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EDITORIAL

Establishing breath as a biomarker platform—take home messages from the Breath Biopsy Conference 2023

Hsuan Chou, Lucy Godbeer and Madeleine L Ball*

Owlstone Medical Ltd, 183 Cambridge Science Park, Milton Road, Cambridge, CB4 0GJ, United Kingdom
* Author to whom any correspondence should be addressed.

E-mail: madeleine.ball@owlstone.co.uk

Abstract

The annual Breath Biopsy Conference hosted by Owlstone Medical gathers together the leading experts, early career researchers, and physicians working with breath as a biomarker platform for clinical purposes. The current topics in breath research are discussed and presented, and an overarching topical theme is identified and discussed as part of an expert panel to close the conference. The profiling of normal breath composition and the establishment of standards for analyzing breath compared to background signal were two important topics that were major focuses of this conference, as well as important innovative progress that has been made since last year, including the development of a non-invasive breath test for lung cancer and liver disease. This meeting report offers an overview of the key take-home messages from the various presentations, posters, and discussions from the conference.

1. Introduction

Blood and urine are examples of well-established biomarker platforms with a multitude of different uses for clinical research and practice, but despite breath also being enriched for informative compounds, there are only a few breath tests currently in use. Breath analysis is well-positioned to address many current unmet needs: for many diseases, diagnosis is often happening too late through a lack of accurate or appropriate early detection tools, and clinical trials for drug development also currently are limited by high cost, lengthy processes, and a high failure rate. These issues could be addressed through better biomarker monitoring tools: breath can be collected in a completely non-invasive manner, is a virtually inexhaustible resource produced almost continually by the body, and contains volatile metabolites (otherwise known as volatile organic compounds or VOCs) that can act as biomarkers of many physiological processes in the body.

There has been fantastic progress in the breath research field in recent years, with new and innovative ways to best utilize the power of breath pushing more breath tests closer to clinical use. Understanding breath as a biological sampling matrix is paramount to discovering, validating, and translating breath-biomarkers. One of the key challenges to overcome that is specific to breath is the inherent dual nature of its composition. Breath is comprised of both volatile compounds that have originated from within the body and those that have been inhaled from the environment. Due to this, robust standardization procedures are of heightened importance, and it is no surprise that calls for better standardization have been repeated in the field of breath research for many years now: how you collect, store, analyze, and interpret the composition of breath is ever influenced by the levels of control that have been implemented over the background sources of compounds [1, 2].

Several different breath analysis techniques are routinely used, all with their own strengths and limitations. The two major categories of technique are 'online' and 'offline' analysis tools, in which breath is introduced directly into the analyzer, or collected separately, stored, and then analyzed later in a laboratory respectively. The breath matrix is complex with a high number of volatile compounds, meaning it requires very comprehensive analytical methods, the most comprehensive that are currently available are offline methods such as GCxGC-TOF. Online analyzers avoid many sampling and storage-related artifacts of offline methods, but there is currently no online analytical technology that is capable of providing the

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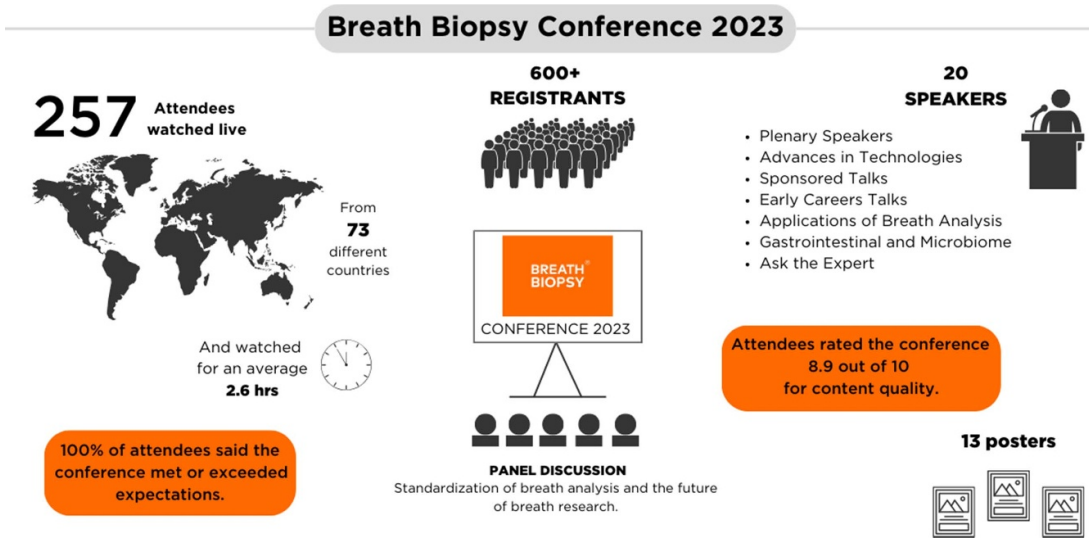

Figure 1. A summary of the Breath Biopsy Conference 2023 hosted by Owlstone Medical.

same level of information. It is important to note that both approaches have particular strengths in clinical practice, and there is no one 'right way' to undertake breath collection and analysis. The advancement and utilization of both techniques in tandem can increase the translatability of breath analysis in the clinic, however, to ensure high-quality data between the different methods developed for breath research, the results must be consistent and reproducible. Therefore, some standardized practices that allow for better cross-validation across different approaches must be established before more breath tests can be developed successfully into clinical tools. Ensuring the implementation of these standardized practices across the entire breath community requires an open forum discussion and the establishment of consensus opinions. In order to provide an additional platform for these discussions alongside other breath science conferences, Owlstone Medical hosts the annual Breath Biopsy Conference.

The Breath Biopsy Conference is an annual two-day conference hosted online, and features keynote speakers, selected presentations, posters, and an expert panel discussion to close the event (figure 1). This year's keynote speakers were Dr Michael Wilde from the University of Plymouth, and Dr Sean Harshman from the US Air Force. The full conference is recorded and is available for all attendees to watch sessions on-demand after the event—a flexible setup that enables those from all over the world to access the content, no matter if they are available to attend live. A total of 613 people registered for the Breath Biopsy Conference 2023 which was attended live by 257 people from 73 countries all over the world. This is an approximate 6% increase in sign-ups from last year, and an increased range of countries that participants are joining from—up from 69 countries in 2022, and 49 countries in 2021. This is a testament to the continuing success of the Breath Biopsy Conference, and alongside other conferences such as the International Association of Breath Research (IABR) Summits reflects the sustained interest in the breath field, and recognition of the potential that breath analysis has to clinical research and practice. This report, in line with previous reports [3–5], will cover the major themes and take-home messages from the conference speakers and discussion sessions to highlight the progress that has been made in breath research, and consensus opinions on how to improve the translation of breath biomarkers toward clinical application. The full recordings of the talks and poster presentations, as well as poster PDFs, are available to watch any time for free.

2. A greater appreciation of complexity: VOCs and metabolite networks underpinning their production

Over the last few decades, researchers have dedicated efforts to understanding the VOCs in exhaled breath, seeking to comprehend their origins, their relevance to physiological processes, and their potential applications in disease diagnostics and treatment monitoring. Although many candidate breath biomarkers have been suggested, very few have successfully translated into clinical settings. This can be attributed, in part, to the challenges of inconsistent findings and the inability to replicate or validate previous results. The lack of standardization in defining breath VOCs and identifying them, coupled with the insufficient understanding of the mechanistic and biochemical pathways in the human body, contributes to these hurdles.

An alternative approach to exploring breath VOCs for disease biomarkers is to adopt a more systematic approach, seeking patterns and understanding how compounds cluster together as opposed to centering around individual VOCs, or a panel of VOCs for disease prediction model development. The global view of VOC patterns and their relation to specific diseases will help narrow down interesting candidate biomarkers, which can then be targeted in validation studies. Categorizing compounds by chemical classes and their directional changes will also facilitate the identification of VOCs that are likely involved in specific metabolic processes but are actually from external sources, as a compound found 'on-breath' (compounds detectable in breath that can be reliably distinguished from background levels via quantification) can originate from both. More importantly, the systemic approach can help better align several informative breath compounds within the same metabolic pathway, increasing the sensitivity and specificity of diagnostic performance. Dr Michael Wilde, one of the two plenary speakers at the Breath Biopsy Conference, delved into the identification of exhaled metabolite sets, providing evidence of breath metabolite networks through a longitudinal study on acute breathlessness in cardio-respiratory patients at triage. The study, encompassing 277 subjects with breathlessness from severe exacerbation of asthma, COPD, heart failure, pneumonia, and healthy controls, utilized multidimensional gas chromatography-mass spectrometry (GCxGC-MS) to analyze samples [6]. Dr Wilde advocated for a pragmatic approach, favoring non-exclusive small signatures of individual chemical markers, and combining them with clinical metrics, such as lung function data.

Rather than focusing solely on single biomarker discovery, Dr Wilde emphasized the importance of exploring networks in datasets. The application of topological data analysis revealed four distinct networks in the five study cohorts, with COPD and pneumonia merged. Similarly, the clinical blood markers overlaid onto the network correlated with the disease groups. To understand the potential significance of branched hydrocarbons in acute exacerbations, Dr Wilde explored ways to seek evidence that suggests these hydrocarbons come from the same biological process (i.e. inflammation). Because volatile metabolites are less annotated than blood metabolites, pathway analysis is not applicable; however, the chemical information of these VOCs can be leveraged to identify similarities in the dataset.

Markers arising from the same pathway are expected to be highly correlated, show set-level significance, and likely share similar chemical properties. While these markers may not all move in the same direction within the pathway, identifying the similarities shared between them is a helpful method to understand VOCs and their involvement in metabolic pathways. To enable metabolite set enrichment and chemical similarity analysis, Dr Wilde grouped VOCs into several metabolite sets based on their shared properties. By plotting the set-level significance against chemical similarities, the visualized nodes representing different metabolite sets provide information on the directional and fold changes, as well as their enrichment significance. These can then be linked back to the core findings within a study.

Additionally, Dr Wilde demonstrated how these metabolite sets can be transformed into a radio-clustered diagram to enhance visualization. The visualization showed that these metabolites are highly correlated by carbon number, functional groups, and substructures, suggesting that they likely originated from the same sources or the same biological responses in the diseases. In the acute breathlessness study, Dr Wilde found significantly enriched, co-expression of C5-7, C8-10, C11-16 hydrocarbons, and C3-5 oxygen-containing VOCs with high chemical similarity, exhibiting the highest change during acute exacerbation. These results provide primary evidence of exhaled VOCs indicative of disease responses measured *in vivo*. The findings were validated through Louvain clustering, a community detection technique, and the results were concordant with the metabolite set enrichment analysis.

Extending beyond the broader understanding of dysregulation in breath metabolite networks across cardiorespiratory exacerbation subtypes, Dr Wilde also provided insights in the later panel discussion on the importance of clustering to identify irrelevant features. Because VOCs in breath are most likely coming from more than one source, it is important to know what influences breath. Through evaluating the systemic differences of VOCs in the dataset, researchers may narrow down interesting compounds by excluding those that correlate with each other but do not make much sense in chemical classification. Controlling factors that may also affect data, including storage time, operator, and major instrument maintenance, remains a challenge in breath research. While it is not always realistic to control every factor, Dr Wilde stresses that identifying potential influences on the data should be put into standard practice. The topic of standardization regarding breath collection was also emphasized by other presenters during the two-day conference, or example, Dr Pedro Vaz presented his work on utilizing exhaled breath in lung cancer screening. Dr Vaz suggested that once patients are enrolled, fasting time, avoidance of cosmetics and perfumes, as well as other factors, should be standardized prior to breath collection. Relevant to the aspect of standardization, the other plenary speaker Dr Sean Harshman discussed the influences of background, sampling room and food VOCs and presented his work on the impact of thermal desorption (TD) tubes on the variability of exhaled breath [7]. While the data indicated there is minimal impact, Dr Harshman mentioned a few caveats in the study and

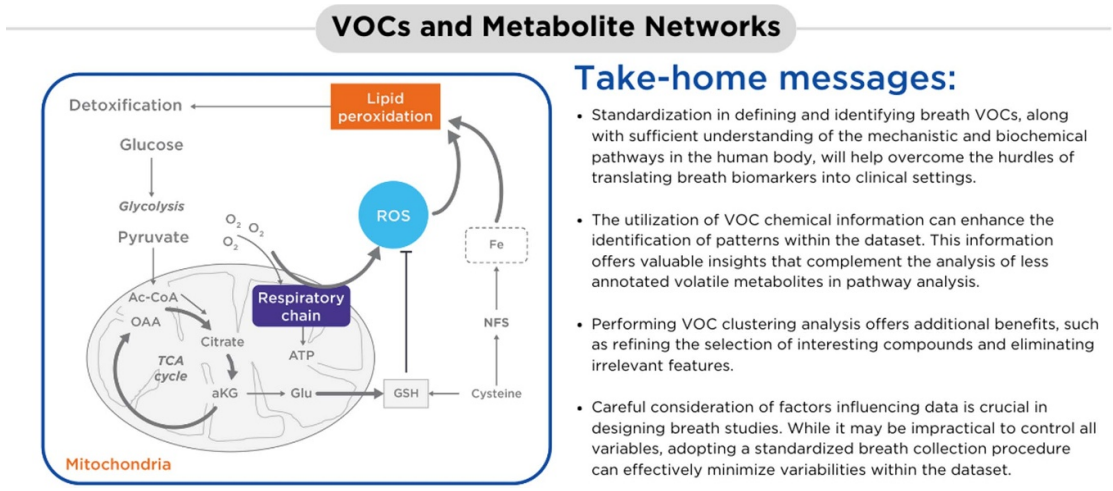

Figure 2. A summary of the take-home messages from the conference that revolved around a greater understanding of VOCs in breath and the metabolite networks that underpin their production. An example metabolite network is indicated in the schematic, particularly highlighting lipid peroxidation, as it is thought to be one of the major sources of certain VOCs in the breath.

suggested backgrounds from both TD tubes and the sampling room should be considered when approaching data analysis. Dr Harshman also mentioned that profiling food VOCs and studying the length of these VOC's presence in breath after consumption will help researchers understand what to expect and what to focus on in the data, addressing the challenge of limiting diet in studies. Other aspects of standardization in breath research are detailed in the later sections.

In contrast to blood metabolites with over a century of study and integration with other data, such as genetics, the understanding of breath volatile metabolites is still at an early stage. Utilizing the well-established metabolomics pathway analysis can facilitate the understanding of breath VOCs, but secondary pathways not yet shown in literature will need to be identified. As a take-home message, Dr Wilde advocated leveraging chemical data to fill knowledge gaps, and once the VOCs of interest are well known, researchers can reverse engineer to uncover pathways not yet explored in literature, progressing the breath research field towards the translation of breath biomarkers in the clinic. Dr Harshman encouraged researchers to assess their own systems thoroughly, including blanks and background samples, to understand how these contribute to the data. Furthermore, he emphasized the importance of continuously evaluating factors affecting exhaled breath collection and analysis to enhance standardization efforts. This is a key take-home message for breath researchers, as this allows for a better understanding of why there might be variation in results between different systems. This is especially important due to the complex metabolite networks that VOCs in the breath originate from, and therefore a greater appreciation for the methodological factors that influence their abundance in the breath will enable improved identification of true biomarkers of disease to ultimately advance breath as a platform for biomarker analysis (figure 2).

3. Breath technology is advancing

While network analysis enhances our understanding of the inter-metabolite relationships of VOCs, the ultimate goal of breath biomarker discovery is to translate the findings into clinical settings. The 2023 Breath Biopsy Conference featured several speakers discussing the advanced techniques for utilizing breath in both pre-clinical and clinical settings. The technologies presented, characterized by sensitivity, accuracy, and robustness underpin the detection and measurement of breath volatiles, showcasing the broad applicability of breath research.

Dr Kerstin Geillinger-Kästle from Boehringer Ingelheim addressed the need to develop methods for sampling breath from pre-clinical animal models to study interstitial lung disease. The minimized inter-individual variability of inbred mice strains and the tightly controlled laboratory environment offer unique advantages for expediting the identification of breath biomarkers. Boehringer Ingelheim collaborated with Owlstone Medical to develop a robust method for characterizing volatile metabolites in exhaled mouse breath. This method, modifying the flexiVent® small animal ventilator, enhances control over signal-to-noise ratios [8]. Filters for the instrument and ambient air are connected to the ventilator by flexible tubing, along with a sorbent tube for breath collection. Additionally, the approach involves comparing mouse breath samples to blank samples within a controlled environment to discern on-breath compounds.

The study utilized 15 healthy mice to establish baselines, identifying 73 on-breath compounds from

the 472 compounds in the mouse breath dataset. The study also incorporated 13 human breath samples, while mouse breath harbors a smaller array of volatile metabolites, the comparison revealed a 29% overlap (57 compounds) between human and mouse breath. These results presented a reliable mouse breath sampling and analysis platform, establishing mice as a viable animal model for a pre-clinical study of breath biomarkers. A second study utilizing a disease model to identify biomarkers of lung fibrosis is currently in progress. Although biomarkers identified in pre-clinical settings must eventually undergo validation in clinical studies, these attributes should streamline the process of biomarker discovery, validation, and eventual clinical utilization.

Several of the presenters at the Breath Biopsy Conference discussed the utilization of exogenously produced volatile compounds (EVOC®) as an innovative and alternative approach for breath-based testing, as it involves the use of a targeted probe to elicit a response from a specific metabolic pathway or process of interest [9]. This concept bears similarities to how ^{13}C-labelled urea is currently employed as a metabolic probe to evaluate *H. pylori* infection within the gastrointestinal tract in clinical settings [10].

In his talk, Dr Luis Mendez from Clinica Alemana demonstrated how limonene, an exogenous volatile compound, could offer new possibilities for non-invasive diagnostics in liver cirrhosis. Cirrhosis is a globally recognized health problem with most cases diagnosed too late for effective treatment, and currently relies on the invasive and costly gold standard of a liver biopsy for disease detection. Increased levels of breath limonene were discovered in a study comparing subjects with cirrhosis to healthy controls [11]. As a result of disease, the liver Cytochrome P450 (CYP) proteins CYP2C9 and C19 are less able to metabolize limonene, resulting in its accumulation. The study also showed reduced levels of limonene in breath after cirrhotic patients underwent a liver transplant. To apply the concept of EVOC probes, Dr Mendez conducted a longitudinal study by administering limonene to 29 subjects with cirrhosis and 29 healthy controls. Subjects fasted with no citrus intake the day prior to sampling, and breath samples were taken at baseline and at intervals after limonene ingestion. The results showed the best prediction model to differentiate cirrhotic patients from healthy controls at 60 min after limonene intake (AUC = 0.9).

Dr Mendez suggests that combining these studies with clinical skills may be the best approach to overcome the diagnostic challenges of liver cirrhosis. Another speaker, Dr Giuseppe Ferrandino, presented the same concept of administering glucose to 10 subjects in a longitudinal study to phenotype gut bacteria through gut microbiome metabolites in exhaled breath. Dr Ferrandino's talk is discussed in more detail in the next section.

EVOC probes can also target enzymes involved in disease-specific metabolic pathways, for example, the targeting of aldo-keto reductase (AKR) in cancer. In many cancer types, increased oxidative stress can lead to overproduction of aldehydes. It has been suggested that the upregulation of AKRs can effectively eliminate these compounds [12]. In a poster presentation, Dr Alexandra Martin from Owlstone Medical demonstrated the potential of AKR-targeted EVOC probes in an *in vitro* study. Dr Martin showed that through the administration of aldehydes as substrates in cancer cell lines, inhibition of AKR activities by using either inhibitors or knockouts resulted in reduced alcohol production. Utilizing this enzyme-targeting strategy, an EVOC probe, D5-ethyl-βD-glucuronide, was developed as a substrate to target beta-glucuronidase, an enzyme known with increased levels in the extracellular space of cancer cells. Upon enzymatic cleavage, D5-ethanol is released, serving as a volatile reporter for cancer screening. This targeted EVOC approach that amplifies altered metabolic signals is currently being applied in the Evolution trial for lung cancer screening.

Like many areas in breath research, the goal of identifying breath VOC biomarkers for lung cancer detection has been challenging. Dr Robert Rintoul from the University of Cambridge walked us through the journey of conducting the LuCID trial- the largest-ever breath biomarker discovery study for lung cancer, to the Evolution trial. Despite numerous breath studies in lung cancer, there are no available biomarkers translated into clinical settings to date [13]. The LuCID study aimed to identify breath biomarkers for the early detection of lung disease, as well as evaluate diagnostic performance in real-life clinical populations. Subjects suspected of having lung cancer and controls were recruited, resulting in 518 cases and 677 controls. These subjects were split into a training cohort and a validation cohort at a 3:1 ratio for data analysis. While a total of 11 compounds differed significantly ($p < 0.05$) between cases and controls in either early or advanced disease in the training or validation cohorts, no compound remained significant after correction for gender, age, BMI, and smoking status. Compared to the 61 compounds found in previous studies, only three were found in this study. The results from the LuCID study suggest that individual breath biomarkers show some association with the presence of lung cancer, especially at advanced stages. However, the combined diagnostic performance of these biomarkers does not exceed that of clinical risk prediction models.

The LuCID study provided valuable insights for improving the breath analysis workflows, and work is currently under preparation for submission. From this, the focus has now transitioned from open discovery to a hypothesis-driven approach. As mentioned in Dr Rintoul's talk, the current targeted EVOC approach that amplifies altered metabolic signals for

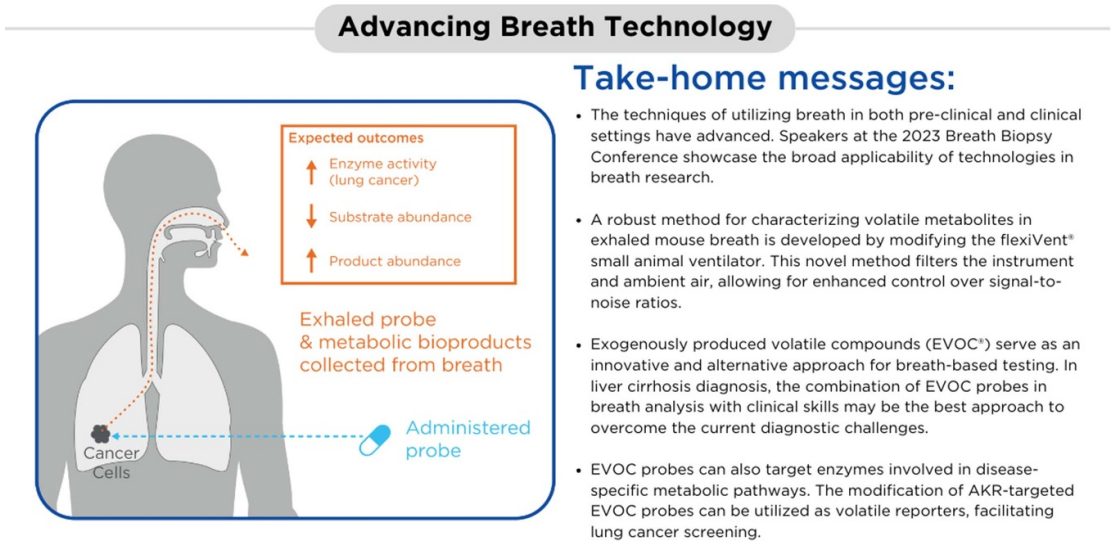

Figure 3. A summary of the technological advances of breath research presented at the Breath Biopsy Conference 2023, and how more innovative uses of breath analysis have pushed the incorporation of breath into clinical use closer. A schematic of the rationale behind EVOC probes presented during this conference is given as an example.

lung cancer screening has been shown to be promising in the phase I (safety, proof of mechanism, and dose-ranging) Evolution clinical study, with 12 lung cancer patients and 19 healthy subjects recruited in the phase lb trial. The phase II study has just started, with an aim to confirm the robustness of the test from the phase l data, as well as to understand the specificity of the EVOC probe in lung cancer. These collective efforts of technology advancements highlighted at the 2023 Breath Biopsy Conference showcase the expanding horizons in breath research. The development of pre-clinical breath sampling in animal models, the application of EVOC probes in liver cirrhosis diagnosis, and the modification of EVOC probes in facilitating lung cancer screening all represent a movement towards more innovative methods of utilizing the power of breath analysis. This lays the foundation for a promising future where breath analysis becomes an integral part of clinical practice (figure 3).

4. Microbial metabolism is a major producer of the VOCs in exhaled breath

Trillions of microbes live within the human body, with the highest concentrations to be found in our gut, where they digest and break down the food we consume. These microbes produce a significant number of volatile metabolites that are exhaled in the breath. The last decade has seen significant growth in both breath research and microbiology research, and the overlap of these two fields has the potential to aid the development of a new era of microbiome studies and clinical tests. At the Breath Biopsy Conference 2023, microbial metabolism was discussed by a range of professionals from across the globe, all of whom highlighted how vital research is in this field.

Dr Giuseppe Ferrandino from Owlstone Medical presented his research on 'Gut Bacteria Phenotyping Using Dynamic Breath Analysis'. The talk began by covering how gut microbiome metabolites can exacerbate certain diseases. For example, carbohydrates can be converted into ethanol and released into the blood, which is a risk factor for non-alcoholic steatohepatitis (NASH). When NASH patients consume carbohydrates, there is a spike in blood ethanol that is not seen in healthy controls. If NASH patients are treated with an antibiotic that kills the microbes in the gut, this spike in blood ethanol is not seen [14]. This demonstrates that gut microbiome metabolism of carbohydrates is the main source of blood ethanol in patients with NASH. Currently, there is no test sensitive enough to measure gut microbiome ethanol production, which means clinical trials aiming to develop new medications for liver disease cannot determine how much ethanol is produced by the gut compared to how much is circulating in the body due to the consumption of alcohol. Because of this gap in the industry, Dr Ferrandino designed a pilot study ($n = 10$) to test whether ethanol produced by the gut microbiome can be measured in exhaled breath. Several metabolites were measured in exhaled breath that were reported to be generated by the gut microbiome, including the short-chain fatty acids associated with many facets of gastrointestinal disease development. However, the focus of this study was ethanol production, and a spike was seen in all tested subjects after the consumption of the substrate. This demonstrates that the fermentation of carbohydrates by the gut microbiome generates ethanol measurable in exhaled breath and that many volatile microbial metabolites of interest can also be measured using breath analysis.

Dr Bruce Johnson from the Mayo Clinic also presented his research at the Breath Biopsy Conference 2023, on 'Volatile Organic Compounds in Exhaled Breath Reflect Physiological Changes in Ultramarathon Runners'. Dr Johnson's study evaluated VOCs in exhaled breath that differed in abundance from 24 subjects before and after running an ultramarathon [15]. The purpose of the study was to identify biomarkers associated with lung injury due to exhaustive exercise. Interestingly, increased levels of acetate, 2,3-butanedione, and 2,3-butanediol were shown in the post-race breath samples, all of which are products of microbial fermentation. This indicated that exhaustive exercise influenced gut microbiome metabolism, and VOCs in breath can be used to evaluate this influence. This is important because professional athletes often experience gastrointestinal symptoms, such as abdominal pain, diarrhea, and nausea. Alterations in the gut microbiome caused by exhaustive exercise can also produce exercise-induced gastrointestinal disorders [16]. Breath analysis therefore provides a non-invasive, easy-to-use avenue to monitor gut microbiome metabolism in athletes to prevent the development of gastrointestinal diseases.

'Establishing Normal Values for Gastrointestinal Hydrogen Sulfide Production in Breath Using Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)' was a poster presented by Dr Anthony Hobson ($n = 25$). The poster discussed how hydrogen and methane breath testing (HMBT) is well-established for the diagnosis of small intestinal bacterial overgrowth (SIBO) and intestinal methanogen overgrowth (IMO). Hydrogen and methane are products of microbial fermentation in the gut, and this fermentation also produces hydrogen sulfide which is associated with diarrhea and inflammation in inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Studies that include patients with IBS and IBD are needed to determine the difference in the level of hydrogen sulfide production from microbial fermentation between the two different diseases, as well as between the diseases and healthy controls. IBS and IBD are disorders with clear unmet needs regarding risk stratification, treatment options, and monitoring. Hydrogen sulfide can therefore be used as a biomarker for IBS and IBD and be developed into a non-invasive breath test to provide better management of the disorders.

Pathogenic microbes responsible for infectious diseases can also result in altered volatile metabolites in exhaled breath, either through altered metabolic pathways in the host, or with certain pathogens like bacteria and fungi, their own endogenous metabolic processes. In December 2019, COVID-19 emerged from Wuhan, China, and resulted in a worldwide outbreak in many countries. Globally, as of today, there have been over 770 million confirmed cases of COVID-19, including nearly 7 million deaths [17]. Research towards screening platforms for COVID-19 infections has been emphasized to provide more sensitive, specific, and reliable tests that are non-invasive and can be accessible to the entire population—of which the suitability of breath analysis has been previously evaluated in detail [18]. At the Breath Biopsy Conference 2023, Dr Lorena Diaz de Leon Martinez presented the poster 'SARS CoV-2 Infection Screening via the Exhaled Breath Fingerprint Obtained by FTIR Spectroscopic Gas Phase Analysis'. In this study, simulated breath was evaluated using acetone, acetaldehyde, and nitric oxide, as the levels of these compounds in the breath have previously been identified as being capable of distinguishing between COVID-19 from healthy controls [19–21]. This provides a proof-of-concept into how breath tests could be used in the future to detect altered levels of VOCs in the breath caused by viral infections affecting metabolic processes. Bacterial infections can also alter the VOCs found in the breath, either by affecting the internal metabolic processes of patients (like viruses) or by the production of compounds from their own metabolism. VOCs such as isoprene, ethanol, methyl mercaptan, and more, have altered levels in the breath of patients with bacterial infections compared to healthy controls [22]. Therefore, breath analysis can be used to diagnose and monitor infections and be used in point-of-care scenarios.

The panel of experts at the Breath Biopsy Conference 2023 was asked how they envision the future trajectory of breath research and its potential, and the consensus was that breath analysis could majorly improve infectious disease research and diagnosis. Dr Brooke Kaiser from the Pacific Northwest National Laboratory stated that she believes infectious diseases, in particular respiratory infections, are where breath research can be useful. Dr Kaiser highlighted that breath analysis can be used to evaluate the body's immune response, identify the metabolic pathways of microbes, and diagnose infectious diseases. Dr Ethan McBride from Los Alamos National Laboratory agreed and added that understanding bacteria profiles in different parts of the body has great potential thanks to breath analysis. Understanding how microbes respond to treatment is another possible avenue too. Dr Micheal Wilde from the University of Plymouth emphasized Dr McBride's point by adding that bacteria are ethically easier to evaluate and test in a laboratory, meaning research and clinical trials can be cheaper and quicker, allowing for a faster understanding of how breath analysis can be used for the diagnosis and management of infectious diseases. However, the results seen in bacteria cultures are not always easy to translate to human and animal samples. Mr Billy Boyle from Owlstone Medical also highlighted that fungal infections may produce unique volatiles which could be very exciting in the identification and diagnosis of those diseases. Although many challenges remain,

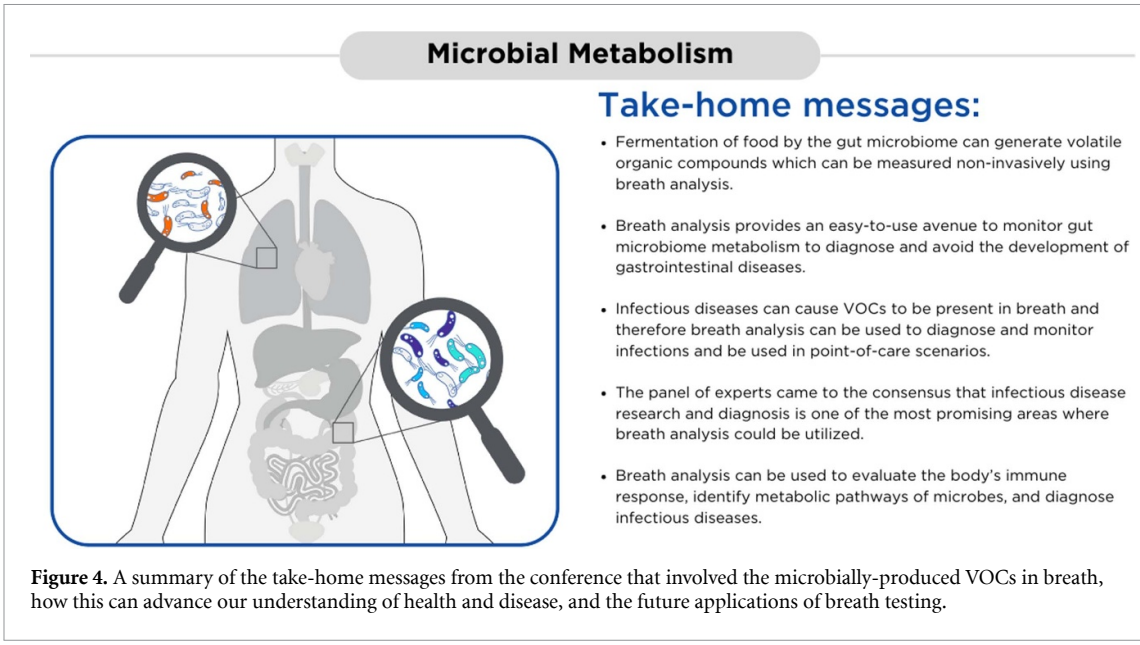

Figure 4. A summary of the take-home messages from the conference that involved the microbially-produced VOCs in breath, how this can advance our understanding of health and disease, and the future applications of breath testing.

breath analysis has great potential to revolutionize how we diagnose and monitor the microbiome, as well as infectious diseases, and the progress so far is very encouraging (figure 4).

5. Achieving better standardization and mapping normal human breath are important goals

The issue of standardization in breath research is commonly cited as a central reason for the difficulty in replicating identified VOCs across different studies and has been named a key goal to address for many years in order to progress breath faster toward its broader use in the clinic. However, what exactly needs to be done to improve standardization in practical terms is still subject to debate. The IABR has supported the drive toward better standardization in breath research for many years, including the Peppermint Initiative to pilot a benchmarking experiment between different breath methods, and is involved in important ongoing work in this area [23, 24]. To establish some consensus and provide more methods of achieving better standardization in breath research, the 'Ask the Expert' panel discussion of the 2023 Breath Biopsy Conference focused on this issue. The CEO of Owlstone Medical Mr Billy Boyle was joined by four world-leading experts in the analysis of human breath: Dr Ethan McBride from the Los Alamos National Laboratory, Dr Brooke Kaiser from the Pacific Northwest National Laboratory, Dr Mangilal Agarwal from Indiana University-Purdue University, and Dr Michael Wilde from the University of Plymouth.

An interactive poll was available for Breath Biopsy Conference attendees to fill in, and one of the key questions asked for feedback on what they considered the most important factor to improve standardization—is it that more people need to be using the same hardware, or does there need to be more analytical standards to ensure data quality? Although opinions varied in the responses, the most popular response supported a focus on standardized analytical procedures rather than the hardware/methodology used to collect and analyze breath samples. It was unanimously agreed by the panel that insights from a broader range of viewpoints, perspectives, technologies, and approaches are important to advance the field as this can give greater confidence in consistent findings, and different platforms are all capable of producing high-quality results that are suited to slightly different applications. However, it is still important to understand what impacts the choice of methodology is having on the resulting breath data, as certain criteria such as breath volume collected benefit can be optimized [25]. Dr Sean Harshman mentioned during his plenary talk that storage, background, and handling of exhaled breath are well understood to be sources of variability, but that the collection tubes themselves can also contribute to the background signal—with his data presented showing that 52% of the tentatively identified compounds from the TD alone being reported in breath samples in the literature [7].

One of the most important issues to address when standardizing analytical procedures is how to deal with inhaled background signals that comprise a significant number of the volatile compounds identifiable in breath samples. This is an unavoidable factor in breath research because the intake of compounds from the surrounding air is intrinsic to the mechanism of breathing, and so must have robust procedures set up to minimize their impact on biological inference. Dr Harshman previously presented data that showed that background contaminants could account for more than 70% of the exhaled

breath signal in their study, highlighting the need for robust, standardized procedures to handle compounds that have originated from the background. The panel members all had very similar approaches, which all revolved around the collection of background air samples to analytically compare to breath samples. Dr Agarwal and Mr Boyle share an approach whereby the background air is sampled before and after breath samples are taken for quantitative comparison. Dr Kaiser discussed her statistical-orientated approach, which involves the collection of many breath and background air samples rather than one-to-one paired samples, and then quantifying what volatiles are enriched in the background and what are enriched in the breath. Dr McBride takes a similar approach and highlighted how important this procedure is, as the secondary electrospray ionization (SESI) system utilized as part of his work can even pick up signals from the floor cleaner used several rooms away from the breath collection room. With the very sensitive equipment needed to detect very small signals in breath, and the inherent dual nature of breath to contain both background and on-breath compounds, comes the need for very rigorous control procedures. Therefore, the consensus from this panel to address background contaminants is that background air should always be collected, with time spent to carefully perform analysis on these samples alongside breath samples. This is especially important when breath sampling is conducted at several different locations, and accordingly, the recommendation is that the corresponding background air should be collected at each sampling site where breath samples are collected to best distinguish what is, and is not originating from the breath.

The handling of chemical identification is a challenge often referred to as the 'bottleneck of metabolomics', and so is a key point of discussion for standardizing breath research. Dr Wilde believes that identifying the compounds in breath is essential at the point of making claims about biologically relevant biomarkers or deciding to remove a compound as an artifact, but that identifying everything is not necessarily needed—particularly background-originating compounds as the process is time-consuming and expensive. Dr Agarwal and Dr Kaiser both agreed with this approach, and Dr McBride continued by mentioning some lessons from multi-omics pipelines that can be instated here, for example using the Metabolomics Standards Initiative (MSI) to produce quantitative criteria for breath compound identification pipelines [26]. There are distinct levels of MSI confidence in identification, with level 1 being the highest confidence as an 'identified compound', and level 4 being an 'unknown compound.' If level 2 'putatively annotated compound' and above can be achieved more often, this indicates stringent requirements have been met and is a practical goal to aim for in breath research. Therefore, including robust MSI identification procedures in the reporting of breath studies can help improve the reproducibility and robustness of the data, which is one of the key goals to achieve better standardization and improve the translatability of breath biomarkers.

Building upon the discussion of the importance of identifying a compound, going a step further, and assigning endogenous vs exogenous origin of identified compounds is a challenge for the breath field. The approach that Dr Kaiser has taken is to take additional complementary breath-based samples such as condensate which can provide protein information to assist in identifying metabolic pathways. Dr McBride reiterated how important this question is, but also stated that this is a separate procedure from chemical identification. While a compound can be identified confidently through meeting stringent MSI requirements, the confirmation of its presence alone may not provide sufficient information as to what biochemical pathways in the body could have produced it. Dr Agarwal stressed that the determination of whether a compound is endogenous or exogenous cannot simply be based on its relative concentration to background alone. For example, compounds like terpenes introduced via the diet can be measured at high concentrations in breath relative to the background, but because they cannot be produced by metabolic processes in the human body, they are still considered exogenous. Dr Wilde brought up using correlational metabolic networks that formed the topic of his plenary talk, as this can assist with identifying these sources—many compounds can potentially come from makeup products such as aldehydes but could also have an endogenous origin, but if these signals are correlating with other exogenously originating compounds this may help with the interpretation. A final important point that was raised by Mr Boyle was not to discard exogenous-originating VOCs as environmental contamination outright, as anything unexpected could have clinical utility—limonene is an exogenous compound from the diet that is reliably considered to be on-breath, that is being utilized as an EVOC® probe with very promising results in liver disease that was discussed as part of earlier talks. However, limonene used to be removed outright during preliminary investigations due to it being classified as an exogenous compound, which raises the issue of needing to distinguish between exogenous on-breath compounds, and exogenous background contamination.

The importance of establishing a reference database for the baseline composition of breath across a population of healthy people cannot be understated, as Dr Kaiser put it: 'Understanding what is normal is critical to understanding what abnormal looks like'. Showcasing how important this development is for breath science, there is a current collaborative effort by many members of the wider breath community

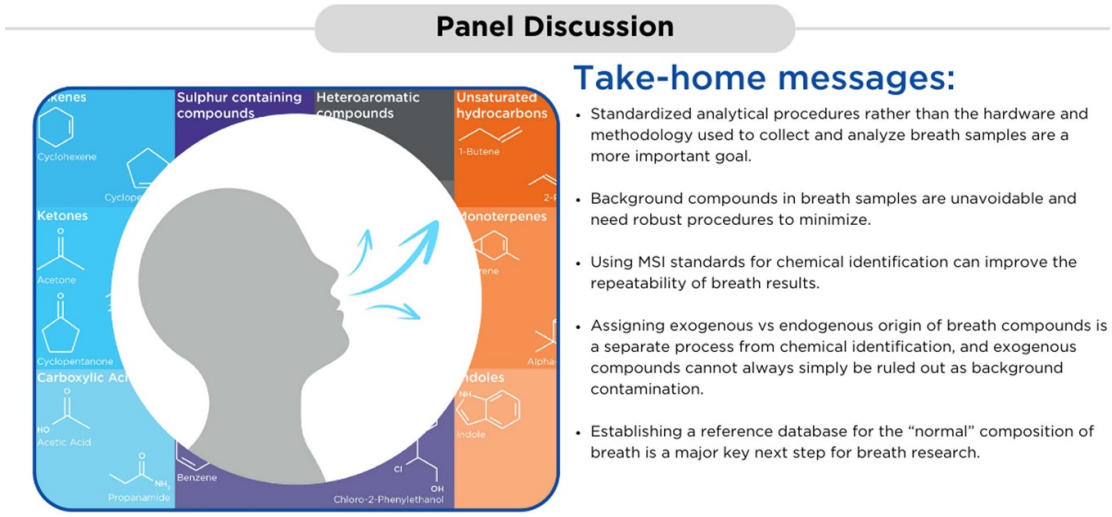

Figure 5. A summary of the consensus opinions generated during the 'Ask the Expert' panel discussion. Key practical tips to improve standardization in breath research were established, and the importance of mapping the baseline of human breath was outlined as a key goal.

to develop the Human Breath Atlas [27], and developing reference databases for normal human breath is a major pursuit for almost all the panel members. One of the questions posed to attendees in the survey was 'Most of the panel are involved in VOC Atlas projects to identify and quantify VOCs in "nominally healthy" subjects. How important do you think it is to do this work and to make it available to the research community?' which was given an average score of 4.5 out of 5—indicating that most people considered it of critical importance. The approach that Dr Kaiser is taking supports the earlier motion that different approaches used to evaluate breath can increase confidence in your data by evaluating where the commonalities and differences are. For this reason, she is working as part of a collaborative effort with the other groups to generate individual lists based on a large number of breath samples from healthy volunteers. The data sets can then be combined and visualized in a Venn diagram of the core breath-based compounds and their levels that are seen in common to all datasets, and then which compounds vary more across methodology and different cohorts. But what fraction of the population do the VOCs need to show up in to be considered part of normal breath? Dr Wilde mentioned the need to consider what is the natural prevalence of VOCs in the population. The example of breath methane was given, in which the number of 'methane-producers' in the population has been estimated to be between 30% and 60% [28]. The process of building these datasets of normal breath will hope to shed light on the different normal prevalences of VOCs, and Mr Boyle mentioned that for the VOC Atlas being built at Owlstone Medical, there are multiple criteria used to distinguish from background compounds, but also must be present in at least 50% of the population. For comparison, Dr Wilde uses a frequency threshold of about 80% per group rather than per individual. As was discussed, these numbers used as thresholds are ultimately arbitrary starting points, and so what the appropriate threshold is to set may become an important point of discussion as these normal human breath database projects advance (figure 5).

6. Conclusions

The Breath Biopsy Conference continues to be a well-attended and enjoyed event and brings together the Breath Biopsy community in a collaborative and open environment to celebrate the successes of the past year, discuss the advances in the field, and discuss consensus opinions to address key challenges. There were several key take-home messages for the breath community that were generated from the various talks, posters, and the Ask the Expert panel discussion.

1. The understanding of breath volatile metabolites is currently at an early stage. It is important to establish standards for defining and identifying breath VOCs to effectively translate breath biomarkers into clinical applications. Leveraging chemical data to address knowledge gaps can reveal previously unexplored pathways, advancing the translation of breath biomarkers in clinical settings.
2. The advancement of pre-clinical breath sampling in animal models, the utilization of EVOC probes for diagnosing liver cirrhosis, and the adaptation of EVOC probes to enhance lung cancer screening collectively show a shift towards more innovative approaches in harnessing the potential of breath

analysis. These developments lay the foundation for a promising future where breath analysis becomes an integral part of clinical practice.

3. Breath analysis provides an easy-to-use and non-invasive avenue to monitor the metabolism of the gut microbiome and can be used to diagnose and prevent gastrointestinal disorders. There are also VOCs present in the breath that are associated with infectious diseases—the metabolic pathways of the microbes and the body's response to them—which represent some of the most exciting future applications of breath analysis in clinical research and practice.
4. The panel discussion of world-leading breath analysis experts discussed how standardized analytical procedures rather than the hardware and methodology used to collect and analyze breath samples are a more important goal (including analyzing background air samples alongside breath samples), and that steps can be taken to achieve this through a wider adoption of MSI chemical identification standards, and more detailed attempts to assign exogenous vs endogenous origin of compounds found in the breath.
5. Establishing a reference database for the 'normal' composition of breath is a major key next step for breath research. This can form the basis of which biomarker discovery for different clinical applications can be fast-tracked and build the foundational data on which breath can be established as a more routinely used sampling medium in clinical use and research.

The next annual Breath Biopsy conference will be held as an online event from the 5th to the 6th of November 2024, and it will be exciting to track the progress of the research presented this year.

Data availability statement

No new data were created or analyzed in this study.

Acknowledgments

The Breath Biopsy Conference 2023 was another exciting and informative event, and we acknowledge all the speakers, attendees, and sponsors of the Breath Biopsy Conference 2023 who made it so successful. There were some truly engaging and insightful presentations, and discussions, and it was a pleasure to join members of the global Breath Biopsy community together. We also acknowledge the organizers of the conference from the Owlstone Medical team, particularly Caitlin Curran, for putting together a brilliant program and making sure the event ran smoothly on the day. Finally, we would like to acknowledge the conference chair, Billy Boyle, for always leading the conference with passion and enthusiasm.

ORCID iDs

Hsuan Chou https://orcid.org/0000-0002-8028-225X
Madeleine L Ball https://orcid.org/0000-0002-6150-0485

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