storage in the body, and could be a confounding factor when analysing the effects of EVOC probes on breath composition. To investigate whether background levels of VOCs can alter the effect of EVOC probes, we measured the background levels of limonene contained in our EVOC probe in a population of 136 subjects who did not receive the probe, and compared them with breath levels of the same compound 30 minutes after ingestion of EVOC probe in three subjects. Our results show interindividual variability in the levels of limonene in breath, likely due to different dietary intake and/or release. Importantly, administration of the EVOC probe led to a significant increase in the breath levels of limonene, generating a separated distribution of breath limonene concentrations (Figure S1). Since these are exogenous compounds, the level of environmental exposure might vary in the population. Administration of EVOC probes at high concentrations would ensure that inter-individual differences due to environmental exposures would become negligible. (Figure S1). With tight control on administration and adsorption kinetics of EVOC probes, this could be a valuable strategy to overcome several aspects of variability, and improve results reproducibility.

Important to realise in this context is that the well described mechanisms governing exhalation of VOCs such as the alveolar gradient, cardiac output, blood:air and blood:fat partition coefficient drive the elimination kinetics of the probe. Whilst some of these are an attribute of the compound itself, others contribute to variability likely to be unrelated to the enzymatic pathway which is being probed. This points toward the relevance of studying these parameters to understand if they need to be controlled and/or limit the applicability of this particular EVOC strategy. This is however a constraint which applies to all breath analysis [101,105] and the signal amplification achieved through administering an EVOC probe may overcome some of these limitations.

Together, these data indicate that administration of EVOC probes results in robust and reproducible detection of the EVOC probes in breath that might be an indication of metabolism *in vivo*. This strategy could help to overcome some of the challenges associated with inter-individual variability often observed in breath analysis.

Discussion

Untargeted approaches for the discovery of endogenous breath VOCs as disease biomarkers are very complex and, after several decades of research, have yet to result in widely adopted clinical applications for disease diagnosis. The use of exogenous VOC (EVOC) probes as a method to assess specific metabolic activities *in vivo* builds on understanding of biological pathways and holds great potential for the development of specific disease biomarkers. EVOC probes constitute a tool for perturbing targeted metabolic enzymes or entire organs, thus increasing the signal-to-noise ratio and potentially helping to overcome interindividual variation.

It is important to emphasise that this perspective article is meant to describe an idea/concept rather than pretend to be a clinical study for diagnostic purposes from which medical or physiological conclusions could be drawn. The goal of the described experiments is to prove the feasibility of the concept. In order to translate such a concept into a medical application, further investigations are required, and all the key-kinetic factors (Gastrointestinal tract/ renal / cardiac / liver metabolism) should be first addressed and determined. Additional studies, including blood and urine analysis, as well as *in vitro* experiments, would be necessary to determine the specificity and reliability of EVOC probe strategies in the assessment of pathophysiological conditions. Moreover, this article aims at inspiring the community to study the assumptions underpinning the concept of EVOC probe strategies towards future targeted analysis. To this aim we have launched a community forum facilitating the exchange of EVOC probes experiences at https://support.owlstonenanotech.com/hc/en-us/community/topics.
Future steps towards the design and development of novel EVOC probe clinical applications should focus on identifying specific diseases or conditions that can be targeted through this strategy. Examples comprise diseases that have a strong metabolic component, and for which effective diagnostic techniques are much needed, such as cancer. Cancer cells are known to undergo profound metabolic changes, some of which appear to be conserved across different cancer types and genetic mutations [106–108]. Identification of metabolic enzymes that are upregulated in cancer cells, compared to the surrounding healthy tissue, could direct the design of EVOC probe strategies for assessing cancer-specific metabolic functions *in vivo*. Of note, this concept is already applied in the clinic by fluoro-deoxyglucose coupled to positron emission tomography (FDG-PET), which exploits upregulation of the glucose transporter GLUT1 by cancer cells, resulting in higher detectable imaging signal [109,110]Applying an analogous approach, design of EVOC probes strategies that target cancer cells could result in increased secretion of specific volatile products in breath.

Compared to currently available stable isotope techniques for breath analysis, EVOC probes would offer the great advantage of multiplexing. All currently available stable isotope probes lead to breath secretion of $^{13}CO_2$, allowing the assessment of only one enzyme at a time. Development of cocktails of EVOC probes that are metabolised by different enzymes/organs, and that can be separately measured in breath, could potentially enable the assessment of multiple enzymatic activities simultaneously. Considering the complexity of most diseases, such an approach could help improving diagnostic accuracy [111,112]. In addition, EVOC probes are VOC that can be directly measured in breath, thus allowing monitoring of the probe itself, together with its metabolic product(s). This aspect allows parallel detection of substrate and metabolite pairs, potentially improving understanding of the kinetics of in vivo metabolism. Finally, EVOC probes do not rely on expensive isotopic labelling of compounds, thus enabling more affordable tools to be applied to breath analysis.

In this article we have presented the promise, limitations and assumptions underpinning EVOC probe strategies. Although this approach holds high potential in terms of targeted VOCs analysis for assessment of hypothesis-driven biological pathways, several limitations and obstacles should be first addressed. For example, oral delivery of the EVOC probe might lead to high biological and/or pre-analytical variability, as difference in absorption, and distribution kinetics, which would induce

heterogeneity in washout curves that might lead to incorrect data interpretation. Secondly, different EVOC probes might have different distribution kinetics and metabolism rates, which would reflect on the length of the test and number of breath samples required to translate data into information. Therefore, in some instances, it might be time consuming and/or inconvenient to the tested subject. Finally, one of the main challenges would be controlling the environmental intake of the EVOC probe and to fully understand the molecular pathways for its metabolism, in order to draw accurate and informative conclusions.

In conclusion, hypothesis-driven approaches deploying EVOC probes hold great potential to further breath research. We strongly believe that detailed understanding of pathophysiological processes complements biomarker discovery research and allows development of targeted EVOC probe strategies which can help push breath biopsy of VOCs from proof of concept to reality. We hope this concept excites the community to collectively assess its potential.

Conflict of interest:

All authors are employees of Owlstone Medical Ltd.

Acknowledgements:

This is an approach as pioneered by the group of Paul Thomas at Loughborough to enable standardised evaluation of different breath analytical tool chains as is the focus of their peppermint consortium

Figure 1

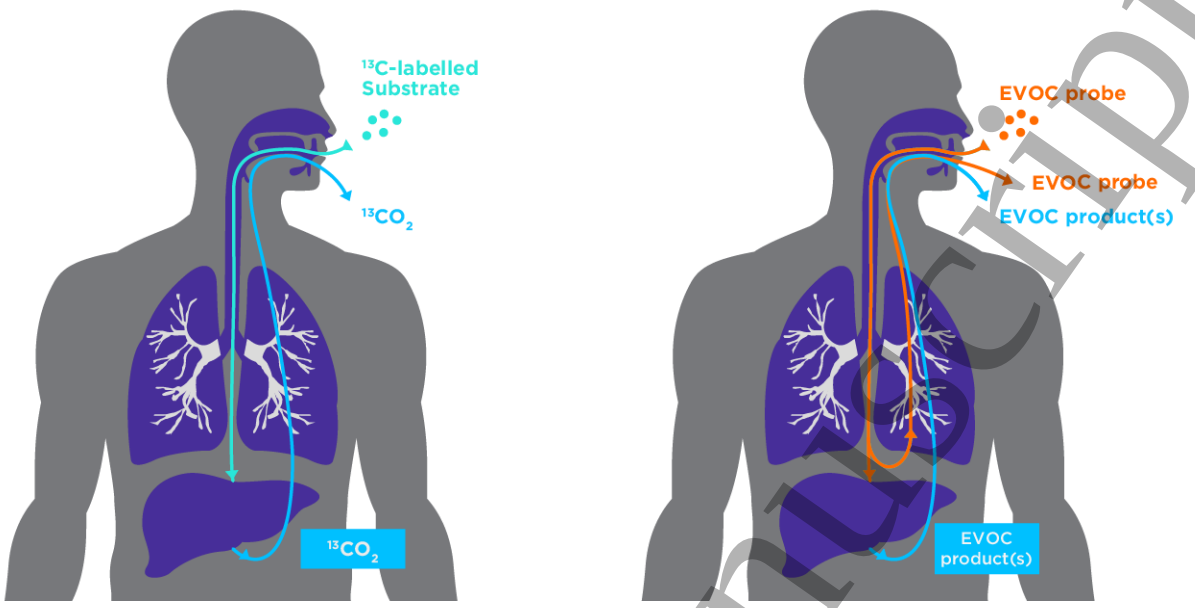
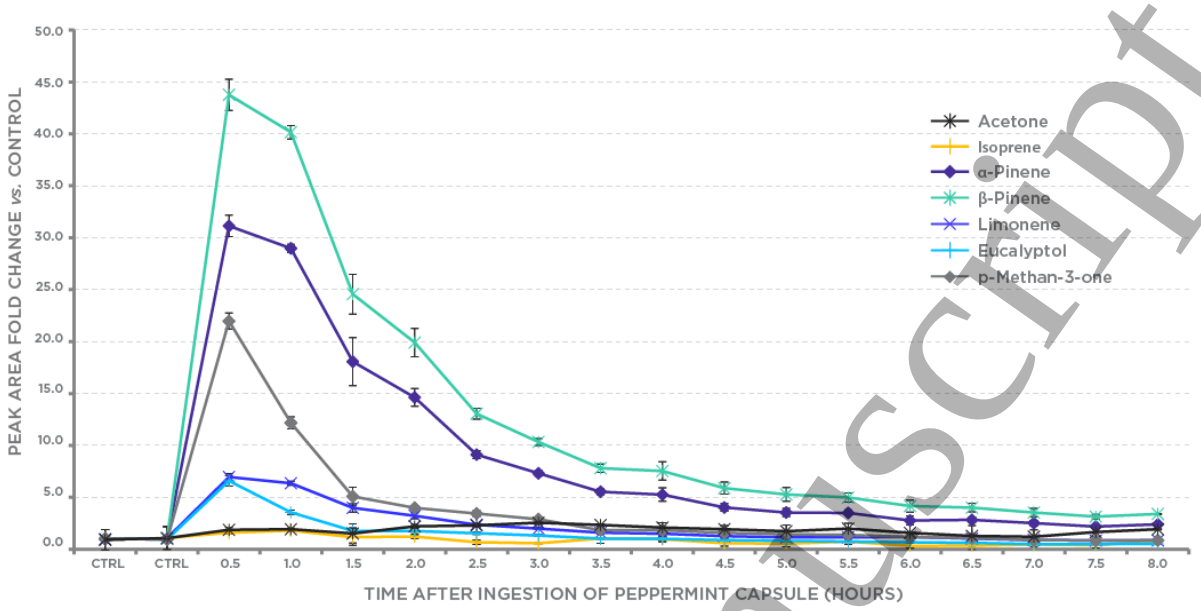


Figure 1. Comparison of stable-isotope labelled compounds and EVOC probes for breath analysis of liver metabolism. Schematic illustration of measurement of liver enzymatic activity using a ^{13}C-labelled substrate (left). Upon administration, the labelled substrate is metabolised by the liver, leading to production of $^{13}CO_2$, which is then secreted through the lungs via breath. Schematic illustration of measurement of liver enzymatic activity using an EVOC probe (right). Upon administration, the EVOC probe is rapidly secreted in breath due to its volatility. At the same time, the EVOC probe is metabolised by the liver and can lead to production of other VOCs, which can be secreted via breath. Enzymatic activity can be assessed by monitoring both the clearance of the EVOC probe itself from the system, and the secretion of EVOC product(s) generated through the enzyme/organ of interest.

Figure 2

A



B

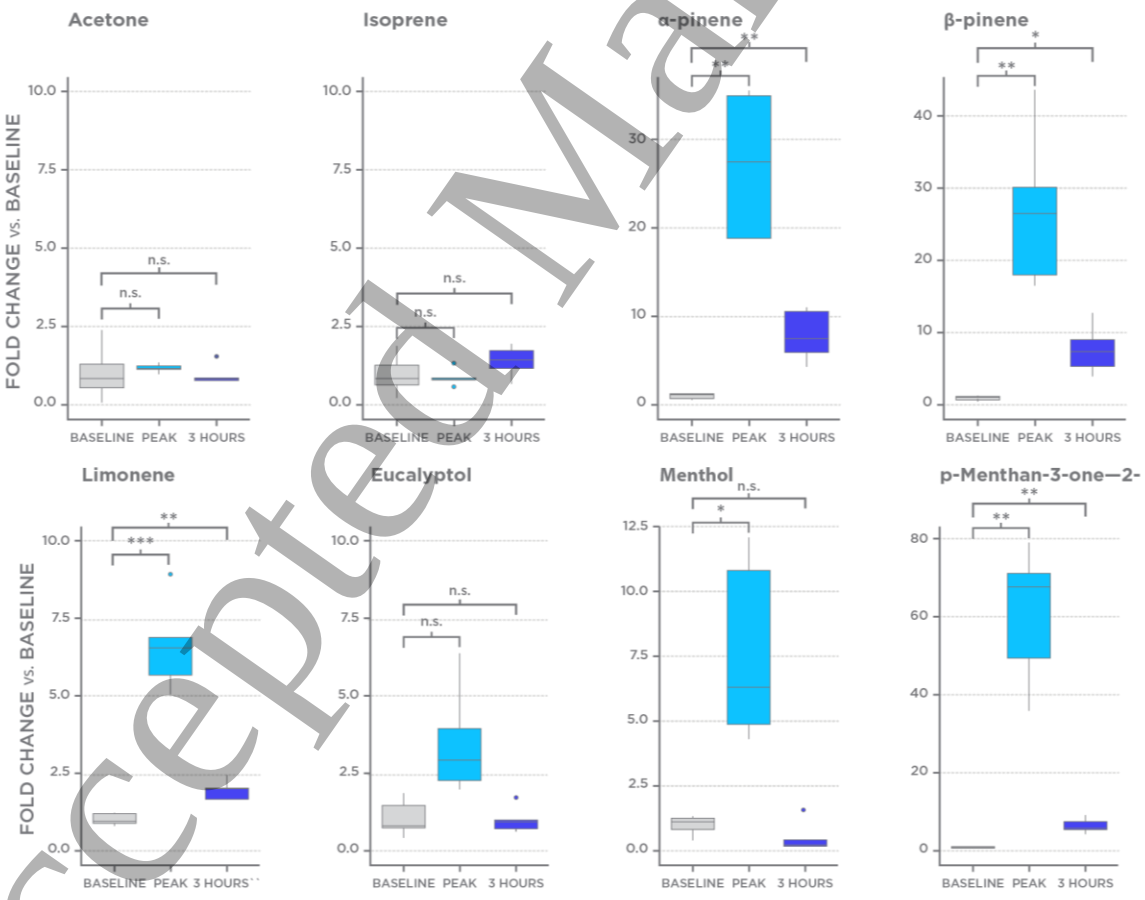


Figure 2. Administration of EVOC probe affects breath composition. **A)** Washout curves of acetone, isoprene,

and different terpenes/terpenoids from one healthy subject at baseline (Ctrl) and after ingestion of the EVOC probe. This experiment was performed 5 times on a single subject, once a week, in which the subject was sampled at baseline, 45 minutes (Peak) and 3 hours after digestion of EVOC probe. Data were normalised on average baseline levels, error bars represents SD of two breath samples obtained simultaneously by the ReCIVA at each time point. **B)** Boxplots of breath levels of acetone, isoprene and different terpenes at baseline (before EVOC), peak (45 min after EVOC) and 3 hours after EVOC, acquired across 5 weeks in one healthy subject. *, **, and *** represent paired t-student p-value < 0.05, 0.01, 0.001, respectively. n.s. = not significant.

Figure 3 (Removed to Supp. Materials)

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