Breath Biopsy®
THE COMPLETE GUIDE
Second Edition
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Introduction

VOCs in Breath

Exhaled breath contains over 1,000 volatile organic compounds (VOCs). Accessible by a non-invasive “Breath Biopsy®”, these VOCs can be used as disease biomarkers for early detection and screening, diagnosis, and precision medicine including patient stratification for therapy selection and treatment monitoring (Figure 1).

VOCs are products of metabolic activity taking place in the body, hence they directly reflect the current state of cells, tissues and the microbiome - providing a rich source of valuable information about the health of an individual.

Metabolic information from VOC biomarker analysis collected during a Breath Biopsy® offers researchers and medical doctors new possibilities beyond those provided by purely genomic approaches. VOCs provide detail on current disease activity in contrast to genomics which provide information only on disease predisposition.

Non-invasive Biomarkers

As exhaled breath sampling is also inherently non-invasive, Breath Biopsy® is preferable to patients over uncomfortable and potentially unpleasant diagnostic procedures, such as stool sampling, blood sampling, and tissue biopsies.
Figure 1. Breath Biopsy® is a non-invasive means to access valuable information about the health of individuals. VOC biomarkers have numerous applications in early detection and precision medicine across a wide range of diseases.
Why Breath?

- Metabolites relate directly to current disease activity
- Breath enables whole body blood sampling
- VOC biomarkers in breath are relevant for a wide range of diseases
- Breath biomarkers have wide-ranging applications in diagnostics and precision medicine
- Sampling is non-invasive and pain-free

Learn more at
owlstonemedical.com/breath-biopsy
Scope of this Guide

In this guide we introduce VOCs, and discuss the scientific basis for the application of VOCs in breath as biomarkers for a wide range of diseases. We provide an overview of the current status of VOC biomarkers in the disease areas of asthma, oncology, inflammatory and infectious disease.

We discuss some of the challenges that have previously limited the potential of breath analysis, and describe how these challenges have now been overcome with the introduction of new technology for breath sampling and analysis.

We will outline the workflows for breath collection and VOC analysis that have introduced Breath Biopsy® as a new platform for early detection and precision medicine. Now, for the first time, it is possible to perform biomarker discovery using breath as a sample type, on a platform robust enough for large scale clinical trials.

Historical Breath Analysis

Clinicians have long recognized that certain breath odors are typical of some diseases, though the biological origin of the smells has often been a mystery. The practice of using this sensory information to identify disease was reported by Hippocrates (460–370 BC) who famously described *fetor oris* and *fetor hepaticus* in writings about breath aroma and disease [1].

One example familiar to doctors for centuries is the fruity smell in exhaled breath of patients with diabetic ketoacidosis. This symptom is due to ketones produced when the body is unable to use glucose due to the lack of insulin. Instead, it breaks down fat as an alternative source of fuel, producing pear scented acidic ketones including acetone [2].

**Nobel Prize-winning Work of Linus Pauling**

The expression of VOCs in breath that are linked to a patient’s disease state offers a powerful, non-invasive approach to identifying illness. The promise of using breath-borne VOCs directly as a tool for diagnosis has been recognized for many years. Twentieth century attempts to quantitatively identify VOCs in breath include those of Nobel Prize-winning chemist Linus Pauling, whose results [3,4] highlighted that exhaled breath contains hundreds of different volatile compounds.

During this era, the first discoveries relating to the origin of certain VOCs in breath were made. This included the identification of small chain hydrocarbons, which were found to be products of lipid peroxidation, possible biomarkers for oxidative stress [5]. Another example is the abundant breath VOC isoprene [6–8], which was discovered in the mevalonate pathway. Its concentration increases significantly during exercise. [9,10].

Breath Analysis in the Modern Era

Since the early discoveries, the field of breath research has benefited from improvements in technologies for the separation and identification of gas phase compounds.
Rising Number of Publications on Breath VOCs

The improvement in analytical technologies and the obvious promise of VOCs as biomarkers for disease has led to increasing numbers of publications relating to VOCs in breath [1], illustrated in Figure 2.

 Technologies for Breath Analysis

Techniques well suited to the analysis of breath VOCs include gas chromatography-mass spectrometry (GC-MS), proton transfer-reaction mass spectrometry (PTR-MS) [11], selected ion flow tube-mass spectrometry (SIFT-MS) [12], arrays of gas sensors (‘eNoses’) and ion mobility spectrometry (IMS) including field asymmetric ion mobility spectrometry (FAIMS) [13-16].

Reliable breath collection is essential for robust analysis of breath VOCs.

Owlstone Medical’s ReCIVA Breath Sampler is the first breath sampler able to provide an out-of-the-box means to reproducibly capture breath samples. ReCIVA samples breath continuously over a few minutes, enabling the VOC biomarkers originating from throughout the body to be accessed non-invasively and with high sensitivity.

Learn more on page 54.

Figure 2. Increase in annual publications in the area of VOCs and breath. Graph shows the results of a Web of Knowledge search for publications including the terms volatile organic compound AND breath.

“We have been using FAIMS for almost five years and have found it able to non-invasively detect a broad range of diseases, including cancer, with high sensitivity and specificity.”

Professor James Covington, University of Warwick
Established Breath Tests

Some breath tests that use volatile biomarkers have already become established in clinical practice (Table 1 shows approved breath tests). However, much of the potential shown by VOC tests has remained untapped due to a lack of reproducibility between studies looking for disease biomarkers. The variability in the results of breath research has stemmed largely from a lack of high quality, standardized breath sampling technology [17,18].

**APPROVED BREATH TESTS**

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<td>Capnography</td>
<td>CO₂</td>
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<td>$^{13}$C-urea breath test for detection of <em>H. pylori</em> infection [19]</td>
<td>$^{13}$CO₂</td>
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<td>Test for neonatal jaundice [20]</td>
<td>CO</td>
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<td>Disaccharide adsorption deficiency and small intestine bacterial</td>
<td>H₂, CH₄</td>
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<td>overgrowth syndromes [21–24]</td>
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<tr>
<td>Monitoring asthma therapy [25–29]</td>
<td>NO</td>
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<tr>
<td>$^{13}$C-octanoic acid Gastric emptying breath test (GBET) [31]</td>
<td>$^{13}$CO₂</td>
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<td>CO testing smokers [32]</td>
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<td>Roadside intoxication tests</td>
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*Table 1. Approved breath tests established in clinical practice [1].*

Breath Analysis in Clinical Trials

Analysis of all clinical trial data [33] shows that between 1997 and 2016 around 450 trials have used some kind of breath analysis in their workflow (download Excel spreadsheet of breath analysis clinical trials). During this time there has been a rise in the annual number of clinical trials starting that used breath analysis, indicating the increasing interest in tests of this type (Figure 3).

*Figure 3. Increasing numbers of clinical trials using breath analysis are being registered. This includes 75 trials analyzing exhaled breath condensate. Data from https://clinicaltrials.gov/. Download the list of trials as Excel spreadsheet.*
The identified clinical trials cover a wide range of conditions, demonstrating the breadth of applications where breath analysis is thought to be useful. A breakdown of the generalized disease category of the trials is shown in Figure 4.

**DISEASE CATEGORIZATION IN 448 CLINICAL TRIALS USING BREATH ANALYSIS**

![Pie chart showing disease categorization](image)

*CANCER*  
*INFLAMMATORY*  
*INFECTIOUS*  
*OTHER (GASTROINTESTINAL)*  
*OTHER (PULMONARY)*  
*LIVER DISEASE*  
*OTHER*

*Figure 4. Breakdown of disease category of 448 clinical trials using breath analysis in their workflow. Data from https://clinicaltrials.gov/. Download Excel spreadsheet including list of clinical trials.*

**The World’s Largest Breath-based Clinical Trials**

Owlstone Medical’s breath sampling and analysis technology is being used in large clinical trials relating to lung cancer [34], colorectal cancer [35] and asthma [36]. It is also already used in more than 100 academic research groups and clinical labs around the world.
Volatile organic compounds (VOCs) are gas phase chemicals that have a high vapor pressure at room temperature. VOCs form part of the composition of exhaled breath, and represent the volatile fraction of metabolites. These are low molecular weight (<1 kDa) compounds produced by the body’s metabolism, and represent the endpoint of gene, transcription and protein expression (Figure 5).

Illnesses can lead to a loss of metabolic balance [37,38], hence monitoring fluctuations in metabolites offers a powerful way to detect, diagnose and monitor disease.

Thousands of breath-borne metabolite VOCs have been identified [39], and they form a library of information about an individual’s health. VOCs are also emitted from other clinical samples types such as urine, feces and saliva.

Breath Biopsy® offers researchers and clinicians relevant information about disease activity because the identity and concentration of VOCs corresponds to current metabolic processes in cells, tissues and the microbiome. This is in contrast to genomic analyses, which only provide information about an individual’s ‘starting blueprint’.

Figure 5. Metabolites provide information about actual disease activity, in contrast to genomics which only provides a ‘starting blueprint’.
Whole body blood sampling

Breath provides a window into a much larger volume of blood. By sampling breath over a few minutes, VOC biomarkers originating from all around the body are captured with high sensitivity.

Learn more on page 18.
Where do Breath VOCs Originate from?

VOCs in exhaled breath have a number of potential sources. They can be produced biochemically by an individual’s cells and tissues (‘endogenous’ VOCs) or be the result of external influences. These ‘exogenous’ VOCs in breath can be the product of metabolism by the body’s own resident microbiome, or may be introduced from the environment, including downstream drug metabolites from therapeutic interventions (Figure 6).

**Endogenous VOC Sources**

Endogenous breath VOCs can arise from metabolic activity in lung and airway tissue, or can have a systemic origin. Systemic metabolite VOCs can originate anywhere in the body. They are carried to the lungs by the circulatory system, where rapid exchange between the blood and air leads to their release into breath (Figure 7). Breath Biopsy® can therefore provide a rapid and non-invasive means to gain detailed insight into the functioning of the whole body.
Biochemical Origins of VOCs

A variety of processes lead to alterations in the VOC profile. The origins of disease-related VOCs are complex, as they can have multiple potential sources. They can originate from metabolic processes taking place within, for instance, a tumor cell, as well as from metabolic changes in the surrounding tissues ‘reacting’ to the cancer’s presence.

Oxidative stress (OS) has been implicated in the initiation and development of many systemic and organ-specific diseases, including cardiovascular, pulmonary, autoimmunological, neurological, inflammatory diseases and cancer [1]. OS is also known to play an important role in the production of VOC biomarkers [40].

OS represents an imbalance between the rate of metabolic production of reactive oxygen species (ROS) and the rate at which the body can detoxify the reactive intermediates and repair the resulting damage (Figure 8).

At the cellular level, higher than normal ROS levels damage key cellular structures like phospholipid membranes, proteins and DNA. This damage causes dysfunction, and can lead to apoptosis or programmed cell death.

VOCs known to result from ROS interactions with cellular structures, include ethane and pentane, nitric oxide and 4-hydroxy-2-nonenal (Figure 8) [40].

As OS is implicated in many conditions, and known to be important in cancer and inflammatory diseases, OS related VOCs in breath are of particular interest.
Exogenous VOC Sources

In addition to VOCs biochemically produced by an individual's cells, breath always contains exogenous VOCs that are present because of external factors including environmental exposure, lifestyle, diet, the microbiome and therapeutic interventions. Due to the importance of these factors in many diseases, exogenous VOCs can provide important clinical information.

Environmental Exposure

Compounds present in an individual's environment can alter the presence and concentration of VOCs observed in breath. VOC biomarkers that typify exposure have been referred to previously as the exposome [41], and have been used to measure and assess exposure to certain chemicals in the environment. For example, breath analysis has been used by the US military to monitor exposure to volatile compounds in jet fuel - in this case, aromatic and aliphatic hydrocarbons served as biomarkers of exposure [41-43].

Lifestyle, Diet and the Microbiome

An individual's lifestyle and diet also affects the VOCs present in their breath. A good example of a 'lifestyle' related exogenous VOC is acetonitrile, which appears in the breath of smokers, but is not produced endogenously [44]. Diet influences breath composition through the production of VOCs from carbohydrates and fatty acid metabolism. These processes produce acetyl CoA, which via separate pathways yields the volatile compounds isoprene and acetone. Isoprene is also an intermediate in cholesterol synthesis, and protein metabolism generates ammonia and nitric oxide [45].
Metabolic processes of the body's microbiota also generate VOCs. This can be from bacterial fermentation of unadsorbed complex carbohydrates and proteins in the gut. This generates gases including methane, hydrogen, hydrogen sulphide and ammonia [45]. VOCs from the microbiome include those produced by pathogenic microorganisms, such as *C. difficile* [46] or *M. tuberculosis* [14], making them valuable for the diagnosis of these infections.

**Drug Metabolites**

Breath analysis has been identified as a useful, non-invasive route to monitoring certain pharmaceuticals (e.g. Propofol) during or after treatment. Measuring specific drug related volatiles can provide a non-invasive means of gaining insight into pharmacokinetics and pharmacodynamics. Breath analysis has also been suggested for the detection of drugs of abuse, which could be useful for roadside intoxication tests [47].
VOCs as Biomarkers

Metabolomics vs. Genomics

Genes act as the blueprint for an individual’s biological functions. Despite years of research focusing on the human genome, and a number of significant advances, genomics has not yet delivered on its full potential to help us understand and cure diseases.

In the last decade, hundreds of genome-wide association studies (GWAS) have been performed to identify regions of the genome associated with a range of human disease. GWAS have failed to account for all the heritability of any disease [48]. Only 10% of the variants that lead to common diseases are located within coding DNA sequences. The remainder are located in non-coding regions of the genome, or intergenic regions.

A recent paper in *Science* highlighted that only 5% of mutations leading to cancer were attributable to inherited mutations, whereas a larger fraction was thought to result from random replication errors occurring during normal DNA replication [49]. In addition, single mutations rarely result in disease - usually multiple mutations are required.

Ultimately, biomarkers based on genomic data provide information about disease predisposition, but can lack detail on current disease activity. In most circumstances, disease is the result of multiple genetic and epigenetic attributes interacting with environmental factors, diet and lifestyle choices, and the microbiome - all of which combine to produce the phenotype, which is most closely reflected by the metabolome.

Metabolomics for Diagnosis and Precision Medicine

An organism’s metabolism is the set of biochemical processes that sustain life. Metabolic activity produces a variety of small molecules known as “metabolites”. The type and quantity of circulating metabolites is central to the function of all living systems: this biochemical composition is also known as the metabolome.
Metabolites make excellent disease biomarkers because alterations to an organism's biological systems caused by disease, genetic mutations, the microbiome or environmental factors change the profile of metabolites produced. Hence, metabolomics has the closest biological proximity to the phenotype of the observed individual, and allows rapid observations of perturbations in this system.

Metabolites can provide early insight into developing disease states because they reflect and magnify perturbations at lower genomic, transcriptomic and proteomic levels several thousand fold [50,51]. As metabolic changes are present at the earliest stages of disease, metabolites offer a route to earlier diagnoses that can save lives.

Additionally, metabolites are ideal for differentiating between related disease states, stratifying patients for therapy response, and monitoring toxicities and other drug interactions.

Breath Biopsy® provides a non-invasive way to capture the volatile fraction of metabolites present in exhaled breath, and allows rapid and detailed insight into the functioning of the whole body, and the metabolic changes associated with disease.
Whole Body Blood Sampling via Breath

Traditional approaches to breath sampling have relied upon collecting a single exhaled breath, providing a small snapshot of VOCs present in the body. New advances in breath sampling technology allow breath to be collected over a longer time period, enabling VOCs originating from the whole body to be collected.

The ability to capture VOCs originating from throughout the body in a breath sample is extremely powerful. Firstly, breath enables “whole body sampling” of the volatile metabolome. Secondly, this makes VOCs in breath applicable as biomarkers for a wide range of diseases, not just pulmonary conditions.

**Whole Body Sampling of Breath Biomarkers for a Wide Range of Diseases**

Each exhaled breath contains a rich spectrum of VOCs derived from the blood, due to efficient exchange of chemicals between the pulmonary and circulatory systems. It takes approximately 1 minute to circulate the total blood volume around the entire body. Every minute, then, VOCs originating from sources all throughout the body reach the alveoli in the lungs where they pass efficiently from the blood into the alveolar air and are then exhaled.

By continuously sampling breath over a few minutes, the profile of VOCs originating from throughout the body can be accessed in a non-invasive way (Figure 9).

**Extended Breath Collection Time for Detection of Low Level VOCs**

Some VOCs collected during a short 1 minute time period will be present at extremely low concentrations. By collecting and pre-concentrating all the VOCs exhaled over an extended time period (e.g. 10 minutes), even low concentration VOCs can be captured and quantified with accuracy.

Owlstone Medical’s ReCIVA® Breath Sampler collects and pre-concentrates VOCs in breath onto Breath Biopsy Cartridges over extended sampling durations. This approach makes Breath Biopsy® sensitive to even low level VOC biomarkers, such as those present at the earliest stages of disease.
Figure 9. The volatile metabolome of the entire body can be accessed by continuously sampling breath over a few minutes. Capturing VOCs from exhaled breath onto Breath Biopsy Cartridges allows the pre-concentration of VOC biomarkers originating from the entire body via the circulatory system.
Breath Biomarkers
for a wide range
of diseases
and applications

From breath you can non-invasively access the volatile metabolome of the entire body, making Breath Biopsy® relevant for a wide range of conditions.

Volatile metabolites in breath are promising biomarkers with numerous applications in clinical diagnostics and precision medicine.

Learn more on page 24.
Breath Biopsy® vs. Liquid Biopsy

Liquid biopsy is the sampling and analysis of blood for the identification of diseases such as cancer. The technique can be used to collect circulating tumor cells or tumor-derived cell-free DNA/RNA in blood. Because liquid biopsy is less invasive compared to traditional biopsy the method has created much enthusiasm. For example liquid biopsy has shown promising results in therapy selection of late stage cancers.

Liquid biopsy suffers from three fundamental challenges:

1. Circulating tumor DNA/RNA (ctDNA/RNA) is present only at limited amounts, especially during the early stages of disease (Figure 10)
2. Blood can only feasibly be sampled in small amounts, which limits the sensitivity of the liquid biopsy approach
3. Detection of a single mutation in ctDNA may not be a reliable indicator of cancer, hence the liquid biopsy approach may suffer from low specificity for early diagnosis of cancer.

Bettegowda et al. [52] reported that less than 50% of patients with Stage I cancer had detectable ctDNA. Notably, the study was conducted using a relatively large volume of plasma (5 ml). This highlights the limitations of liquid biopsy, especially for early detection and screening applications.

**LIMITATIONS OF LIQUID BIOPSY**

![Graph](image)

**Sensitivity**

Metabolite VOC biomarkers are altered at the earliest stages of disease. Breath Biopsy® can capture and detect these ‘early warning’ VOCs that are present at extremely low concentrations. This is possible because, unlike blood, breath can be sampled on demand in effectively unlimited volumes.

By sampling for an extended period of time (~10 mins) using the ReCIVA Breath Sampler, VOCs are concentrated onto Breath Biopsy Cartridges. Pre-concentration of VOC biomarkers greatly increases the sensitivity of Breath Biopsy® compared to liquid biopsy techniques, enabling detection of VOC biomarkers present at the onset of disease.
Analytical advantages
Breath Biopsy® also offers practical advantages over liquid biopsy. The analysis of VOCs can be accomplished in a matter of minutes making it rapid and cost-effective. Liquid biopsy on the other hand requires significant sample preparation and lengthy workflows to analyze nucleic acid by PCR or sequencing.

Monitoring Changes in Breath VOCs Over Time
Single measurements of metabolites provide only a snapshot of an individual’s current state. While this provides useful information, point measurements do not give information about the baseline abundance of metabolites for that individual.

The Breath Biopsy platform can be used to capture multiple breath samples over time, uncovering detailed changes in the concentration of volatile metabolites and drug-related compounds present in breath.

Breath Biopsy for Pharmacokinetics and Dynamics
The breath VOC profile includes VOCs produced during the metabolism of pharmaceuticals and other xenobiotics. Observing changes in the type and concentration of metabolites over time after the administration of pharmaceuticals is a useful tool in drug pharmacokinetics and drug compliance studies, enabling changes in levels of metabolites to be investigated non-invasively via breath.

Making multiple measurements over time allows the detection of changes in metabolite concentration that can, for instance, indicate whether a patient is complying with a medication regime or provide information about how a particular drug is being metabolized by the patient.

Breath Biopsy can be used to measure longitudinal changes in exhaled VOCs for monitoring:
• Drug activity
• Drug compliance
• Pharmacokinetics
• Therapy response
In the above study, VOCs in breath were monitored following ingestion of a surrogate pharmaceutical, in this case a peppermint oil capsule, demonstrating that Breath Biopsy can be used to observe the decrease in target compounds over time using repeated, robust breath collection and analysis over a period of 8 hours (Figure 12).

Analysis of breath captured 30 minutes after consumption of the peppermint capsule shows a large increase in the VOCs α-pinene, β-pinene, limonene, eucalyptol and (±)-menthol compared to baseline pre-ingestion controls captured immediately prior to taking the capsule (Figure 12). The most abundant of these peppermint-related compounds are α-pinene, β-pinene and limonene. Limonene was present at part-per-trillion (ppt) concentrations.

Breath collections made every 30 minutes after this initial capture show a consistent decrease in the target VOCs over time. (Figure 12). Captures made from 6.5 hours after consumption show the levels of the target VOCs decreasing to baseline levels. All of the target compounds display a similar washout curve over time.

**What is Pharmacobreathomics?**

In pharmacobreathomics, the breath VOC (volatile organic compound) profile is used to study how a drug interacts with the body. This new field combines pharmacology (the science of drugs) and breathomics (the study of VOCs in breath) to develop precision medicine tools that aim to deliver the right drug, to the right patient, at the right time.
Exhaled breath represents a vast untapped resource of chemical information. It contains over 1,000 volatile organic compounds, which reflect metabolic activity and the state of cells and tissues. VOCs can therefore act as convenient biomarkers.

The clinical applications for non-invasive, breath-based VOC biomarkers are wide ranging, with numerous applications in precision medicine and early detection.

**Breath Biopsy® Applications**

- Early detection and screening
- Precision medicine:
  - Companion diagnostics
  - Complementary diagnostics
  - Patient stratification
  - Therapy response biomarkers
  - Toxicity prediction
- Clinical diagnostics and prognostics
- Disease monitoring
VOCs originating from all parts of the body are captured in breath, making Breath Biopsy® applicable to a wide range of diseases including:

- Cancer
- Inflammatory disease
- Infectious disease
- Metabolic disease
- Cardiovascular disease

**Research case studies**

Learn about how our academic and clinical partners are applying VOC biomarkers in their research across a range of disease areas.

[www.owlstonemedical.com/case-studies](http://www.owlstonemedical.com/case-studies)

Figure 13: VOC biomarkers in breath have been reported for a large number of diseases in the literature [53,54], highlighting the wide disease relevance of Breath Biopsy®.
Novel Biomarkers

Until recently, the identification of robust VOC biomarkers in breath has been hampered by the lack of standardized sampling and analytical methods. Progress is now being made by the advent of new technologies for the reliable collection of patient breath samples (ReCIVA Breath Sampler) and advances in VOC analysis technology (Breath Biopsy® analytical platform).

Breath and the metabolome are under-explored in a clinical context, this means VOC biomarkers identified in breath are likely to be novel, which may allow their utilization as companion or complementary diagnostics.

Advances in breath sampling technology allow Breath Biopsy® to be embedded into early stage clinical trials. This allows pharmaceutical companies to discover and use biomarkers early in the drug development process and may help to maximize the chance of of achieving regulatory approval.

Precision Medicine Applications

Volatile metabolites are closely linked to disease activity, which makes them ideal for capturing the biological variability of disease. As a result, VOC biomarkers have found use in precision medicine applications that depend on a detailed insight into the biological variability that underlies disease processes.

For example, VOCs in breath have been used to stratify asthma patients into groups which are either responsive or non-responsive to a particular therapy (see page 30). The Stratification of Asthma Treatment by Breath Analysis (STRATA) was supported by the NHS and Innovate UK to further explore the usefulness of VOCs in asthma stratification.

Similarly, VOC biomarkers offer a non-invasive means to accurately stratify inflammatory bowel disease patients to assist with therapy selection (see page 45).

If VOC biomarkers can be identified that predict therapy response and treatment efficacy it can lead to the development of diagnostic breath tests for identification of responders.

Breath Biopsy® Applications in Precision Medicine
Download the Drug Discovery World article

owlstonemedical.com/ddw
DNA provides the “blueprint” for disease predisposition, but metabolites give information on the current state of tissues and cells, and the health of the individual.

Altered metabolism occurs at the earliest stages of disease, and magnifies changes at the genomic, transcriptomic and proteomic levels several thousand fold.

Learn more on page 16.
Clinical Trials Patient Matching

VOC biomarkers can be used to develop companion diagnostics to screen and stratify patients for inclusion in clinical trials. By selecting the patients most likely to respond to the particular therapy, this approach enables smaller and more cost effective clinical trials.

More importantly, stratifying patients for inclusion in clinical trials will lead ultimately to more successful clinical trials, and maximize the chance of gaining regulatory approval for therapies that would otherwise fail due to health economic pressures.

There is evidence that VOCs can be used to classify asthma patients by inflammatory subtype, highlighting the potential use of Breath Biopsy® as a companion diagnostic for therapy stratification (see also page 30).

Drug Metabolites and Disease Monitoring

Breath Biopsy® has potential applications in disease and treatment monitoring. Comparing pre- and post-treatment breath tests could provide confirmation of therapy efficacy by detecting changes in disease-related biochemical activity.

Tracking changes in the VOC profile in breath over time could provide an early warning of changes in the underlying disease state.

Administration of drugs also results in changes in metabolites which can be accessed via a Breath Biopsy®. Breath Biopsy can therefore be a tool to evaluate the drug’s pharmacokinetics and drug compliance (see page 23). There is also evidence that metabolite changes may relate to drug-induced toxicity [55].

Early Detection and Screening of Cancer

Breath is available in an effectively unlimited supply, which provides analytical advantages compared to liquid biopsy techniques (see also page 22). Breath Biopsy® shows great promise for applications including early detection and screening, where the goal is to detect biomarkers present at the earliest stage of disease.

The ability to concentrate exhaled chemicals by sampling over a few minutes, enables the detection of biomarkers at very low levels in a way that is not possible with other sampling modes such as blood tests. This has the potential to dramatically improve test sensitivity across a range of diseases.

The completely non-invasive nature of breath sampling will allow clinicians to greatly increase compliance rates, which can be problematic in the case of diagnostic procedures which are less pleasant, more invasive and carry risks to patients.
Asthma and COPD

An estimated 334 million people worldwide have asthma [56], but a lack of stratifying diagnostics means that current guidelines advocate a ‘trial and error’ approach, which results in increased healthcare costs, prolonged periods of poor disease control, and an increased risk of exacerbations. Breath analysis offers the possibility of a rapid, straightforward and non-invasive method to stratify patients into receiving the right therapy and monitor what dosage they need.

Asthma management is focused on achieving control of symptoms to minimize the risk of future exacerbations. Many patients do not respond sufficiently to treatment, however, so their symptoms are not kept adequately under control. In the UK, 4.4% of patients fail to respond to standard therapies even at high doses, and account for more than half of asthma costs to the NHS.

Precision Medicine for Asthma

The treatment of asthma can be challenging because the condition consists of a number of complex, overlapping, phenotypes (Figure 14, top row) with similar symptoms, but which require different treatments.
Chronic airway inflammation has diverse origins and triggers, and can be broken down into a range of different subtypes of pulmonary inflammation. These inflammatory subtypes (Figure 14, second row) can be further divided into different endotypes, with different underlying molecular mechanisms or responses to treatment.

There is now a considerable move towards precision medicine in asthma, as highlighted by the Lancet Respiratory Asthma Task Force and others [57,58]. By identifying different disease endotypes or treatable traits, precision medicine addresses asthma’s underlying heterogeneity, enabling the right treatment to be given to the right patient at the right time.

The last decade has seen the approval of biological drugs such as XOLAIR® targeted at specific pathways relevant to inflammatory subtypes, yet their approval for clinical use has been delayed due to the high cost of the treatment, combined with the difficulty of identifying patients with the correct asthma phenotype who would benefit from the drug.
Volatile Metabolites - Biomarkers for Precision Medicine

Differences in the metabolic profile of individuals with asthma compared to healthy controls has already been detected in serum - even in steroid naïve patients with mild asthma [59]. The differences shown in this case relate both to the severity of the disease, and also to the steroid treatment.

An increasing body of evidence shows that volatile metabolites in exhaled breath are promising biomarkers that relate to metabolic changes caused by inflammation of the airways [60].

“A systematic review with meta-analysis and recent prospective studies favored exhaled volatile organic compounds as one of the most promising biomarkers in asthma diagnosis and monitoring.” Pité et al. [60]

Breath Biopsy enables you to analyze volatile metabolites in exhaled breath, providing a new non-invasive approach to characterize different disease endotypes.

Breath Biopsy® for IBD diagnosis and stratification

One breath-based biomarker, fractional exhaled nitric oxide ($F_eNO$), is already being used to support asthma diagnosis. $F_eNO$ reflects a protective biochemical pathway of the lungs, but lacks specificity, as it is a single biomarker affected by many processes other than asthma.
Studies have demonstrated that volatile organic compounds (VOCs) in exhaled breath can outperform F<sub>e</sub>NO and lung function tests as biomarkers when discriminating between asthmatics and healthy controls [61]. It has also been shown that VOCs can be used to discriminate with a high degree of accuracy between asthmatic children and those with transient wheezing [62].

**Discrimination of Asthmatic vs. Non-Asthmatic Patients with Breath Biopsy**

We’ve examined the ability of VOCs analyzed using our Breath Biopsy platform to discriminate between asthmatic and non-asthmatic patients (Figure 15). As you can see, even in a heterogeneous clinical trial population, which included individuals with a wide variety of pulmonary conditions, the VOC profile discriminates well between patients with or without a diagnosis of asthma.

![DISCRIMINATION OF ASTHMATIC vs. NON-ASTHMATIC PATIENTS](image)

*Figure 15. Breath VOCs analyzed using Owlsone Medical’s Breath Biopsy platform were used to build a classifier which could discriminate between patients with or without a diagnosis of asthma. The Receiver Operator Characteristics (ROC) curve shows an area under the curve of 0.92. The patient population included individuals with a wide variety of pulmonary conditions, participating in a larger clinical trial.*
**Distinguishing Inflammatory Subtypes in Asthma**

There is evidence that breath VOCs can be used to classify patients by asthma phenotype with high accuracy. For example, Ibrahim et al. [63] provide evidence that VOCs in breath correlate with different inflammatory subtypes in asthma, as defined by sputum cell counts.

VOC biomarkers in breath could therefore enable different phenotypes of asthma defined by different inflammatory responses to be distinguished.

**Breath Biopsy for Distinguishing Controlled vs. Uncontrolled Asthma**

Reliable biomarkers to identify patients with uncontrolled asthma, or to predict exacerbations, would inform treatment decisions and facilitate disease management.

The profile of VOCs in exhaled breath can be used to distinguish between patients with controlled and uncontrolled asthma (Figure 16), which is an important aspect of a patient’s asthma phenotype.

---

*Figure 16. Breath VOCs analyzed using Owlstone Medical’s Breath Biopsy platform were used to build a classifier which could identify individuals with self-reported uncontrolled asthma with 85% sensitivity and 98% specificity. The Receiver Operator Characteristics (ROC) curve shows an area under the curve of 0.88. A subpopulation of individuals participating in a larger clinical trial were included in this analysis.*
It has also been reported that breath VOCs analyzed using our FAIMS technology can potentially predict loss of control in asthma [64]. In this study the Lonestar VOC Analyzer, was shown to outperform sensor array type eNoses and gas chromatography-mass spectrometry (GC-MS).

**VOCs for Treatment Stratification**
A reliable tool to identify whether the patient is likely to respond to a particular treatment would be an important development.

**Predicting Steroid Responsiveness**
Van der Schee et al. that found that VOCs in breath are able to predict responsiveness to steroid treatment in steroid-naïve, mild to moderate asthma patients [65]. With an area under the curve of 0.88, VOCs outperformed both \( F_{iNO} \) and measurements of eosinophil cells from sputum samples.

**Breath VOCs Differentiate Treatment Groups**
Promising initial results from the U-BIOPRED (Unbiased BIOmarkers in PREDiction of respiratory disease outcomes) consortium project show that VOCs measured using a Lonestar VOC Analyzer could be used to stratify asthmatic patients into treatment subgroups. For example, VOCs discriminated between anti-IgE-treated XOLAIR and non-treated severe asthma patients with 83% accuracy [66,67].

"**BREATH VOCS DISTINGUISH ANTI IgE-TREATED XOLAIR®/OMALIZUMAB AND NON-TREATED SEVERE ASTHMA PATIENTS WITH 83% ACCURACY USING OWLSTONE MEDICAL’S TECHNOLOGY**"

**Summary**
Promising results indicate that VOCs in exhaled breath reflect specific molecular processes that underlie chronic inflammation in asthma, making them ideal biomarkers for precision medicine and treatment stratification.

By enabling the non-invasive collection and analysis of (VOCs) in exhaled breath, Breath Biopsy provides a new approach to discover biomarkers that could characterize different asthma endotypes and predict the effectiveness of treatment.
Pulmonary arterial hypertension (PAH) is a progressive cardiopulmonary disease characterized by extensive occlusion of small to mid-sized pulmonary arterioles. The gradual increase in arterial pressure leads to the development of high resistance, leading eventually to right-sided heart failure and death. PAH is considered to be a heterogeneous group of conditions including forms of the disease with unknown causes, known as idiopathic PAH (IPAH).

PAH is often diagnosed in its advanced stages, reducing the effectiveness of available treatments. The complex pathological mechanisms involved in PAH could be a source of VOCs in breath or urine, present at earlier stages of the disease [68] (Figure 17). The excessive cell proliferation associated with local hypoxia promotes glycolysis as an energy source, which results in production of ketones and alcohols. Additionally, increased cholesterol metabolism causes higher levels of isoprene, and reactive oxygen species (ROS) from inflammation oxidize proteins and fatty acids, releasing various hydrocarbons. These VOC biomarkers offer a potential route to earlier diagnosis, which could improve survival rates and clinical outcomes.

Detecting IPAH with Lonestar VOC Analyzer

Owistone Medical performed a pilot study to assess the ability of the Lonestar VOC Analyzer to distinguish between IPAH patient and control urine samples. The ATLAS headspace sampler was used to analyze urine samples (10 IPAH and 10 Controls) in a randomized sequence. Multiple features were found to be present in FAIMS spectra for IPAH and control subjects’ urine.
The 3 dimensional raw data acquired from the Lonestar was transformed and multivariate data analysis was performed using SIMCA (Sartorius Stedim, Sweden).

A supervised approach Partial Least Squares - Discriminant Analysis (PLS-DA) showed a clear separation between the two groups of urine samples, indicating differences in the raw data which are detectable by the Lonestar (Figure 18, top panel)

The bottom panel in Figure 18 shows an example box plot for one of the discriminant features, demonstrating the difference across the two groups.

**Figure 18.** Top panel: partial Least Squares - Discriminant Analysis (PLS-DA) of. Bottom panel: example box plot for one of the discriminant features.
Cancer has many complex and interacting causes, including genetic predisposition, mutations in the genetic code, lifestyle, diet and many other environmental factors. As metabolites are the conclusion of gene, transcription and protein expression, they reflect gene-protein-environment interactions. This unique position means they can provide an accurate representation of the ‘metabolic phenotypes’ of different cancers [69].

**Cancer Metabolites**

Cancer cells display altered metabolism from day one. The mechanisms by which oncogenes reshape a range of metabolic pathways to meet the nutritional demands of cancer cells is beginning to be elucidated [70].

One of the best described metabolic changes is the “Warburg effect”: cancer cells have glycolytic rates up to 200 times above normal cells, even when sufficient oxygen is present. This metabolic change is key to cancer survival as it provides the building blocks a growing tumor requires. The Warburg effect is a major contributor to a cancer’s volatile signature [71] and also affects the profile of VOCs in breath [72].

Cancer specific metabolite VOCs, observable by Breath Biopsy®, are likely to be related to changes in cancer cell metabolic programming. In addition, changes to VOC concentrations in the breath of cancer patients are likely to result from other processes, such as local inflammation and increased oxidative stress (see also the section “Biochemical origins of VOCs” on page 13), and knock-on effects of the disease on the body.

Since altered metabolites are present in the first stages of disease, this enables earlier detection and diagnosis. As disease prognosis is greatly dependent on cancer stage and tumor type, these factors make metabolites ideal biomarkers for cancer.

The search for VOC signatures associated with cancer has produced studies looking at breath VOCs related to many cancers, including lung, breast, colorectal, gastric, ovarian, liver, head and neck and malignant mesothelioma [73].

**Lung Cancer**

Lung cancer is the most common cancer worldwide and also one of the deadliest, with around 1.8m cases and 1.6m deaths each year. It has one of the lowest 5-year survival rates of all cancers, at just 17.7% in the US.
Lung cancer is the most common cancer worldwide and also one of the deadliest, with around 1.8m cases and 1.6m deaths each year. It has one of the lowest 5-year survival rates of all cancers, at just 17.7% in the US.

Exhaled breath is an excellent source of VOC biomarkers originating from airway and lung tissues. This makes breath analysis an ideal approach to the detection of metabolites relating to cancer cells in the lungs.

Several studies have found evidence that lung cancers can be detected using VOCs in breath [75]. However the lack of sampling standardization and underpowered clinical trials hindered the introduction of a breath test for lung cancer.

**REPORTED VOC LUNG CANCER MARKERS**

<table>
<thead>
<tr>
<th>Volatile Candidate Lung Cancer Markers</th>
<th>CAS number</th>
<th>Approximate concentration in Lung Cancer Patient Breath</th>
<th>Concentration detected using FAIMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butane-2,3-dione</td>
<td>431-03-8</td>
<td>30 - 50 ppb</td>
<td>&lt; 2 ppb</td>
</tr>
<tr>
<td>Isoprene</td>
<td>78-7-95</td>
<td>80 - 140 ppb</td>
<td>&lt; 0.4 ppb</td>
</tr>
<tr>
<td>Limonene</td>
<td>138-86-3</td>
<td>30 - 50 ppb</td>
<td>&lt; 2 ppb</td>
</tr>
<tr>
<td>Methanol</td>
<td>67-56-1</td>
<td>100 - 150 ppb</td>
<td>&lt; 2 ppb</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>75-05-8</td>
<td>2 - 60 ppb</td>
<td>&lt; 2 ppb</td>
</tr>
<tr>
<td>Acetone</td>
<td>67-64-1</td>
<td>450 - 650 ppb</td>
<td>&lt; 1.5 ppb</td>
</tr>
<tr>
<td>Acetamide</td>
<td>60-35-5</td>
<td>30 - 50 ppb</td>
<td>&lt; 3 ppb</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>50-00-0</td>
<td>30 - 50 ppb</td>
<td>&lt; 2 ppb</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>78-93-3</td>
<td>30 - 50 ppb</td>
<td>&lt; 1 ppb</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>100-52-7</td>
<td>30 - 50 ppb</td>
<td>&lt; 2 ppb</td>
</tr>
<tr>
<td>Acetophenone</td>
<td>98-86-2</td>
<td>30 - 50 ppb</td>
<td>&lt; 2 ppb</td>
</tr>
<tr>
<td>Methyl-propyl sulfide</td>
<td>3877-15-4</td>
<td>30 - 50 ppb</td>
<td>&lt; 1 ppb</td>
</tr>
</tbody>
</table>

Table 2. Lung cancer VOC biomarkers reported in the literature [44,76–83], their approximate concentrations in lung cancer patient breath, and the concentrations at which they have been detected using the Lonestar VOC Analyzer (FAIMS).

A screening approach using the Breath Biopsy® platform offers the promise of non-invasive early diagnosis of lung cancers. At present, the scientific consensus is that no single marker can be used to accurately detect lung cancer. Instead, combinations of lung cancer-specific VOC biomarkers and changes in the concentrations of compounds normally found in healthy breath make accurate detection of malignant cells possible.

Owlstone Medical’s FAIMS technology is sensitive enough to detect VOC biomarkers identified in previous studies [44,76–83], at concentrations relevant for human breath (Table 2).

To make lung cancer breath tests a reality, Owlstone Medical’s LuCID (Lung Cancer Indicator Detection) project is currently underway. LuCID includes highly powered clinical trials for early detection of lung cancer, and is funded by an SBRI Healthcare development contract.
Colorectal cancer

Colorectal cancer (also referred to as bowel cancer) is the fourth most common form of cancer in the US, with around 135,000 new cases and 50,000 deaths each year. It is the second most common cause of death from cancer in the US, and has a mortality rate of around 35% within five years of diagnosis.

As with many other types of cancer, the stage at which colorectal cancer is diagnosed has a huge impact on the patient’s prognosis. Patients identified at Stage I have around a 90% five-year relative survival rate, which drops to 13.5% if the cancer has metastasized (stage IV) [84].

To increase the number of early diagnoses, the way in which patients are screened for colorectal cancer needs to be improved. Existing non-invasive colorectal cancer screening methods such as gFOB (guaiac based fecal occult blood) and the now more common FIT (Fecal Immunochemical Testing) show relatively low sensitivities (FIT sensitivity = 66 - 88%, specificity = 87 - 96%). FIT also lacks sensitivity to advanced adenoma (27 - 41%, specificity = 91 - 97%).

A major drawback of current screening techniques is that patients have to provide stool samples. Globally, the uptake of colorectal cancer screening utilizing fecal samples varies from 60% in the UK to 8% in the US, and is as low as 1 and 2% in China and India respectively (Figure 19).
Figure 19. The percentage of the population screened for colorectal cancer using FIT (Fecal Immunochemical Testing) varies globally. Screening based on fecal testing typically suffers from poor compliance due to poor patient acceptability.

PERCENTAGE OF POPULATION SCREENED FOR COLORECTAL CANCER USING FECAL TESTING

USA
8% of 89 million population screened with FIT
Age range*: 50-74 years old

UK
60% of 10 million population screened with FIT
Age range*: 60-75 years old

FRANCE
42% of 19 million population screened with FIT
Age range*: 50-69 years old

GERMANY
18% of 28 million population screened with FIT
Age range*: >56 years old

ITALY
27% of 16 million population screened with FIT
Age range*: 50-69 years old

SPAIN
14% of 11 million population screened with FIT
Age range*: 50-69 years old

JAPAN
17% of 75 million population screened with FIT
Age range*: >40 years old

CHINA
1% of 564 million population screened with FIT
Age range*: 40-70 years old

INDIA
2% of 175 million population screened with FIT
Age range*: 50-69 years old

*Defined by the age range obtained from identified source for national screening program or recommendations.
There is a clear need for a new, non-invasive screening system with the potential for high patient compliance and low cost. Studies have previously identified at least 15 discriminating VOCs that can be used to identify between patients with and without colorectal cancer (e.g. [85]). It has also been shown that breath VOCs can be used to differentiate patients with colorectal cancer from those with other cancers, specifically breast, lung and prostate [86].

There is also evidence that VOCs from urine can be used to discriminate between patients with and without colorectal cancer [87]. Arasaradnam et al. found that Owlstone Medical’s FAIMS technology can be used to differentiate between patients with colorectal cancer and healthy controls, with a sensitivity of 88%, by rapid, non-invasive analysis of volatile organic compounds (VOCs) in urine (Figure 20 and Figure 21).

The authors recruited 133 volunteers, 83 of whom had confirmed colorectal cancer. VOC analysis was performed using an Owlstone Medical Lonestar VOC Analyzer fitted with an ATLAS headspace sampling system. These results show that detection of colorectal cancer’s VOC profile with FAIMS from patient urine samples can prove valuable as part of a non-invasive screening program for colorectal cancer.

![FAIMS Spectrum of VOCs in Urine](image)

**Figure 20.** Lonestar VOC Analyzer FAIMS spectrum from urine sample of patient with colorectal cancer.
To investigate the performance of both breath and urine VOCs in a larger at risk population of using FAIMS to screen for colorectal cancer, Owlstone Medical have launched a 1,400 patient clinical trial in collaboration with Warwick University and the University Hospital Coventry and Warwickshire NHS Trust. Known as InTERCEPT, the trial aims to evaluate the accuracy of both breath and urine VOC biomarkers in the detection of colorectal cancer at an early stage, when patient survival rates are at their highest.

**InTERCEPT**
The world’s largest breath-based study for early detection of colorectal cancer

owlstonemedical.com/intercept

Figure 21. To determine whether patients with colorectal cancer could be distinguished from healthy volunteers using the Lonestar VOC Analyzer (FAIMS), Arasaradnam et al. used Fisher Discriminant Analysis on FAIMS data collected from urine samples of patients with colorectal cancer and controls. They found that it was possible to use the entire VOC profile measured with FAIMS to detect colorectal cancer cases with 88% sensitivity.
Esophageal-Gastric Cancer

Esophageal and stomach cancers are amongst the most commonly occurring cancers with 456,000 and 952,000 new cases respectively diagnosed globally in 2012 [88]. Early stage esophageal-gastric cancer is usually associated with non-specific symptoms. Identifiable symptoms often only occur once the disease is in an advanced state, with reduced prospects of long-term survival. Improving long-term survival by detecting the cancer earlier is challenging as existing detection techniques are invasive and expensive hampering their use on the vast number of patients who present with upper gastrointestinal symptoms of unclear origin.

To look for VOCs that might be used as disease biomarkers at an early stage, Sung Tong-Chin et al. [89] collected breath from esophageal-gastric cancer patients with the ReCIVA Breath Sampler. Using a cross-platform mass spectrometry strategy, they measured VOC profiles in cancer patient breath. Figure 22 shows the relative intensity of VOC composition in patients with esophageal-gastric cancer. Further analysis is required to find features in the VOC profiles that might allow discrimination of the early stage disease in the patient population.

Figure 22. Box and whisker plot of the relative intensity of VOC composition in exhaled breath of esophageal-gastric cancer patients analyzed by TD-GC-MS.
The term ‘inflammatory disease’ covers an array of disorders and conditions that are characterized by the body’s complex inflammatory reaction to harmful stimuli, including irritants, pathogens and damaged cells.

Acute inflammation is the body’s initial protective response, and involves movement of leukocytes to the affected tissues. Prolonged, chronic inflammation, leads to a progressive shift in the type of cells present, resulting in simultaneous destruction and healing of tissues.

Inflammatory abnormalities underlie a vast variety of human diseases, including asthma, many autoimmune diseases and are also implicated in the development of cancer and heart conditions.

**Inflammatory Bowel Disease**

Inflammatory bowel disease (IBD) is a group of autoimmune diseases primarily affecting the colon and small intestine. The principal types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). IBD is common, and affects approximately 1 - 1.3 million people in the US [90].

The cause of IBD is not entirely understood, but it is widely believed to be the result of complex interactions between an individual’s genetic susceptibility, environmental triggers (e.g. diet, lifestyle, etc.) and the influence of an individual's gastrointestinal bacterial colonies.

At present the diagnosis of IBD and the differentiation of disease types takes a multi-modal approach that includes clinical, histological, serological, radiological and endoscopic observations [91].
Accurate disease stratification is vital for determining the best treatment strategy, but at present 10 - 15% of patients elude correct categorization. Accurate phenotyping often requires endoscopy of the intestine, which high cost, uncomfortable, has high non-compliance rates and can result in complications such as perforation of the bowel [92,93].

Metabolic VOC biomarkers offer a non-invasive route to the accurate stratification of IBD patients. They also offer a potential means of determining which patients will develop more severe disease phenotypes, and who will benefit most from expensive biological and/or immunomodulating agents [94,95].

**Breath Biopsy** for IBD Diagnosis and Stratification

To determine whether breath VOCs offer an effective route to diagnosis and stratification of patients with IBD, researchers have used Owlstone Medical’s FAIMS technology to see if patients’ breath VOC profile can be used to distinguish IBD patients from healthy controls, and to also distinguish those with Crohn’s disease (CD) and ulcerative colitis (UC) [16].

A total of 76 subjects were recruited for the study, of which 54 had histologically confirmed IBD (29 UC and 25 CD) and 22 were healthy controls. The predictive performance of the data from FAIMS analysis of exhaled breath samples was studied using a pipeline consisting of wavelet transformation, feature selection and a sparse logistic regression classifier. This was used to classify samples and calculate sensitivities and specificities as part of a 10-fold cross-validation.

The box plot below (Figure 18) shows the predictive power of IBD (UC and CD) vs. controls, UC vs. controls, CD vs. controls and finally UC vs. CD.

---

*Figure 23. Predictions of classifier to different combinations of diseases and controls (UC – ulcerative colitis, CD – Crohn’s disease, V – volunteer (healthy control)). The study used the Lonestar VOC Analyzer (FAIMS) to analyze VOC biomarkers in breath [16].*
The analysis showed that patients with IBD could be distinguished from control patients using FAIMS analysis of VOCs in breath samples with a sensitivity of 74% (95% confidence intervals (CI): 0.65–0.82) and specificity of 75% (95% CI: 0.53–0.90), p-value $6.2 \times 10^{-7}$. The AUC (area under curve) was 0.82 (95% CI: 0.74–0.89) (Figure 24).

FAIMS could distinguish those with UC from those with CD with a sensitivity of 67% (95% CI:0.54–0.79) and specificity of 67% (95% CI: 0.54–0.79), p-value $9.23 \times 10^{-4}$. The AUC was 0.70 (95% CI: 0.60–0.80).

The study confirms the utility of FAIMS exhaled VOC analysis to distinguish IBD from healthy controls, and UC from CD. It conforms to other studies using different technology, whilst affirming exhaled VOCs as biomarkers for diagnosing IBD.

Figure 24. Receiver operator curve (ROC) plot of IBD (UC and CD) vs. healthy controls. The study used the Lonestar VOC Analyzer (FAIMS) to analyze VOC biomarkers in breath [16].
Liver Disease

The link between liver diseases and characteristic breath aromas has been known to clinicians for thousands of years. Hippocrates (460–370 BC) famously described *fetor hepaticus* in his treatise on breath aroma and disease [1].

Liver diseases including non-alcoholic fatty liver disease and viral hepatitis can progress to cirrhosis and eventually hepatocellular carcinoma. Early diagnosis and treatment of liver diseases is important because progression is common and has a significant risk of mortality.

As a major organ involved in dealing with waste metabolites, the liver is a significant source of metabolic VOCs that can be excreted in breath. Breath Biopsy® offers a non-invasive way to measure liver disease activity and gather vital metabolic information for earlier diagnoses.

**Hepatic Encephalopathy**

Owlstone Medical’s FAIMS technology has already been utilized to analyze the breath of patients with hepatic encephalopathy (HE). HE is a neuropsychiatric condition which occurs when the liver cannot adequately remove toxins from the blood. It can occur suddenly in people with acute liver failure but is more often seen in those with chronic liver disease.

The prevalence of minimal HE (the mildest detectable class of HE under the West Haven criteria) is reported in 30% – 84% of patients with liver cirrhosis. This has profound effects on daily functioning with nearly 50% of minimal HE patients potentially unfit to maintain employment. The condition can be treated effectively using Rifaximin, and earlier detection can prevent liver disease progression and hospitalization.
In the pilot study [15], the chemical fingerprint of breath samples from patients with HE and controls was analyzed using a Lonestar VOC Analyzer fitted with an ultraviolet ionization source. 42 patients were recruited: 22 patients with HE (13 covert and 9 with overt HE) and 20 healthy controls.

Extracted data was analyzed using a pipeline based on a 2D wavelet transform with thresholds to remove background noise. This was followed by feature selection to identify key variables (using a Wilcoxon rank-sum test applied separately to each feature), with the resultant feature set used to separately train four classifiers (sparse logistic regression, Random Forest, Support Vector Machine and Gaussian Process).

FAIMS analysis of exhaled VOCs was able to classify HE patients from controls with a sensitivity and specificity of 88% (0.73–0.95) and 68% (0.51–0.81) respectively with an AUC of 0.84 (0.75–0.93).

This pilot study provides evidence that breath VOC biomarker analysis has potential as a diagnostic aid in distinguishing HE of all grades from healthy control subjects, and further suggests potential for VOC biomarkers as an aid to distinguish between the covert and overt forms of HE.

ReCIVA® Breath Sampler
For reliable, standardized breath collection

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Infectious disease mortality can be lowered by correctly targeting antimicrobial drugs early on in the progress of an infection [96]. At present, most treatment is untargeted due to a lack of adequate diagnostics.

Targeting requires knowledge of the causative bacterial, viral, or fungal pathogen, but culturing has limited sensitivity and can take many days [97]. Other techniques like gram-staining and direct cellular examination are fast but lack sensitivity and specificity, and don’t provide information about exact strain or antimicrobial targeting.

Improving diagnostic techniques to help better target infectious diseases has the potential to save lives and help tackle antimicrobial resistance. One way of doing this is to identify bacteria based on markers linked to their metabolisms, which are distinct to those of their hosts.

Bacteria produce VOCs not commonly generated by human metabolic processes that could be used to identify bacterial strain, improving the potential for drug targeting [98–101]. These VOCs are present in exhaled breath, making Breath Biopsy® a useful, non-invasive approach to infectious disease diagnosis.

**Tuberculosis**

There are around 8.6 million new cases, and 1.3 million deaths globally from tuberculosis (TB) every year, a disease caused by the bacterium *Mycobacterium tuberculosis*. Diagnosis is still mostly performed by trained technicians examining sputum samples under a microscope [102].

Treatment and prevention of TB in low-income countries is hampered by the lack of cheap, simple and accurate diagnostic tests. The sputum test has a sensitivity of only 62%, and unable to distinguish between different *Mycobacterium* species [103]. Patients who are suspected of having TB but who test negative using the sputum ZN-smear pose a diagnostic problem.

Metabolites have been implicated as biomarkers for TB [104], and breath analysis using technologies like FAIMS have the potential to provide a simple diagnosis that could revolutionize TB detection.

A recently published pilot study by Sahota *et al.* [14] found that Owlstone Medical’s Lonestar VOC Analyzer (FAIMS) was able to distinguish between healthy controls and patients with either pulmonary or extra-pulmonary tuberculosis. The latter type of the disease is traditionally more difficult to diagnose. Sahota *et al.* analyzed the FAIMS data from patient breath samples using a wavelet feature extraction process followed by a random forest classifier, to identify features in FAIMS spectra. This allowed discrimination between disease-positive and controls with a sensitivity of 81% and a specificity of 79%.
Figure 26. Classification probabilities for the control and TB groups in pilot study using a Lonestar VOC Analyzer by Sahota et al. Applying a Wilcoxon rank-sum test to the sets of classification probabilities from the two groups, a p-value of 2.89E6 is calculated, showing that there is a highly statistically significant difference between the control and TB groups. The study used the Lonestar VOC Analyzer (FAIMS) to analyze VOC biomarkers in breath [14].

Figure 27. Receiver Operator Curve (ROC) plot from Sahota et al. (AUC = 0.92; 95% CI: 0.84, 1) for breath analysis of patients with TB. The study used the Lonestar VOC Analyzer (FAIMS) to analyze VOC biomarkers in breath [14].
Detection of TB in Patients with Negative ZN-smear Tests

A team from the Pulmonology Department at the Academic Medical Centre, University of Amsterdam were interested to test whether breath biomarkers could detect TB in patients suspected to have TB, but who displayed negative ZN-smear tests.

Owlstone Medical's Lonestar VOC Analyzer (FAIMS) was included in a proof-of-concept study. They showed that tuberculosis can be diagnosed using this system via chemical markers in exhaled breath. Importantly, they also found that TB could be detected in the patients with negative ZN-smear results.

The results of these pioneering studies indicate that FAIMS has potential as a technology that can be applied to breath testing for infectious diseases including TB.

Lonestar VOC Analyzer
Based on proven FAIMS technology

owlstonemedical.com/lonestar

Clostridium Difficile

*C. difficile* is a bacterium that can infect the bowel, causing diarrhea. It commonly affects the elderly and patients who have recently received antibiotics. In recent years, the infection has become increasingly prevalent, and more severe forms of the disease have emerged. More serious symptoms include severe pseudomembranous colitis and toxic megacolon. Large hospital outbreaks have required ward closures and extensive infection control measures.

While the application of breath VOC biomarkers has not yet been investigated for *C. difficile* diagnosis, there is evidence for the utility of VOCs emitted from stool samples. VOC biomarkers emitted from stool samples could accurately distinguish *C. difficile*-positive stool samples from healthy samples.

Both healthy and *C. difficile*-positive stool samples were analyzed using a Lonestar VOC Analyzer (FAIMS) fitted with an ATLAS headspace sampler (Figure 28, [46]. A total of 213 samples were analyzed, of which 71 were confirmed as *C. difficile*-positive by microbiological analysis.
Training and test samples (n=135) were used to identify which characteristics discriminate between positive and negative samples, and to build machine learning algorithms interpreting these characteristics.

The best performing algorithm was then validated on new, blinded validation samples (n=78). The predicted probability of *C. difficile* (as calculated by the algorithm) was compared with the microbiological test results (direct toxin test and culture).

Using a Random Forest classification algorithm, FAIMS had a high discriminatory ability on the training and test samples (AUC of 0.91 (95% confidence interval (CI): 0.86–0.97)). When applied to the blinded validation samples, the AUC was 0.86 (0.75–0.97). For samples analyzed ≤7 days of collection (n=76), diagnostic accuracy was even higher (AUC of 0.93 (0.85–1.00), Figure 28).

A cutoff value of 0.32 for predicted probability corresponded with a sensitivity of 92.3% (95% CI: 77.4–98.6%) and specificity of 86.0% (78.3–89.3%). For even fresher samples, discriminatory ability further increased.
This pilot study shows that FAIMS analysis of unprocessed stool samples can differentiate between C. difficile-positive and negative samples with high diagnostic accuracy. FAIMS analysis is also quick, easy to use and relatively cheap.

Figure 29. The Receiver Operating Characteristic (ROC) curve for the (toxin and culture) negative and (toxin and culture) positive samples. Samples analyzed within 7 days are included. Stool samples were analyzed using a Lonestar VOC Analyzer (FAIMS) fitted with an ATLAS headspace sampler [46]

This pilot study shows that FAIMS analysis of unprocessed stool samples can differentiate between C. difficile-positive and negative samples with high diagnostic accuracy. FAIMS analysis is also quick, easy to use and relatively cheap.

ATLAS Headspace Sampler
Combine with Lonestar VOC Analyzer to sample liquid/solid biospecimens

owlstonemedical.com/atlas
How to Analyze VOC Biomarkers

The era of modern breath testing started in 1971, with the work of Nobel Prize winner Linus Pauling [3]. Since then, hundreds of scientific papers have been published suggesting the presence of VOC biomarkers across a range of diseases (Figure 13). There is limited agreement, however, in identified biomarkers within a disease across these published studies.

The identification of robust VOC biomarkers in breath has been hampered by sampling and analytical challenges [17]. In addition, small patient numbers in pilot studies and the lack of blinded validation studies has impeded the introduction of VOC biomarkers into the clinic.

At Owlstone Medical we have worked to understand and overcome the challenges of breath analysis, and set up robust workflows for analysis of VOCs in breath to enable breath biomarker studies within our clinical pipeline and Breath Biopsy Services.

Owlstone Medical’s Breath Biopsy platform has introduced breath as a new diagnostic modality. Now, for the first time, it is possible to perform robust biomarker discovery using breath.
Modern breath testing commenced in 1971, with the work of Nobel Prize winner Linus Pauling. Hundreds of scientific papers published suggesting the presence of VOC biomarkers across a range of diseases.

**SOME PUZZLING QUESTIONS**

1. Why is there very little agreement in identified biomarkers within a disease?
2. Why is breath testing not used routinely in clinical setting?

**SOME HISTORICAL CHALLENGES**

- unreliable breath sampling hardware and protocols for robust, repeatable sampling.
- high end, expensive spectrometer vs low performance eNose.
- different analytical techniques required in biomarker discovery and clinical translation.
- study design and size - small patient numbers in pilot studies and lack of blinded validation studies.

**WITHOUT SOLVING THESE YOU CAN’T HAVE CONFIDENCE IN INITIAL BIOMARKER DISCOVERY AND VALIDATION**

Figure 30. Historical challenges that have hampered breath analysis, leading to the development of new technologies that enable the identification of robust VOC biomarkers in breath.

Overcoming Challenges in Breath Sampling and Analysis

Historically the robust and reproducible analysis of VOCs in breath has posed many technical and analytical challenges which has led to limited agreement between different studies.

Table 3 provides an overview of the historical challenges faced by breath researchers, and the solutions now available through technological advances in breath sampling and analysis.
### Table 3. Overview of historical challenges to the adoption of breath sampling, and solutions offered by Owlstone Medical’s Breath Biopsy® technology.

<table>
<thead>
<tr>
<th>Breath sampling</th>
<th>Historical challenges</th>
<th>Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Lack of standardized breath collection device</td>
<td>ReCIVA™ Breath Sampler:</td>
</tr>
<tr>
<td></td>
<td>• Variation in volume of breath collected</td>
<td>• Reliable, reproducible breath collection</td>
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<tr>
<td></td>
<td>• Contamination with exogenous VOCs from environment</td>
<td>• Collects a defined breath volume</td>
</tr>
<tr>
<td>Tedlar® and other polymer storage bags:</td>
<td>• Limited sensitivity due to collection of single exhaled breath</td>
<td>• Samples VOCs continuously over ~10 minutes for increased sensitivity</td>
</tr>
<tr>
<td></td>
<td>• Water condensation on inner surface of bag adsorbs VOCs</td>
<td>• Collects breath samples onto Breath Biopsy Cartridges</td>
</tr>
<tr>
<td></td>
<td>• Ingress of external VOCs through bag</td>
<td>• Hydrophobic sorbents in Breath Biopsy Cartridges minimize water interference</td>
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<tr>
<td></td>
<td>• Contamination with VOCs from bag</td>
<td>• CASPER Portable Air Supply minimizes external VOC contamination</td>
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<tr>
<td></td>
<td>• Carryover of VOCs when re-using bags</td>
<td></td>
</tr>
<tr>
<td>Breath analysis</td>
<td>High-end analytical instruments e.g. MS:</td>
<td>Microchip chemical sensor (FAIMS):</td>
</tr>
<tr>
<td></td>
<td>• Expensive and bulky</td>
<td>• Easier to operate than MS</td>
</tr>
<tr>
<td></td>
<td>• Inter-instrument variability</td>
<td>• Superior robustness and reproducibility for target VOCs in complex biological samples</td>
</tr>
<tr>
<td></td>
<td>• Expertise required to operate correctly</td>
<td>• Improved stability, sensitivity and selectivity compared to eNose systems</td>
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<tr>
<td></td>
<td>• Limited throughput</td>
<td>• Small size compared to MS</td>
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<tr>
<td></td>
<td>‘Electronic Nose’ (eNose):</td>
<td></td>
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<tr>
<td></td>
<td>• Limited temporal stability</td>
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<td></td>
<td>• Poor chemical selectivity</td>
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### Study Design

As with any ‘omics technique, the identification of robust VOC biomarkers in breath typically involves three stages:
1. Discovery
2. Verification
3. Validation

The above stages encompass the studies involved in discovering the optimal classifier, verifying the classifier in a larger patient cohort, and performing an external blinded validation in a larger patient cohort representative of the intended use population, to validate both the VOC biomarker algorithm and the analytical instrumentation that together comprise a diagnostic test ready for regulatory approval.

Key in designing a study is to understand the intended clinical deployment scenario. In principle, the inclusion and exclusion criteria for subjects should match the clinical scenario as closely as possible to maximize external validity of the identified biomarkers. However, some general concepts need to be taken into consideration when designing a study aiming to identify breath biomarkers.
Pre-analytical Variables and Confounding Factors

Challenges related to exhaled breath analysis include variability introduced by variation in the breath sampling method (e.g. sampling total vs. alveolar breath, or single-breath vs. fixed-time/-volume) effect of expiratory flow and breath hold, type of sample collection materials, VOC recovery, and sample pre-treatment.

It is vital that all breath samples in the study should be collected using a robust, reproducible, standardized breath sampling protocol (see section on “Reliable collection of breath samples“ on page 59).

In addition, a number of factors have the potential to contribute to the profile of VOCs in exhaled breath, including ambient inspired volatiles, effect of humidity, food/medications, exercise, smoking, and comorbidities (Figure 26).

In the case of pilot or proof-of-concept studies, one approach is to attempt to control these variables, to minimize their potential impact on the ability to detect breath biomarkers. However this may not be the most useful approach, for the following reasons:

1. Signals obtained in the pilot study may not be borne out in subsequent verification studies including larger patient cohorts where the potential variables may be less well controlled.
2. Specifying stringent conditions to minimize variation may pose challenges for implementation of the test into clinical practice.
3. Many of the factors contributing to the VOC profile may be randomly distributed across cases and controls, hence appropriate statistical techniques can prevent them from having a big impact on diagnostic performance.
4. Factors contributing to the VOC profile may actually form part of the signal, and improve the clinical relevance of the biomarker profile.

Three preventative measures can be taken to minimize the impact of potential variables on your study outcome, and maximize the ability to detect a biological signal:

1. Selection criteria for subjects should reflect the actual population in which the test is to be used as closely as is practically feasible. This means potential confounders are less likely to impact biomarker discovery.
2. Potential confounding factors should be considered and recorded in the Case Report Form (CRF) accompanying the study. It is crucial to assess the systematic bias or confounders that are unevenly distributed between case and control groups that might impact the biomarker signal. Clinical background data can be used to assess the accuracy with which the breath biomarkers can identify the presence of certain confounders.
3. Signal-to-noise ratio should be maximized by reducing background noise (e.g. using CASPER Portable Air Supply), and using a well standardized and validated breath collection method and analytical pipeline.
FACTORS THAT CAN CONTRIBUTE TO THE VOC PROFILE

Figure 31. Factors that can contribute to the VOC profile [105]. It is crucial to assess systematic bias or confounders that are unevenly distributed between case and control groups that might impact the biomarker signal, hence potential confounding factors should be detailed in a Case Report Form (CRF) accompanying the study.

Cohort Size

Many breath analysis studies reported in the literature involve small patient cohorts, which limits the statistical significance of the findings, and makes it challenging to investigate the impact of possible confounding factors.

The sample size needed to reach statistical significance depends both on the inter-sample variation, and on the expected amplitude of differences the study is intended to show. In a cohort including a diverse set of human individuals, the population is likely to be fairly heterogeneous with a high degree of biological variation.

If the goal is to identify very small differences in VOC levels between sub-groups of the cohort, then a large number of samples may be required. A power analysis can be used to evaluate the number of samples needed to detect an effect of a given size.

Patient Population

Care should be taken to select not only the optimal cohort size, but also the appropriate patient population. This patient population is likely to vary according to the stage of the study.
Initial discovery or pilot studies may involve more clearly defined and homogeneous groups. Whilst this increases chances of finding a signal, there is a trade off with the external validity of these results which should be carefully considered. Trying to focus on a population representative of the intended use population is key to minimize this risk.

In some cases, e.g. screening, the target condition only occurs infrequently in the population in which the breath test is to be used. In these cases, recruiting an enriched population is often an effective way of running the discovery phase of the study. However, in order to obtain the evidence required for regulatory approval, the validation study will always need to be in the intended population.

Ideally a study would focus on the ‘intention to diagnose’ population during early test development. This prevents embarking on follow-up studies that turn out to be false negative as a consequence of population differences between the studies.

**Breath Analysis Workflow**

Owlstone Medical has developed services to facilitate the discovery and targeted analysis of VOC biomarkers.

**BREATHE BIOPSY DISCOVERY METHODOLOGY**

![Diagram of Breath Biopsy Discovery Methodology]

Figure 32. Schematic representation of the general process of Breath Biopsy.

**Reliable Collection of Breath Samples**

Reproducibility is key for any analytical technique, and standardization of breath collection is key to enable the breath diagnostics field to perform robust breath biomarker discovery for a range of medical diagnostics applications.

To address the challenges in sampling breath, Owlstone Medical formed the Breathe Free Consortium, a group of 100 multi-disciplinary experts in breath diagnostics, in order to jointly develop the ReCIVA Breath Sampler as the industry standard for the reliable collection of breath samples.
The ReCIVA Breath Sampler provides a much-needed alternative to containers such as Tedlar® bags which have been shown to result in the loss of sampled VOCs [106]. The ReCIVA Breath Sampler collects breath samples onto Breath Biopsy Cartridges, which can be analyzed immediately or stored for later transport under ambient conditions to an off-site laboratory for analysis.

Sampling breath using ReCIVA and Breath Biopsy Kits ensures reproducible collection of VOCs (Figure 33 and Figure 34), unlike other commonly used collection media, such as Tedlar® bags [106].
Importantly, the ReCIVA Breath Sampler also allows VOCs in breath to be sampled continuously over a ~10 minute time period. This enables the pre-concentration of low concentration VOCs from much larger breath volumes than can be practically captured using a bag. Hence, ReCIVA facilitates the collection of VOCs originating from throughout the body, and makes it possible to detect even low level VOC biomarkers, such as those present at the earliest stage of disease (see also page 18).

Pressure and CO$_2$ sensors in the ReCIVA Breath Sampler provide real-time monitoring of patient breathing, allowing specific breath fractions to be selected (e.g. enriched bronchial vs. end-tidal). ReCIVA is designed to be used with the CASPER Portable Air Supply, to minimize the risk of contamination from external VOCs.

Named Invention of the Year in the 2017 Top 50 in Digital Health Awards, ReCIVA is the first breath sampler able to provide an out-of-the-box means to reproducibly capture breath samples for analysis.

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**Figure 34.** D-limonene in breath samples collected on independent VOC sample tubes (1 and 2) using ReCIVA and analyzed using TD-GC-FAIMS (Lonestar VOC Analyzer) shows high reproducibility within a single breath sample. Small changes in D-limonene peak area are observed between different breath samples (A and B, performed on the same individual), which is to be expected because some VOCs in breath do vary over time.

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How does ReCIVA® compare to Tedlar® bags?

Find out here:

[www.owlstonemedical.com/reciva-vs-bags](http://www.owlstonemedical.com/reciva-vs-bags)
ReCIVA is CE marked and is in use in over 100 academic research groups and clinical labs around the world. In addition, a pediatric ReCIVA Breath Sampler enables breath collection from children as young as 5.

ReCIVA is currently being deployed as part of the NHS funded lung cancer detection project (LuCID) - the world’s largest breath-based study ever undertaken for early cancer detection (Learn more on page 37).

Breath Biopsy® Services
VOC biomarker discovery and targeted analysis
owlstonemedical.com/services

VOC Biomarker Discovery

Owlstone Medical's highly skilled data scientists identify discriminating features from mass spectra generated by TD-GC-MS analysis of breath samples. Identified features are combined with patient clinical and outcome data from our Breath Biopsy Digital Biobank to develop biomarker classification methods (see page 66).

GC-MS is the Gold Standard for VOC Analysis

Out of the available techniques, GC-MS provides highly specific information on the qualitative and quantitative presence of VOCs in exhaled breath, making it the gold standard for the untargeted analysis required in biomarker discovery. Excellent long term stability makes GC-MS capable of reproducibly measuring potential VOC biomarkers at the low concentrations present in exhaled breath (Figure 35). The technique is also selective enough to allow the elimination of possible interferences.

Other approaches such as electronic nose (eNose) systems that use arrays of non-selective chemical sensors to detect VOCs are far more restricted. These systems often suffer from unwanted chemical interactions between VOCs and the sensors, poor intra-device repeatability, limited temporal stability and poor chemical selectivity.

The Breath Biopsy platform has been designed specifically for the detection of low concentration VOCs.

High reproducibility is achieved in part through standardized breath collection parameters controlled by the ReCIVA Breath Sampler, such as fraction and volume of breath collected. The platform also includes the CASPER Portable Air Supply which minimizes interference from unwanted VOCs in ambient air.
Figure 35: Three concentrations of Methyl Salicylate were loaded onto separate Breath Biopsy Cartridges and analyzed using TD-GC-MS. The three QCs were tracked over time to assess the variation.

Sensitivity is increased by minimizing external interferences. This is achieved through a rigorous cleaning and conditioning process of all components of our Breath Biopsy kits which include Breath Biopsy cartridges and masks.

Our robust analytical workflow ensures that samples, instruments and consumables undergo standard procedures and stringent QC checks. Owlstone Medical’s Breath Biopsy laboratory is ISO-accredited and dedicated to analyzing breath samples only.

Together these measures lead to highly reproducible and reliable analysis of VOCs in breath.

Target VOC Analysis

Once biomarkers of interest have been identified Owlstone Medical’s targeted VOC analysis offers a robust and reproducible way to quantify the VOCs for validation studies.

Analysis at Owlstone Medical’s Breath Biopsy lab can be performed using TD-GC-MS or TD-GC-FAIMS (field asymmetric ion mobility spectrometry).

FAIMS distinguishes ionized VOCs according to differences in the speed that they move through a buffer gas under the influence of an oscillating electric field [107].

Over $70 million has been invested in developing Owlstone Medical’s FAIMS technology. It has been rigorously validated and is used globally in military and industrial applications.

Owlstone Medical has incorporated its FAIMS technology into the Lonestar VOC Analyzer for medical applications, enabling laboratories with the right expertise to run their own targeted analysis.
Quality Control

Consistency in the breath collection and VOC analysis workflow is key to obtaining reliable data for biomarker discovery. This section provides an overview of how to monitor the workflow to identify outlier samples and sources of technical variation.

Quality Checks During Breath Collection

To ensure a successful breath collection using the ReCIVA Breath Sampler, it is important to verify that the Breath Biopsy mask fits well by checking that there is no air escaping from the mask around the nose or mouth.

The pressure and CO₂ levels within the ReCIVA are monitored throughout collection to warn the operator of leaks from the mask, hyperventilation, hypoventilation, shallow or otherwise irregular breath profiles that negatively impact VOC collection. Feedback is given in real-time to correct poor collection technique. Breath profiles and sampling time are also stored along with other patient metadata to inform subsequent data analysis.

Occasionally, it may not be possible to collect the required volume of breath in the collection time specified for the project. In this case, the breath sample can still be analyzed, but this sample is flagged in case the reduced breath volume may affect the results.

Quality Control During VOC Analysis

Successful VOC biomarker discovery requires a robust data set, where levels of individual VOCs are measured with a high degree of accuracy. Some VOC biomarkers may be present at extremely low levels, therefore it is important that analytical techniques are highly sensitive, with low background levels.

Owlstone Medical has implemented QC checks monitoring a range of instrument parameters on a daily and weekly basis, to ensure that VOC analytical workflows are highly sensitive, accurate and reproducible (Figure 35).

Breath Biopsy cartridges are checked prior to breath collection, and blank samples are regularly tested, to ensure that any background is below a threshold level (Figure 37). A set of standard compounds with a range of concentrations is used to check the linearity (Figure 36), limit of detection and signal-to-noise ratio.

The VOC profile of each patient sample is also inspected for the presence of compounds that is expected to be present above certain thresholds in a patient breath sample. This QC serves as an initial check that the analysis has been successful, before initiating data analysis.

“My group have collaborated with Owlstone for several years in the discovery of biomarkers for a range of diseases including colorectal cancer, inflammatory bowel disease and tuberculosis. We are very impressed with the capability of Owlstone’s new non-invasive technology.”

Professor Ramesh Arasaradnam, University of Warwick and Consultant Gastroenterologist at UHCW
Figure 36. Calibration plot of D-limonene standard applied to Breath Biopsy cartridges at a range of amounts and measured using TD-GC-FAIMS (Lonestar VOC Analyzer). Excellent linearity is observed, enabling accurate quantification down to 0.2 ng on-column mass which equates to around 70 parts per trillion (ppt) in breath. Good precision is demonstrated; data shown is the mean of measurements from 4 - 6 different VOC sample tubes analyzed on 4 different days (error bars represent 1 standard deviation).

Figure 37. Comparison of a breath sample containing D-limonene, a blank collect sample and an ambient air sample. All samples were collected using the ReCIVA Breath Sampler and analyzed using TD-GC-MS. The blank collect sample was collected mimicking normal breath collection a using the CASPER Portable Air Supply. The ambient air sample was collected without the CASPER Portable Air Supply. The graph highlights the importance of measuring ambient VOCs to distinguish unavoidable external signals from those resulting from breath VOCs. Further it shows the advantage of minimizing contamination of external VOCs to reduce background noise.
In order to identify VOCs that are potential biomarkers for disease, the raw spectrometry data must be processed and analyzed. To facilitate this process, we have developed a data analysis pipeline that takes raw spectrometry data, patient clinical and outcome data, develops classification algorithms, and ultimately outputs a classification algorithm that uses multiple biomarkers to accurately identify patients by disease presence, therapy response or outcome (Figure 38).

**Data Analysis**

In order to identify VOCs that are potential biomarkers for disease, the raw spectrometry data must be processed and analyzed. To facilitate this process, we have developed a data analysis pipeline that takes raw spectrometry data, patient clinical and outcome data, develops classification algorithms, and ultimately outputs a classification algorithm that uses multiple biomarkers to accurately identify patients by disease presence, therapy response or outcome (Figure 38).

**VOC DATA ANALYSIS PIPELINE**

- **Raw Data**
  - Build statistically comparable and balanced training and test/blind data sets

- **Training Set**
  - **Data Pre-Processing**
    - Time calibration and feature extraction
  - **Feature Table**
    - Feature transformation and feature selection
  - **Classification Algorithm Development**
    - Using machine learning approach

- **Test/Blind Set**
  - **Data Pre-Processing**
    - Time calibration and feature extraction
  - **Feature Table**
    - Feature transformation and feature selection
  - **Apply Classifiers to Test/Blind Set**
    - Evaluate sensitivity, specificity and statistical significance

- **Breath Biopsy Digital Biobank**
  - Classifiers are verified *in silico* using patient VOC profiles in our Breath Biopsy digital biobank

- **Apply Classifiers to Blind Set**
  - Evaluate sensitivity, specificity and statistical significance

*Figure 38. The VOC data analysis pipeline.*
Training and Test Data Sets

The first step is to build training and test data sets that are statistically comparable, where any confounding factors are represented equally. Both training and test data sets then undergo unsupervised data pre-processing to generate calibrated retention times and identify features (peaks in the FAIMS or mass spectrum data, or absence thereof) that can be used for classifier development. Any features that are system-related are excluded.

Classifier Development

Using the training data set, a machine learning approach is taken to train a learning algorithm and develop classifiers using 10-fold stratified cross validation [108]. The classifiers are applied to the test data set, and a systematic study of different sources of bias is performed, in order to identify any sources of technical variation and to verify that the classifier is robust to confounding factors. The features and classifiers are then further refined through an iterative process.

Classifier Evaluation

In order to select the best performing classifier, classifier performance is evaluated in terms of sensitivity, specificity and area under the ROC curve (Figure 40). In the case of on-going studies, we also assess whether the number of patients is sufficient for statistical significance, or whether the study would benefit from recruitment of additional patients.

IDENTIFICATION OF FEATURES FROM RICH SPECTRAL FAIMS DATA

Figure 39. Top: Total ion chromatogram. Middle: Mass spectrum for limonene at 22 minutes. Bottom: Extracted ion chromatogram for limonene ion (m/z = 93).
In silico Verification
At Owlstone Medical we use our Breath Biopsy Digital Biobank containing thousands of anonymized patient VOC profiles to verify classification algorithms in silico. We assemble virtual patient populations defined by demographics, comorbidity, and outcome, and apply the classifier to gain early evidence of likely clinical performance, without the need for additional samples or studies. This step can also identify potentially confounding variables or relevant sub-populations that may not have been adequately represented in the original trial population.

Verification
Analysis of candidate VOC biomarkers is performed in an independent patient cohort to verify the performance of the classifier. In this situation, the same data analysis pipeline will be applied to analyze the list of candidate biomarkers identified in the biomarker discovery phase, and further refine the classifier.

Validation
Following successful verification of the classifier, the classifier is locked down and an external blinded validation is performed in a larger patient cohort representative of the intended use population, to validate both the VOC biomarker algorithm and the analytical instrumentation that together comprise a diagnostic test ready for regulatory approval.
From collection of high quality breath samples to the discovery and validation of VOC biomarkers

Breath Biopsy® Kits

Learn more at owlstonemedical.com/breath-biopsy-kits
Bring Breath Biopsy® to your own research.

Collect, discover and validate breath-based biomarkers for cancer, inflammatory and infectious disease with our Breath Biopsy platform.

Get in touch with us to arrange a consultation to discuss your project: owlstonemedical.com/contact
Integrate Breath Biopsy into your Clinical Trial

Collect Breath Samples Using the ReCIVA Breath Sampler

Simply lease a ReCIVA Breath Sampler for the duration of your study, purchase Breath Biopsy Kits and collect breath samples in clinic. The ReCIVA Breath Sampler allows breath to be captured over a few minutes and pre-concentrated onto the Breath Biopsy Cartridge, enabling VOCs present even at low levels to be detected.

By leasing the ReCIVA Breath Sampler and collecting samples using our QC-checked Breath Biopsy Cartridges and Masks, you can be confident that the breath samples collected will be of the highest quality throughout your study.

- Reliable, reproducible breath collection for VOC biomarker analysis
- High patient safety and comfort
- Includes CASPER Portable Air Supply to minimize contamination of breath samples by external VOCs
- Used in conjunction with Breath Biopsy Kits containing Breath Biopsy Cartridge and Mask
- In use in the world’s largest breath-based clinical trials, at over 100 clinical sites around the world

owlstonemedical.com/reciva
Many researchers take advantage of Owlstone Medical's Breath Biopsy Services and send the Breath Biopsy Cartridges for analysis in our Breath Biopsy clinical laboratory. Alternatively, samples can be analyzed in your own laboratory using the Lonestar VOC Analyzer, or TD-GC-MS.

**Send Breath Biopsy Cartridges to Owlstone Medical for Analysis**

Our Breath Biopsy Services enable academics, clinicians and pharmaceutical companies to identify novel breath biomarkers for diagnostics and precision medicine applications including therapeutic response, patient stratification and drug toxicity prediction.

Our exceptional analytical platforms, rigorous quality standards and experienced staff deliver reliable, reproducible and rapid results.
Discovery or Targeted VOC Analysis

Our Breath Biopsy Services include global analysis of breath VOCs for biomarker discovery applications, or targeted analysis of specific VOCs of interest. Our experts perform a comprehensive analysis of VOCs in breath analysis using our Breath Biopsy platform including gold standard TD-GC-MS.

**Did you know?**

Our Breath Biopsy Digital Biobank is the world’s largest collection of anonymized breath VOC profiles matched to phenotype. Our data science team use the Breath Biopsy digital biobank to aid the discovery of novel biomarkers using proprietary machine learning algorithms.

**Breath Biopsy Services Workflow**

- **Consult**
  Our team of clinical and technical experts advise on all aspects of study design and sample collection, assist with ethics approval and access to our clinical trial network.

- **Breath Collection**
  We install the ReCIVA Breath Sampler in clinic and provide training and on-going support for optimal breath collection using Breath Biopsy Kits.

- **Breath Analyze**
  Breath Biopsy Cartridges are sent to Owistone Medical and analyzed using our Breath Biopsy analytical platform including gold standard TD-GC-MS.

- **Discovery or Targeted Analysis**
  Our experts identify discriminating features from spectral data and develop robust classification algorithms using machine learning algorithms. Target VOCs are identified and quantified.

- **Report**
  An in-depth report provides the results of our analysis including publication-ready figures. Discovery analysis includes in silico verification of the classifier using the world’s largest Breath Biopsy digital biobank. Target VOC analysis includes compound identification and quantification using our Breath Biopsy Digital Biobank.

*Figure 42. The Breath Biopsy Services workflow*
Quality Assurance

Extensive QC checks are part of our Quality Management System, which covers the entire analysis workflow starting with preparation of the Breath Biopsy Cartridges and Masks for breath collection. We perform a number of daily and weekly QC checks to monitor a range of instrument parameters, and use blanks and standard compounds to monitor background, sensitivity, limit of detection and signal-to-noise ratio.

Clinical Project Management

We offer CRO capabilities to set up and run clinical trials, drawing on our experience of running VOC biomarker clinical trials as part of our own clinical pipeline. We have an extensive network of clinicians, hospitals, and research centers, that can facilitate access to large patient populations and provide access to trial nurses if required.

Our Services include everything from consultation on the optimal study design, to biomarker discovery, verification and external blinded validation of the diagnostic test algorithm and analytical instrumentation in the context of the clinical use case, ready for regulatory approval (Figure 43).

<table>
<thead>
<tr>
<th>Discovery</th>
<th>Verification</th>
<th>Validation</th>
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<tr>
<td>Analyze full spectrum of VOCs in a small cohort and develop classifier.</td>
<td>Analyze candidate VOC biomarkers to verify classifier performance in a larger cohort.</td>
<td>Validation of defined VOC biomarker algorithm and analytical instrumentation in large patient cohort representative of the intended use population.</td>
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In silico verification using Owlstone Medical’s Breath Biopsy Digital Biobank.

In silico verification using Owlstone Medical’s Breath Biopsy Digital Biobank.

Application for regulatory approval

Figure 43. Stages of a typical VOC biomarker discovery and validation project. During discovery and verification, the classifier is further verified in silico using a population assembled from Owlstone Medical’s Breath Biopsy digital biobank of anonymized breath VOC profiles matched to phenotype. Following successful verification of the classifier, the classifier is locked down and an external blinded validation is performed in a larger patient cohort representative of the intended use population, to validate both the VOC biomarker algorithm and the analytical instrumentation that together comprise a diagnostic test ready for regulatory approval.
Analyze Breath Biopsy Cartridges in your own Lab using the Lonestar VOC Analyzer

Breath Biopsy Cartridges can be analyzed in your own laboratory using TD-GC-MS, or the Lonestar VOC Analyzer.

The Lonestar VOC Analyzer is based on FAIMS technology (Field Asymmetric Ion Mobility Spectrometry), and enables the analysis of Breath Biopsy samples using TD-GC-FAIMS.

- Easy to use instrument for the detection of VOC biomarkers in breath
- Rapid, sensitive and selective VOC analyzer
- High intra-device repeatability
- Used in conjunction with Breath Biopsy Collection Kits

owlstonemedical.com/lonestar

Breath Biopsy Kits
From collection to discovery and targeted VOC analysis

owlstonemedical.com/breath-biopsy-kits
Further reading

Search for publications, posters etc. on the resources page:
https://www.owlstonemedical.com/resources/

Research case studies involving VOC biomarkers:
https://www.owlstonemedical.com/science-technology/research-case-studies/

Drug Discovery World Article: Breath based biomarker detection:

Whitepaper: Application of Breath Volatile Organic Compounds (VOCs) in Asthma:
https://www.owlstonemedical.com/download/asthma-whitepaper/

Innovations in Pharmaceutical Technology - Breath Diagnostics:

Breath Analysis Products and Services Overview:

ReCIVA Breath Sampler flyer:

ReCIVA Breath Sampler FAQs:
https://www.owlstonemedical.com/products/reciva-faq/

Application Note comparing Breath Biopsy Cartridges and Tedlar® bags:

Lonestar VOC Analyzer brochure:
https://www.owlstonemedical.com/media/uploads/files/Lonestar_Medical_Brochure_3_lTyi7e.pdf

Whitepaper: Headspace sampling:

Ebook: Simulating human breath: The complete guide to generating VOC and humidity calibration standards:

FAIMS microchip chemical sensor technology:
https://www.owlstonemedical.com/science-technology/faims-technology/

LuCID Clinical Trial for early detection of lung cancer:
www.owlstonemedical.com/clinical-pipeline/lucid/

LuCID Infographic:
References


Our mission:
to save 100,000 lives and $1.5B in healthcare costs.

Our vision:
to be the global leader in Breath Biopsy for early disease detection and precision medicine.

Owlstone Medical has developed a unique breathalyzer for disease. The company’s vision is to become the global leader in non-invasive breath tests for early disease detection and precision medicine across cancer, inflammatory disease and infectious disease with the aim of saving 100,000 lives and $1.5 billion in healthcare costs.

Owlstone Medical’s Breath Biopsy® platform is creating a new industry category, based on the routine detection and analysis of volatile organic compound (VOC) biomarkers in breath, which has the potential to revolutionize healthcare. The award winning ReCIVA Breath Sampler is the first standardized breath collection device designed to capture the VOC biomarkers present in breath, which are then analyzed using Owlstone Medical’s Breath Biopsy services and products.

Owlstone Medical has an active clinical pipeline and is currently developing tests for lung and colorectal cancer, two of the most common cancer killers worldwide, and for asthma stratification by therapeutic response. Owlstone Medical also offers Breath Biopsy® products and services to academic, clinical and pharma partners who want to develop breath-based diagnostics for their own applications.

Keep up to date with Breath Biopsy®
Join the LinkedIn Group

linkedin.com/groups/8594987
OWLSTONE MEDICAL LTD

Cambridge, UK
183 Cambridge Science Park
Milton Road
Cambridge CB4 0GA

T: +44 (0)1223 428200
F: +44 (0)1223 428201

London, UK
Unit 19
205 Richmond Road
London E8 3FF

T: +44 (0)2036 385656

www.owlstonemedical.com