WHITE PAPER

Breath Biopsy® OMNI®: Advanced Global Breath VOC Analysis

BREATH® BIOPSY OMNI®
Owlstone Medical
Novel Insights

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About Breath Biopsy OMNI

Keywords: Breath Biopsy, VOC analysis, non-invasive, biomarkers, volatile organic compounds, GC-MS

Abstract

Breath Biopsy OMNI is the most advanced service for global analysis of volatile organic compounds (VOCs) on breath. Developed and optimized to maximize compound detection, OMNI seeks to facilitate the discovery and validation of breath biomarkers by enabling sensitive, reliable and reproducible detection of VOCs across a broad dynamic range. OMNI employs unique methods to assess variability between samples including the exclusion of background signals originating from ambient VOCs.

OMNI is a complete end-to-end solution incorporating expert support for study design, data analysis and interpretation as well as specialist technology for breath collection and analysis. Samples are collected using the Breath Biopsy Collection Station consisting of ReCIVA Breath Sampler, CASPER Portable Air Supply and the Breath Biopsy Collect Software. Together these devices learn patient breathing patterns and selectively collect VOCs from specific breath fractions while also collecting real-time monitoring data and excluding key sources of ambient background VOCs.

Analysis is performed using high resolution accurate mass (HRAM) mass spectrometry, enabling high compound resolution and detection. The resulting data is reviewed, processed and analyzed by our experienced, specialist team of biostatisticians and translational biologists to identify relevant signals and provide detailed biological interpretation helping to relate your results to relevant pathophysiology.

As a demonstration of OMNI's capabilities, we show how it is being used to investigate chronic liver diseases and share results highlighting the consistent detection of limonene and other compounds as indicators of cirrhosis.

Key Points

- OMNI is the most advanced global breath VOC analysis service
- It is a complete solution for breath collection and analysis including expert study design and reporting, as well as collection and analysis technologies
- It can support research aiming to characterize disease endotypes and therapeutic responses for early detection, precision medicine and drug development
- Data are high confidence, reliable and reproducible

Introduction

Healthcare systems worldwide are in critical need of better ways to detect, monitor and treat diseases. Year on year, a growing and aging global population means that more people are becoming ill, placing increasing strain on limited healthcare resources. Early detection and precision medicine have emerged as two prominent areas that have great potential to save lives and reduce costs by improving the efficiency of how we diagnose and treat illnesses.

Achieving these improvements is dependent on finding reliable biomarkers that can be conclusively related to disease pathophysiology and that can be accurately detected through suitable testing methods. Over recent years, volatile organic compounds (VOCs) on breath have attracted growing interest as a promising biomarker source that may be relevant for a wide range of clinical applications and that offer several advantages compared to more standard clinical methods.

Advantages of Breath Testing

Over 1,000 different VOCs have been detected in human breath and these have both endogenous and exogenous origins (Figure 1). Endogenous VOCs are typically the

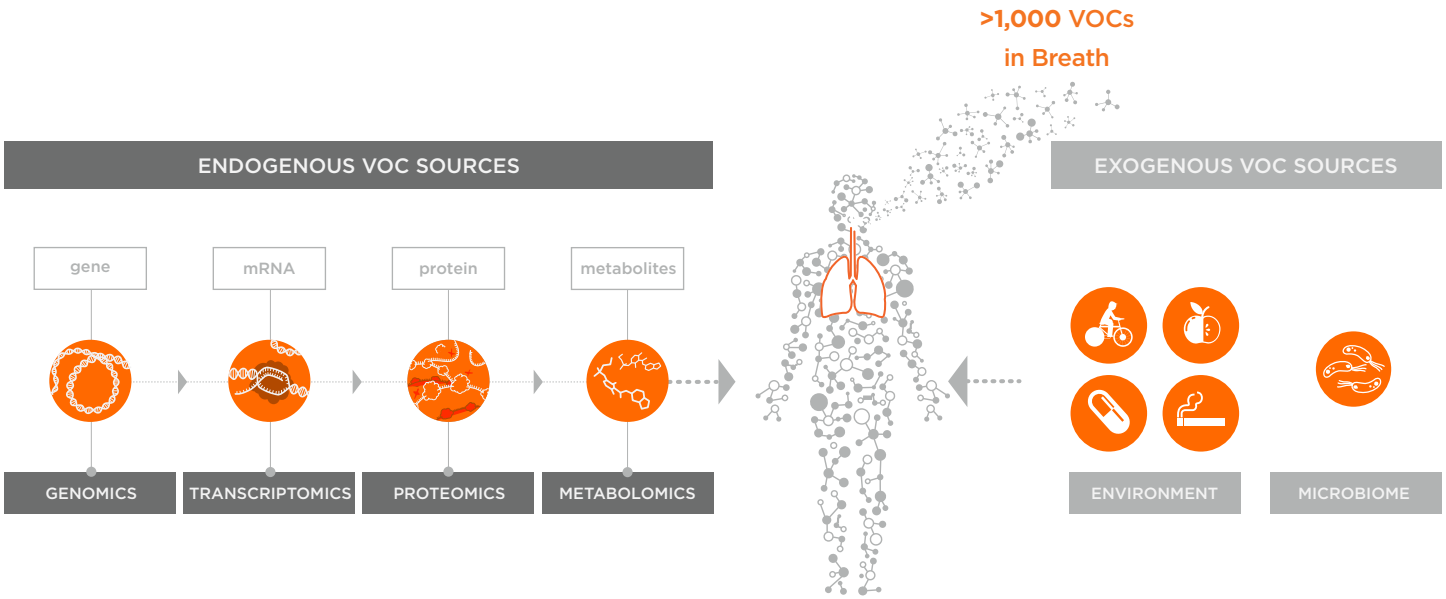

Figure 1. Volatile organic compounds (VOCs) from endogenous and exogenous sources can be detected on breath. VOCs produced by metabolic processes all over the body are carried to the lungs by the blood. Detecting VOCs on breath can reveal metabolic changes associated with a wide range of illnesses and is suited to early detection and identification of phenotypes that can guide precision medicine. The challenge is to develop a high sensitivity, reproducible approach for VOC analysis that can help to identify and validate prospective VOC biomarkers with clinical relevance.

product of metabolic processes and, as such, changes in their abundance could reflect a wide range of biological changes including the onset and progression of disease. Other VOCs are exogenous and arise on breath as a result of e.g. dietary intake, inhalation or even via the skin. Some exogenous VOCs are detectable directly in breath or are metabolized into products that are suitable for breath monitoring.

Breath can contain VOCs which arise throughout the body. VOCs are typically small molecules which can be carried in the blood and exchange readily into air in the lungs. As such, biomarkers relevant to illness anywhere in the body could be detectable on breath. Unlike tissue biopsy, this means that breath can provide a much more comprehensive overview of health throughout the body and not just in a small sample of cells.

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The most notable advantage of breath testing is that collection can be completely non-invasive, which makes it pain-free, easy to use and well tolerated by patients (Figure 2) [1]. Furthermore, collection devices, such as the Breath Biopsy ReCIVA® Breath Sampler, collect breath during regular tidal breathing which means samples can be collected from a wide range of patients, even those with breathing difficulties or reduced lung function. Unlike other sampling matrices such as blood or urine, breath has a virtually unlimited supply, which means it is compatible with high frequency serial sampling and longitudinal monitoring.

Making Progress Toward Breath Biomarkers

Studies have been published investigating the potential of breath biomarkers in a range of contexts including cancers, respiratory diseases, liver diseases, neurological conditions, gastrointestinal disorders and many more. While the majority successfully report candidate biomarkers, few have progressed these beyond the stage of small size pilot studies.

The lack of evidence to support progressing biomarkers further is largely due to the diversity of methods used and types of results reported within the field. Making progress depends on finding an approach to breath analysis that produces consistent, reliable and reproducible results. Additionally, while many studies report tentative identities for detected VOCs based on library matching, few validate these assignments. Creating a system that can progress breath research into

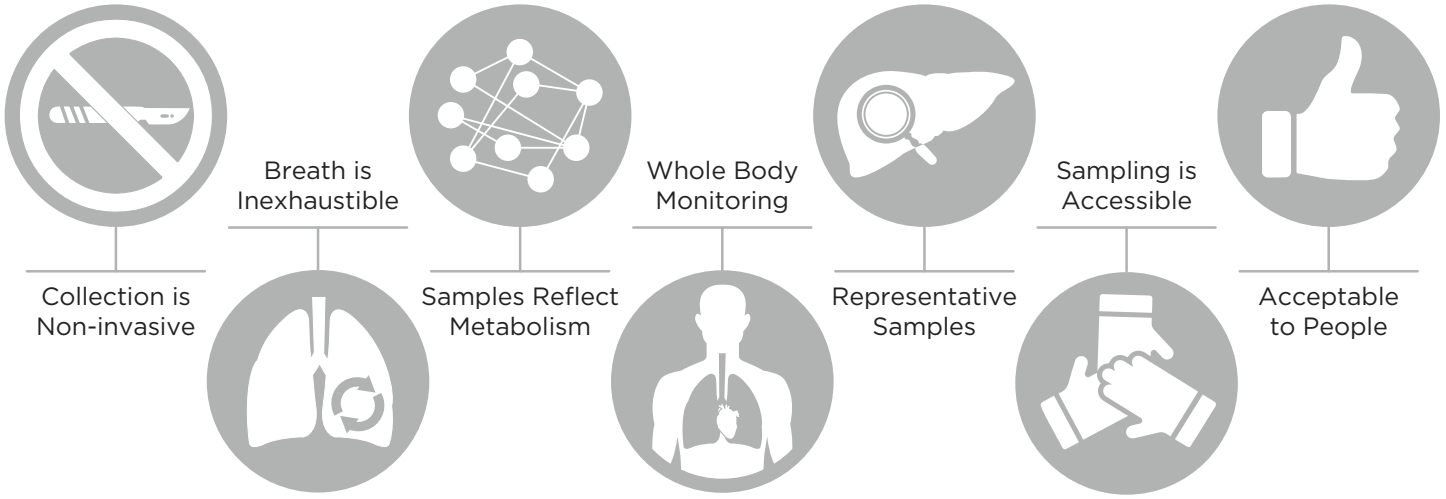

Figure 2. Distinguishing features of breath analysis relevant to developing clinical tests to guide disease diagnosis and treatment.

large-scale validation studies requires advances across all stages of sample collection and analysis.

As a gas, breath samples can be particularly challenging to collect and, relatively speaking, include a high level of noise due to VOCs present in inhaled air. To date, the majority of breath collection systems either do not seek to control for variable background contamination or rely upon restrictive and expensive clean air supplies to minimize ambient contamination. ReCIVA was developed with leading breath researchers, aiming to minimize contamination from the device itself while also standardizing sample collection. It works in conjunction with the CASPER® Portable Air Supply to exclude contaminants while remaining adaptable to all kinds of clinical trial and academic sampling needs.

For analysis, methods based on gas chromatography mass spectrometry (GC-MS) are the recognized gold standard, largely because they enable the detection of diverse, chemically distinct compounds within complex mixtures with high sensitivity and across a large dynamic range. Additionally, GC-MS analysis permits accurate quantitation of VOCs and can be used to confirm biomarker identities with high confidence.

WHAT'S THE BEST METHOD FOR BREATH ANALYSIS?:
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Accurate biomarker identification is a critical stage in the process of developing clinically viable breath tests. Knowing the identity of biomarkers can be used to demonstrate how they relate to disease processes, therefore providing biological evidence to support their use in disease detection and monitoring. Notably, this is relevant when seeking regulatory approval. Furthermore, knowing the properties of relevant biomarkers allows clinical detection devices to be designed and optimized to be specific and sensitive to relevant compounds. Finally, identifying biomarkers allows the use of standards to calibrate detection systems ensuring consistency across devices and avoiding analytical drift over time.

Breath Biopsy OMNI provides an end-to-end pipeline for robust collection and global analysis of VOCs on breath. Based on high resolution accurate mass (HRAM) GC-MS technology, the system has compound detection capabilities across six orders of magnitude and with sensitivity as low as parts per trillion. Here we present an overview of the OMNI system and demonstrate some of its capabilities and a few example applications.

Method

OMNI supports the full study process including reliable capture and analysis of VOCs from human exhaled breath (Figure 3).

Breath is collected using ReCIVA Breath Sampler, a unique standardized reliable and reproducible breath sampling device, allowing sampling of selected fractions of exhaled breath. ReCIVA operates in partnership with CASPER Portable Air Supply, which provides low background inhaled air during sampling, minimizing environmental impact and reducing sample variability caused by ambient VOC signals. Controlled by Breath Biopsy Collect software, ReCIVA captures exhaled VOCs onto adsorbent tubes, allowing easy storage and transport of samples for analysis. Samples are sent to our Breath Biopsy Laboratory and thoroughly dry purged to remove excess water from the tubes prior to analysis.

After recovering the VOCs from the tubes using thermal desorption, the sample is then separated using gas chromatography (GC) and analysed with HRAM MS. The chromatographic and spectrometric data is processed using a tailor-made feature extraction and data processing workflow, allowing statistical analysis and biological interpretation of the study results.

Distinguishing Features of Breath Biopsy OMNI

Study design

To answer key scientific and clinical questions, the design of the study should take into account several important features, including sample size, inclusion and exclusion criteria, choice of the most relevant control group(s) and a deep understanding of potential confounders. Our clinical and translational scientists help to address all of these considerations, providing support from idea generation, through to study design and clinical protocol writing.

QUESTIONS TO ASK DURING STUDY DESIGN:
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Power calculations

Power calculations help to estimate the statistical power of your study design in relation to desired clinical performance. Our biostatisticians offer advice on minimum sample sizes needed to reliably identify

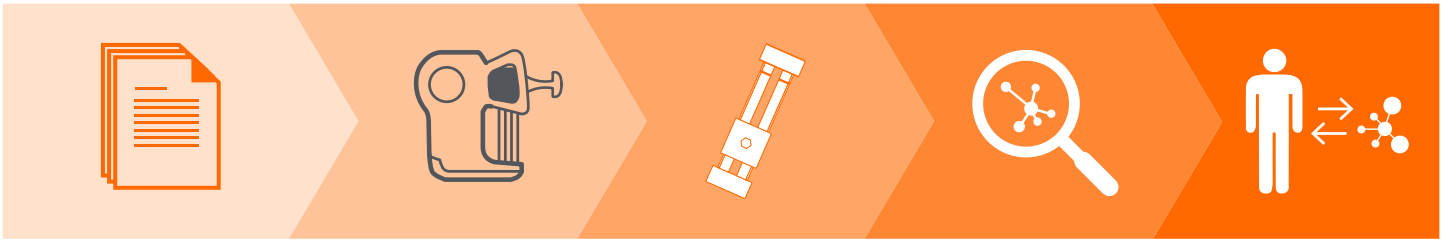

Figure 3. Breath Biopsy OMNI is a complete, end-to-end solution for global breath VOC analysis. In addition to an award-winning collection system and robust HRAM GC-MS analysis capabilities we include expert support for study design and management, statistical analysis and biological interpretation. We have several reporting and analysis options for you to choose from.

meaningful changes in the abundance of exhaled VOCs and can help to assess the expected variability within populations of interest.

Sample collection

Breath samples are obtained during regular tidal breathing using ReCIVA, making continuous sample collection convenient and comfortable for most subjects, including those with breathing difficulties or reduced lung function (Figure 4). While the subject is breathing normally through the mouthpiece, software controlled pumps within the device selectively sample fractions of the exhaled breath, adsorbing VOCs onto 1/4" by 3 1/2" inert-coated stainless-steel tubes containing the sorbent materials Tenax TA/carbograph 5TD. Pumps are regulated in response to pressure sensors that learn and respond to a subject's breathing pattern. Each sample includes four separate tubes, typically pairs of tubes are pooled to increase sensitivity.

On average, OMNI breath sampling takes around 15 mins. During sampling, inhaled air is provided via CASPER, which removes over 90% of ambient VOC content, minimizing contamination and consequently reducing the risk of false discovery. Our optimized collection method involves collecting VOCs from 1250ml of exhaled air from the lower breath fraction (dead space excluded). This combination of the volume and fraction provides the best signal to background ratio of on breath VOCs and reduces the measured variability. While these are our optimized settings, they can be adjusted for specific applications. For example, higher breath fractions may be more suitable to studies of oral diseases.

Blank sample collection

To assess the undesired sources of VOCs, representative background samples (process blanks) are also collected with ReCIVA. These blank samples contain air representative of inhaled air from the sampling pathway, which is captured directly onto the sorbent tubes. The sampled air still passes through CASPER and ReCIVA as normal. This ensures that each blank sample includes all possible sources of VOCs other than breath, making it possible to assess signal to background for each detected VOC.

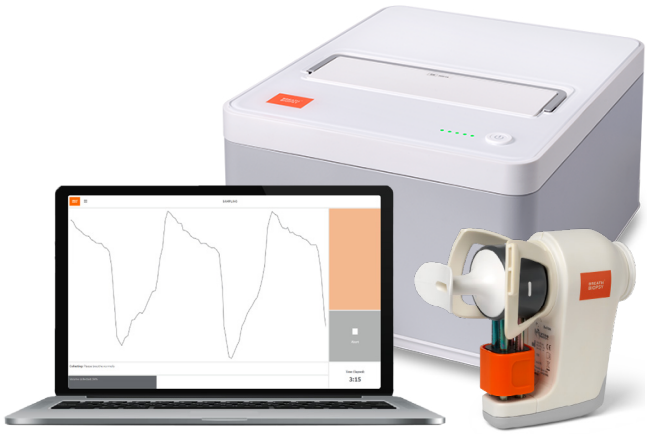

Figure 4. The Breath Biopsy Collection Station. VOCs are captured during tidal breathing by ReCIVA Breath Sampler (Right), CASPER Portable Air Supply (Top) reduces background signals and the Breath Biopsy Collect software controls and monitors the system during breath collection.

Quality assurance

Sample integrity and resulting analysis is monitored and assessed at all stages to assure reliable results.

- Sorbent tubes undergo a thorough purging and reconditioning process between uses. In each cycle, a subset of tubes are analyzed to ensure suitable reconditioning. All tubes are also tracked throughout their lifetime to avoid contamination caused by carryover between studies.

- The consistency of the sampling process is carefully monitored to assure collection of the desired volume, pump functionality and correct breath fraction selection. For some issues, the software can detect and provide on-screen error reports for users. We perform other checks in the Breath Biopsy Laboratory and report these through our Clinical Study Delivery team.

- Following dry purge, samples are stored in tubes with brass end-caps to limit sample degradation during long-term storage. Samples are batch analyzed to minimize the impact of analytical variation on results.

- All samples are injected with eight internal standards, which are deuterated to ensure that they are unique and do not mask compounds from the sample itself. Standards allow each sample to undergo individual quality control to detect any analytical issues as well as to assess overall analytical performance.

- 52 calibration curves on compounds relevant to breath are generated in each analytical sequence to allow monitoring of GC-MS device performance.

- Our expert biostatisticians and translational biologists perform a range of quality control checks during data analysis and reporting.

Analysis with HRAM TD-GC-MS

During analysis, VOCs are first recovered from tubes using a thermal desorption system to be focused onto a cold trap. The cryofocused analytes are then desorbed and purged by helium into the GC column (TraceGOLD TG-624SilMS; Thermo Fisher Scientific), for separation of the analytes in the matrix. Mass detection is performed using a HRAM Q Exactive™ GC Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific)

VOC identification

Detected compounds can be assigned identities on the basis of matching to libraries of standard compounds. For some VOCs, mass spectra and retention times can be compared to the Breath Biopsy HRAM Library, which has been developed using the same analysis technology, so offers a more reliable comparison to OMNI data. Where IDs cannot be assigned from the HRAM Library, tentative assignments can be made from the NIST Standard Reference Database. Definitive assignment of compound identities and absolute quantitation for the most promising biomarker candidates can be explored as additional tasks using reference standards.

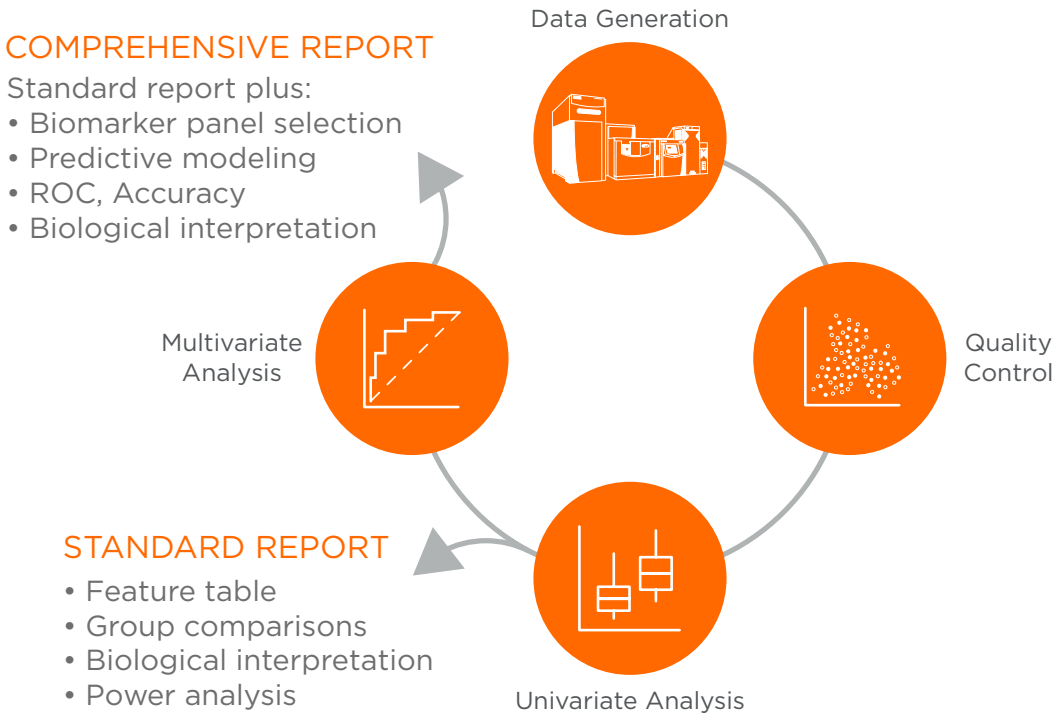

Figure 5. Breath Biopsy OMNI analysis and reporting options. Standard reporting includes univariate analysis with associated analysis. This can be further expanded with multivariate analysis for comprehensive reporting. Choose the option that best suits the goals of your study and to complement your in-house capabilities.

Options for Deliverables/Reporting

Depending on the overall scope of your study, available in-house resources, and the specifics of your study design, we can offer a range of reporting options for your results (Figure 5). This can vary from a custom univariate and multivariate analysis with biological interpretations.

Prior to analysis, the OMNI feature extraction process translates raw GC-MS data into descriptions of compounds detected in each sample that is available for your own statistical analysis, data interpretation and identification of potential biomarkers. Feature extraction for OMNI is a custom process developed in house to increase the quality and reliability of the results and to support more accurate interpretation. Feature extraction produces a feature table, included in our reports, containing a list of all detected VOCs and their relative quantities in each of the samples when present.

1. Standard report

To answer the scientific and clinical questions of the study, a bespoke statistical analysis can be performed. Univariate analysis describes the potential of individual VOCs to differentiate between study groups. We can then support this with biological interpretations highlighting reported associations between identified VOCs and relevant pathophsyiological processes.

2. Comprehensive report

Additional multivariate analysis of VOCs and pattern recognition models can be applied to assess feasibility of VOC combinations as a composite biomarker.

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Results & Case Studies

Designed for Reproducibility and Consistency

OMNI was developed to allow reliable, broad-ranging investigation of exhaled breath VOCs, increasing the chances to find clinically valuable biomarkers. OMNI's capabilities and performance are measured based on two key metrics, maximized detection and reduced analytical variability. Specifically, we aimed to maximize the number of detectable VOCs on breath and to minimize the introduced variability. These goals help to achieve reliable assessment of differences in VOCs that are most likely to be biologically relevant.

To enable this, we perform breath or blank (BoB) studies, a relatively simple study design intended to provide a standardized scientific approach to breath research that can be used in conjunction with any breath collection and analysis workflow. Irrespective of approach, BoB studies enable comparable reporting regarding the number of detectable on breath VOCs and can be used to assess intra and inter subject variabilities.

FIND OUT MORE ABOUT BoB STUDIES:
owlstonemedical.com/bob-studies

The hallmark of a BoB study is the inclusion of sufficient representative blank samples alongside breath samples, typically involving repeat sampling from a small number of healthy volunteers. These blanks (ranging from breath: blank ratio of 5:1 to 3:1) would allow the assessment of all undesired sources of VOCs including from the collection and analysis devices themselves and the ambient environment during collection. This comparison of breath vs. blanks allows the discrimination of VOCs originating from breath (i.e. on breath) from other VOCs that are likely to be contaminants ensuring that only relevant VOCs are included for detailed analysis.

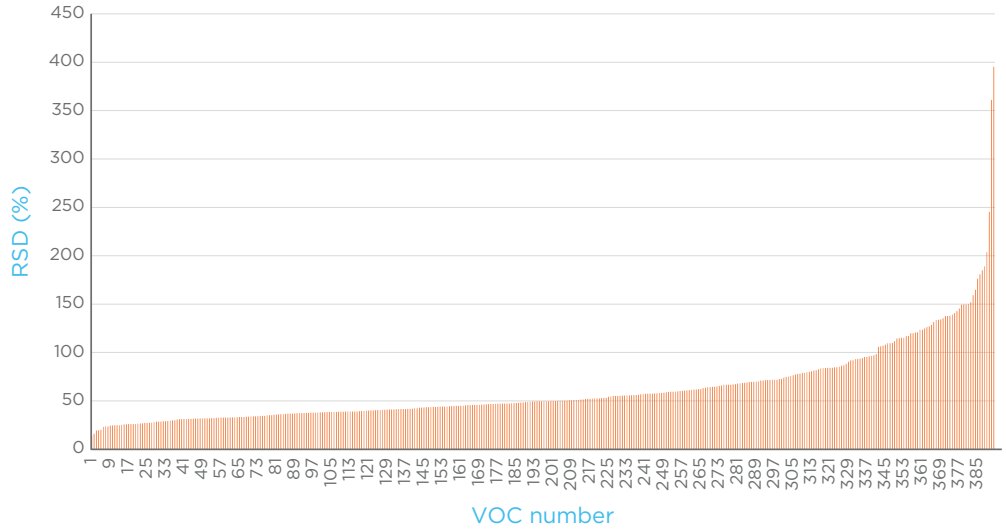

Figure 6. Breath Biopsy OMNI is optimized to provide consistent, reproducible results for breath analysis. In trials involving four healthy volunteers, the median intrasubject RSD for on breath VOCs was 26-36%. Across all samples, the median intersubject RSD was 49% across 392 VOCs.

Standard	RSD(%)
IS1	9.7%
IS2	13.0%
IS3	9.3%
IS4	10.3%
IS5	7.5%
IS6	5.2%
IS7	3.6%
IS8	3.4%

Table 1. RSD for the eight internal standards (IS1-8) included for quality control in all Breath Biopsy samples.

On breath VOCs are defined with high confidence (99.7%) as achieving a signal measured on breath that is at least three standard deviations above the average abundance in blank samples.

In one instance, BoB analysis was applied on 57 breath samples, obtained from four different volunteers, with an equal number of representative blanks. The method detected a median of 1,454 VOCs per sample of which a median of 517 were shown to be on breath.

Relative standard deviation (RSD) was a calculated as a measure of variability between samples (within volunteer) and between volunteers. When calculated for each of the 4 volunteers (intrasubject), the median RSD of on breath VOCs ranged between 26-36%, while when considered as a cohort (intersubject), the median RSD was 49% across 392 on breath VOCs (Figure 6). For the eight internal standards included in each sample, RSD ranged from 3.4% to 13% (Table 1).

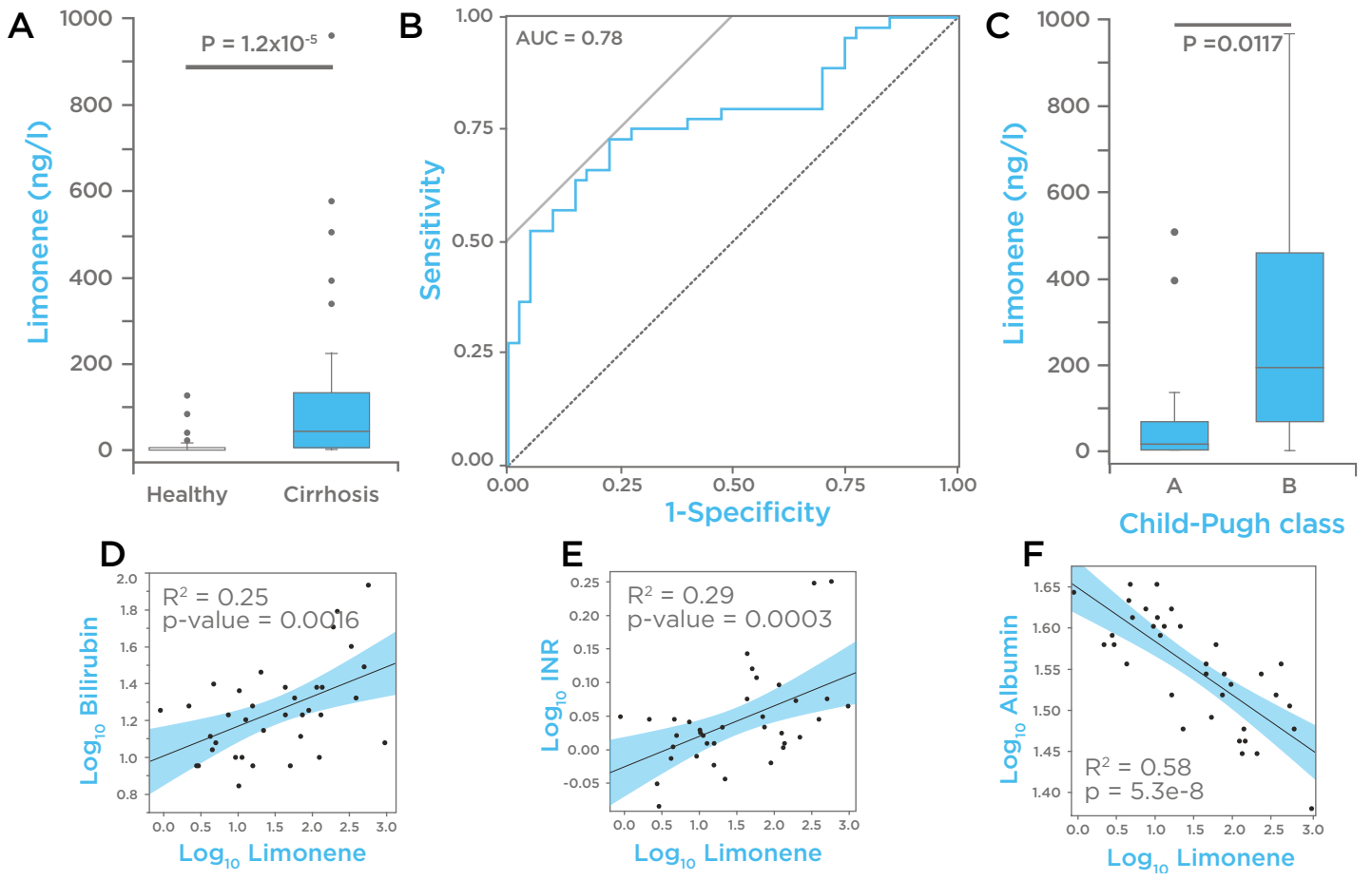

Figure 7. Examining limonene as a breath biomarker for chronic liver disease. Results from a targeted analysis of limonene on breath performed by Ferrandino et al. [3]. (A) Limonene was significantly elevated on the breath of cirrhosis patients compared to healthy controls. (B) Limonene was used to produce a model with AUC=0.78 for cirrhosis detection. (C) Limonene abundance on breath also appeared to reflect disease severity as measured by Child-Pugh class, and (D,E,F) correlated with blood biomarkers of liver function.

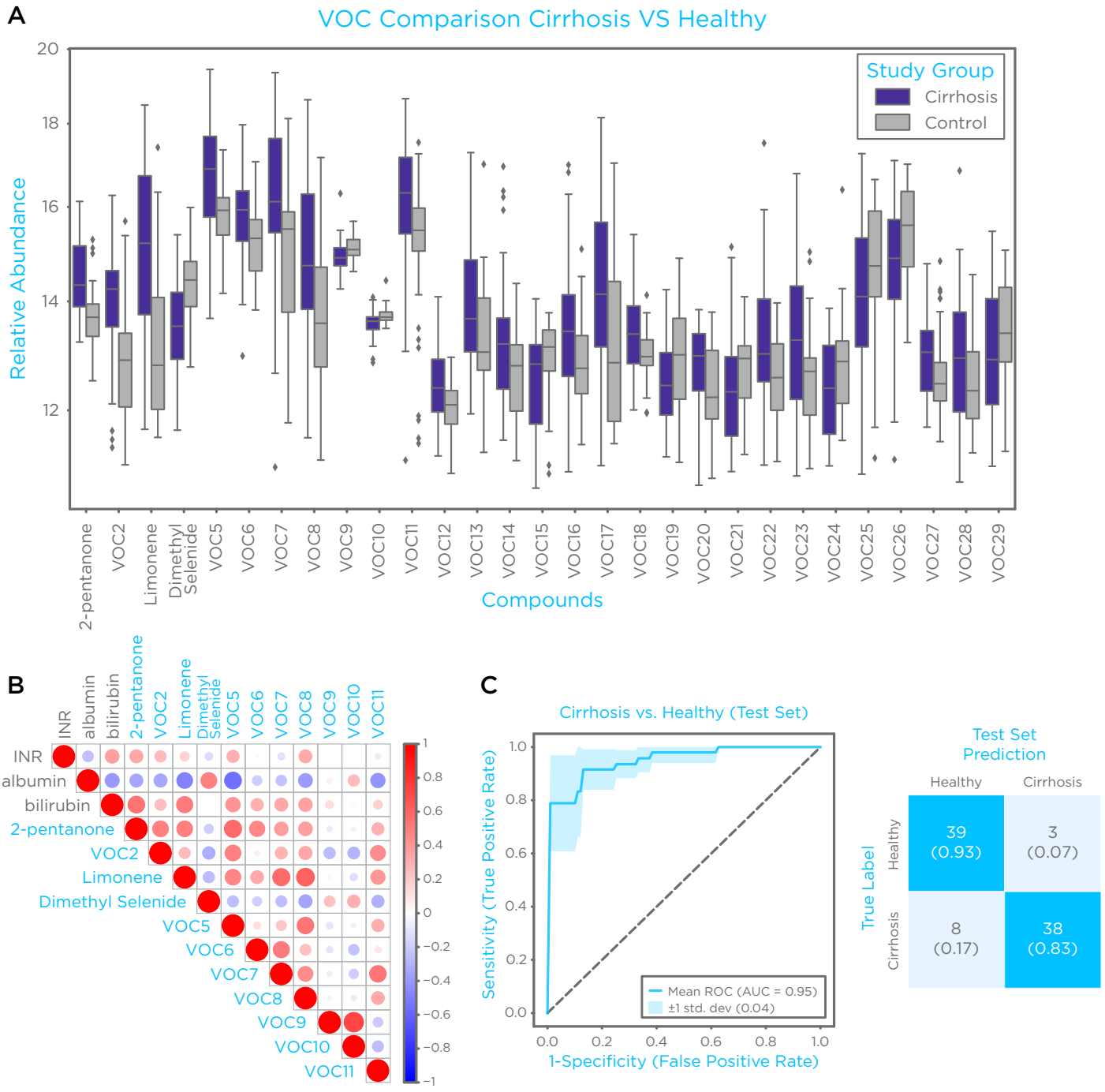

Figure 8. Breath Biopsy OMNI Global VOC Analysis of Cirrhosis vs. Healthy Controls. Paper in production. (A) 29 VOCs on breath identified as significantly different between cirrhosis and healthy controls including limonene and other previously reported compounds (2-pentanone and dimethyl selenide). (B) Correlations between blood biomarkers (INR, albumin and bilirubin) and 11 of the most significant identified VOCs. (C) Models built using measurements of all 29 VOCs applied to a test dataset resulted in a mean AUC of 0.95 with 83% true positive rate and just 7% false positives.

Breath Biopsy in Action – Detecting Chronic Liver Diseases

OMNI represents an evolution of Breath Biopsy, which is being used for a range of applications by researchers worldwide. One of our current projects aims to develop a breath test to detect non-alcoholic steatohepatitis (NASH) an early stage of chronic liver disease. In some countries liver disease is estimated to affect over 30% of adults yet early detection is currently a challenge and the gold standard for detection is an invasive tissue biopsy [2]. Such is the need in this area that several leading pharmaceutical organizations are developing novel therapies for the treatment of NASH.

We have already published a targeted pilot study [3] showing how breath testing targeting the terpene limonene can help to detect cirrhosis as an extreme liver disease phenotype with larger effect sizes. In cirrhosis, limonene correlated with disease severity (Child-Pugh score) and blood-based biomarkers of liver function (bilirubin, albumin, prothrombin time (INR) (Figure 7). We have since extended this work by performing global VOC analysis of breath from 46 people with cirrhosis and 42 healthy controls. The study identified 29 VOCs that differed significantly between groups, of which 11 also correlated with blood biomarkers (Figure 8).

READ OUR CASE STUDY ON FERRANDINO ET AL. (2020):
owlstonemedical.com/liver-case-study

Notably, limonene was identified as one of these 11 compounds validating our targeted work and previous work by others [4-6]. In parallel, a study by Cleveland Clinic using Breath Biopsy looked at cirrhosis alongside

instances of cancer in the liver and also identified related compounds elevated on breath [7]. In particular, two terpene compounds were detected and shown to correlate with another surrogate measure for disease severity (MELD score).

VIEW A RESEARCH POSTER ON OUR WORK WITH CLEVELAND CLINIC:
owlstonemedical.com/cc-poster

This global analysis work demonstrates the capability of Breath Biopsy to identify biorelevant compounds. Limonene and several other VOCs from this study are now being examined further to investigate the potential to administer them as probes for targeted assessment of liver function, known as **EVOC® Probes**.

To explore other applications of Breath Biopsy, visit the Resources section of the Owlstone Medical website.

Conclusion

There is a critical need for clinically-relevant breath biomarkers to support improved early detection and precision medicine across a wide range of medical specialisms. Breath represents a rich source of potential biomarkers that offers additional benefits in terms of ease of use, patient comfort and potential for testing in home or primary care settings.

Previously, development of breath tests has been limited by a lack of standardized methods and result reporting. This has resulted in many proposed biomarkers but few that have successfully advanced through validation. It is as a result of this observation that Breath Biopsy OMNI has been developed, in consultation with leading researchers and with an expert method development team, to provide a complete end-to-end solution for breath collection and analysis that maximizes reliable detection while minimizing sources of variability.

In the process, we have developed a method-independent study design that can be used to assess and quantify key aspects of method performance through the incorporation of relevant blanks. This has been applied to inform all stages of development and continues to be used to enable incremental improvements and evolution of the method based on the latest data and technical capabilities.

OMNI is available as comprehensive global breath VOC analysis service through Owlstone Medical. Whatever application you are seeking biomarkers for, contact us to discuss integrating Breath Biopsy OMNI into your work and to discuss your study design with our expert team.

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Add Breath Biopsy OMNI to your Research

OMNI is available worldwide for clinical trials and academic research applications. If you are interested in finding non-invasive biomarkers for applications including early detection, precision medicine or drug development, get in touch to discuss studies with our specialist team.

To get started, scan the QR code to visit the OMNI webpage and find out more about its features and capabilities.

Contact us to find out more about breath biomarkers relevant to your area of interest and to discuss adding Breath Biopsy to your research.

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