Targeted breath analysis: exogenous volatile organic compounds (EVOC) as targeted metabolic probes in Breath Biopsy
Breath Biopsy® - Reshaping healthcare in the 21st Century

OUR MISSION:
TO SAVE 100,000 LIVES & 1.5B IN HEALTHCARE COSTS.

OUR VISION:
THE GLOBAL LEADER IN BREATH BIOPSY FOR EARLY DETECTION AND PRECISION MEDICINE.

APPLICATIONS
EARLY DETECTION AND DIAGNOSTICS
PRECISION MEDICINE

TARGETING BILLION DOLLAR MARKETS IN LUNG CANCER, COLON CANCER AND RESPIRATORY DISEASE
What are VOCs? Endogenous and Exogenous

ENDOGENOUS VOC SOURCES

- gene
- mRNA
- protein
- metabolites

GENOMICS | TRANSCRIPTOMICS | PROTEOMICS | METABOLOMICS

EXOGENOUS VOC SOURCES

- Environment
- Microbiome
Breath Biopsy: Whole Body Metabolome Sampling

- Collects VOCs originating from airways tissues and blood - providing both local and systemic disease information
- Unparalleled sensitivity for the detection of disease biomarkers in breath
Where are we in “Breathomics”? 

• 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

• Maturity of breath sampling hardware and protocols for robust, repeatable sampling.

• High end, expensive spectrometers 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
Collect samples anywhere and analyze in central lab or near patient

- ReCIVA Breath Sampler for reproducible collection of specific breath fractions
- Breath Biopsy Cartridge captures every VOC from breath and can be shipped without special handling
- Rapid, sensitive and selective VOC analysis based on proven FAIMS sensor technology
- Analysis in central lab or at point of care
In development Breath Biopsy hardware - ReCIVA Breath Sampler, CASPER Air Supply, Breath Biopsy kits, mobile sample collection station
Breath Biopsy Products and Services

Lease ReCIVA Breath Sampler and purchase Breath Biopsy Kits to collect breath samples.

Send Breath Biopsy Cartridges for analysis using Owlstone Medical’s Breath Biopsy Services.

Analyze Breath Biopsy Cartridges in your own lab with the Lonestar VOC Analyzer.
Breath Biopsy Discovery Methodology

SELECT PATIENTS → BREATH SAMPLING → GC-MS ANALYSIS → BUILD FEATURE TABLE

EVALUATE CLASSIFIER → TEST CLASSIFIER → TRAIN CLASSIFIER
Volcano Plot: significant features and fold change

A Volcano plot enables quick visual identification of those features that display large-magnitude fold changes between classes, that are also statistically significant. There are two regions of interest in the volcano plot: those points that are found in the top-left or top-right areas of the plot represent features that display large magnitude fold changes as well as high statistical significance (log$_{10}$ p-value based on Wilcoxon rank sum). Statistical significance can be determined using either Benjamini-Hochberg, or by the calculated Bonferroni correction (0.05 divided by the number of features, in this case 0.05 / 475 = 0.000105). Both cut-offs are shown as horizontal lines in the volcano plot. Features that with a negative fold change between the classes (reduced in Non-smokers compared to Smokers), and significant p-values above the Benjamini-Hochberg cut-off are shown in green. Green dots are features that with a positive fold change between the classes (increased in Non-smokers compared to Smokers), and significant p-values above the Benjamini-Hochberg cut-off.

<table>
<thead>
<tr>
<th>Feature</th>
<th>log2 fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMF58</td>
<td>-2.502</td>
<td>2.57E-20</td>
</tr>
<tr>
<td>RMF57</td>
<td>-1.117</td>
<td>4.79E-15</td>
</tr>
<tr>
<td>RMF38</td>
<td>-2.587</td>
<td>3.32E-14</td>
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<tr>
<td>RMF44</td>
<td>-4.012</td>
<td>1.24E-11</td>
</tr>
<tr>
<td>RMF53</td>
<td>-4.130</td>
<td>1.49E-11</td>
</tr>
<tr>
<td>RMF115</td>
<td>-2.045</td>
<td>3.77E-11</td>
</tr>
<tr>
<td>RMF19</td>
<td>-2.135</td>
<td>3.81E-11</td>
</tr>
<tr>
<td>RMF113</td>
<td>-2.325</td>
<td>5.92E-11</td>
</tr>
<tr>
<td>RMF124</td>
<td>-2.437</td>
<td>2.79E-10</td>
</tr>
<tr>
<td>RMF93</td>
<td>-6.096</td>
<td>6.66E-10</td>
</tr>
<tr>
<td>RMF63</td>
<td>-4.892</td>
<td>1.19E-08</td>
</tr>
<tr>
<td>RMF86</td>
<td>-1.072</td>
<td>4.88E-08</td>
</tr>
<tr>
<td>RMF75</td>
<td>-4.852</td>
<td>6.06E-08</td>
</tr>
<tr>
<td>RMF9</td>
<td>-1.328</td>
<td>1.26E-07</td>
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<td>RMF125</td>
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<td>RMF142</td>
<td>-1.865</td>
<td>6.06E-07</td>
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<td>RMF162</td>
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<td>1.08E-06</td>
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<td>RMF21</td>
<td>-1.507</td>
<td>2.91E-06</td>
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<td>RMF31</td>
<td>-1.246</td>
<td>2.95E-06</td>
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<tr>
<td>RMF158</td>
<td>-1.763</td>
<td>3.15E-06</td>
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<tr>
<td>RMF126</td>
<td>-2.901</td>
<td>5.01E-06</td>
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<td>RMF146</td>
<td>-3.275</td>
<td>1.01E-05</td>
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<td>RMF153</td>
<td>-1.818</td>
<td>1.17E-05</td>
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<tr>
<td>RMF123</td>
<td>-1.606</td>
<td>2.25E-05</td>
</tr>
<tr>
<td>RMF43</td>
<td>-3.641</td>
<td>4.47E-05</td>
</tr>
</tbody>
</table>
Box Plot: Feature RMF58

On the volcano plot (left), red dots are features that with a negative fold change between the classes (reduced in Non-smokers compared to Smokers), and significant p-values above the Benjamini-Hochberg cut-off. Green dots are features that with a positive fold change between the classes (increased in Non-smokers compared to Smokers), and significant p-values above the Benjamini-Hochberg cut-off. See [here](#) for an explanation of the volcano plot. The red dot with black outline represents feature RMF58.

The box plot (upper right) shows the distribution of peak area measured for feature RMF58 in non-smokers vs. smokers. See [here](#) for an explanation of the box plot.

The table shows $p$-value, log$_2$ fold change between classes (non-smokers vs. smokers) and Tentative ID for feature RMF58. Negative fold changes are highlighted in red, positive fold changes in green. Statistically significant $p$-values below the Benjamini-Hochberg cut-off are shown in yellow. Measured spectra are compared against the NIST unit mass spectral library in order to assign a tentative ID to each feature. Please note the tentative ID is likely to be inaccurate, so additional structural elucidation is required to confirm compound identity (available, subject to additional fee).
On the volcano plot (left), red dots are features that with a negative fold change between the classes (reduced in Non-smokers compared to Smokers), and significant p-values above the Benjamini-Hochberg cut-off. Green dots are features that with a positive fold change between the classes (increased in Non-smokers compared to Smokers), and significant p-values above the Benjamini-Hochberg cut-off. See here for an explanation of the volcano plot. The red dot with black outline represents feature RMF57.

The box plot (upper right) shows the distribution of peak area measured for feature RMF57 in non-smokers vs. smokers. See here for an explanation of the box plot.

The table shows p-value, log2 fold change between classes (non-smokers vs. smokers) and Tentative ID for feature RMF57. Negative fold changes are highlighted in red, positive fold changes in green. Statistically significant p-values below the Benjamini-Hochberg cut-off are shown in yellow. Measured spectra are compared against the NIST unit mass spectral library in order to assign a tentative ID to each feature. Please note the tentative ID is likely to be inaccurate, so additional structural elucidation is required to confirm compound identity (available, subject to additional fee).
The box plots above visualize the distribution of the peak area of the top 25 features. The top 25 features were selected based on the p-value of the fold change between classes, non-smokers vs. smokers. The horizontal line within the box represents the median peak area of the feature. The box displays the upper and lower quartiles of the data, while the error bars represent the maximum and minimum peak areas (excluding outliers). Outliers are represented by dots.
Random Forest is a non-supervised machine learning algorithm used for classifier building. K fold validation is a method of cross-validation used to test the model's ability to predict new data that was not used in estimating it, in order to flag problems like overfitting or selection bias, and to give an insight on how the model will generalize to an independent dataset. Multiple rounds of cross-validation are performed using different partitions, and the validation results are combined over the folds to give an estimate of the model's predictive performance.

The ROC curve shows the trade-off between sensitivity (or True Positive Rate, TPR) and specificity (1 – False Positive Rate, FPR). Classifiers that give curves closer to the top-left corner indicate a better performance. As a baseline, a random classifier is expected to give points lying along the diagonal (FPR = TPR). The closer the curve comes to the 45-degree diagonal of the ROC space, the less accurate the test.
Random Forest: Model Evaluation

Confusion matrix describing the performance of the Random Forest Classification Model to classify the samples (top). The figures describe the prediction probabilities of individual samples (bottom left), and box plots of each class (bottom right). Dashed line represents the Non-smoker threshold (0.5).
Enabling a broad range of applications across cancer, inflammatory and infectious disease

<table>
<thead>
<tr>
<th>Application</th>
<th>Patient Numbers</th>
<th>Sens./Spec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>N=133; 63 colon; 50 controls</td>
<td>88/60</td>
</tr>
<tr>
<td>Pelvic radiation disease</td>
<td>N=23</td>
<td>90/90</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory Bowel</td>
<td>N=62; 48 with IBD</td>
<td>74/88</td>
</tr>
<tr>
<td>Inflammatory Disease (IBD)</td>
<td>N=14 controls</td>
<td>74/75</td>
</tr>
<tr>
<td>IBD</td>
<td>N=76; 54 with IBD and 22 healthy controls</td>
<td></td>
</tr>
<tr>
<td>Coeliac</td>
<td>N=47; 27 with histological confirmation of coeliac disease; 20 controls with irritable bowel syndrome</td>
<td>85/85</td>
</tr>
<tr>
<td>Bile acid diarrhoea</td>
<td>N=110; 23 with bile acid diarrhoea; 42 with ulcerative colitis; 45 symptomatic controls</td>
<td>85/90</td>
</tr>
<tr>
<td>Eosinophilic airway</td>
<td>N=52; 27 with eosinophilia</td>
<td>Accuracy: 85%</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma/COPD</td>
<td>N=78</td>
<td>No reported accuracy</td>
</tr>
<tr>
<td>Infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td>N=213; ~71 with C. diff positive by microbiological analysis</td>
<td>92/86</td>
</tr>
<tr>
<td>Hepatic encephalopathy (HE)</td>
<td>N=42; 22 with HE and 20 healthy controls</td>
<td>88/68</td>
</tr>
</tbody>
</table>

>100 peer reviewed papers and scientific posters - [link](#)
Measurement of liver enzymatic activity using $^{13}\text{C}$-labelled substrate

- $^{13}\text{C}$ labelled substrate is administered (e.g. orally, intravenously) and metabolised by the liver
- Leads to production of $^{13}\text{CO}_2$ secreted through the lungs via breath
Breath tests determine the rate of labeled carbon dioxide to estimate enzyme activity, organ function or presence of disease.

Mitochondrial:
- **1-13C-Methionine**
  - Exclusively metabolised by hepatic mitochondria
  - Concentration of $^{13}$CO$_2$ exhaled correlates with liver disease severity, traces appear within 20 mins.

- **Sodium 1-13C-octanoate**
  - Undergoes liver mitochondrial beta-oxidation
  - Impairment of mitochondrial beta-oxidation has been reported with several liver diseases such as nonalcoholic steatohepatitis (NASH)

Cytosol:
- **L-[1-13C]phenylalanine**
  - Quantitatively monitors the rate of hepatic phenylalanine metabolism
  - Abnormal elevation of the plasma concentrations of the aromatic amino acids, phenylalanine and tyrosine are seen in the liver disease

CYP450:
- **13C-Methacetin**
  - Completed within 60 minutes
  - Undergoes extensive liver first pass clearance
  - Cytochrome P450 IA2 converts methacetin via O-dealkylation to acetaminophen and 1-$^{13}$CO$_2$

- **[13C]Caffeine**
  - Highly specific for P4501A2 isoenzyme activity
  - Samples collected over 60 mins ratio of $^{13}$CO$_2$ to $^{12}$CO$_2$ indicates enzyme activity
Breath Biopsy tests for measuring liver function: $^{13}$C-Methacetin Breath Test (MBT)

- Safe, simple and accurate test for diagnosing chronic liver disease in patients.
- Enables differentiation between different stages of chronic liver diseases.
- MBT can reliably distinguish between early cirrhotic and non-cirrhotic patients with 95% sensitivity and 97% specificity.

**SUMMARY**

Background: The $^{13}$C-Methacetin breath test enables the quantitative evaluation of the cytochrome P450-dependent liver function.

Aims: To find out whether the breath test is feasible in noncirrhotic patients with chronic hepatitis C in the early stages of fibrosis.

Methods: Sixty-one healthy controls and 81 patients with chronic hepatitis C underwent a $^{13}$C-Methacetin breath test. In all patients, liver biopsy was performed. The liver histology was classified according to the histology activity index (HAI).

Results: Delta over baseline values of the patients at 15 min significantly differed from controls ($-0.2 \pm 0.7 \text{ vs.} -0.3 \pm 0.8 \text{ mmol/mL, P < 0.05}$). The cumulative recovery after 30 min in patients was $11.4 \pm 4.8\%$ and in healthy controls $13.8 \pm 2.8\% (P < 0.002)$.

Conclusions: The noninvasive $^{13}$C-Methacetin breath test reliably distinguishes between early cirrhotic (Child A) and noncirrhotic patients, but fails to detect early stages of fibrosis in patients with chronic hepatitis C.
Measurement of liver enzymatic activity using $^{13}\text{C}$-labelled substrate

- $^{13}\text{C}$ labelled substrate is administered (e.g. orally, intravenously) and metabolised by the liver
- Leads to production of $^{13}\text{CO}_2$ secreted through the lungs via breath

+ Powerful strategy for assessing metabolic phenotypes / organ function
- Test is **invasive** if intravenous administration required, and needs to take place in clinic
- **Cannot administer multiple probes** - only one enzyme can be assessed at a time
- Labelled isotope probes **require regulatory approval**
- Labelled isotope is **very expensive**
Exogenous VOCs on breath

ENDOGENOUS VOC SOURCES

- gene
- mRNA
- protein
- metabolites

GENOMICS
TRANSCRIPTOMICS
PROTEOMICS
METABOLOMICS

EXOGENOUS VOC SOURCES

ENVIRONMENT
MICROBIOME
Dietary sources of Trimethylamine (TMA) and acute alcoholic hepatitis

Hepatic flavin monooxygenase (FMO) family of enzymes, FMO3 convert trimethylamine (TMA), a volatile organic compound which smells like rotting fish, into trimethylamine N-oxide (TMAO), an odorless stable oxidation product which contributes to the atherosclerosis in humans. Subjects with chronic liver disease, in general, have impaired capacity to convert TMA into TMAO. Furthermore, alcohol consumption in patients with alcoholic liver disease induces bacterial overgrowth and increases gut permeability and the translocation of bacteria-derived lipopolysaccharides from the gut to the liver. It therefore may be desirable to determine whether the amounts or concentration of volatile compounds in a biological sample, for example, a breath sample correlate with the diagnosis of liver disease.
High Concentration of Exogenous VOC Limonene Associated with Liver disease

Research Paper

Volatile Biomarkers in Breath Associated With Liver Cirrhosis — Comparisons of Pre- and Post-liver Transplant Breath Samples

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a School of Physics and Astronomy, University of Birmingham, Birmingham B15 2TT, UK
b Department of Hepatology, University Hospital Birmingham NHS Trust, Birmingham B15 2TT, UK
c Critical Care and Anaesthesia, University Hospital Birmingham NHS Trust, Birmingham B15 2TT, UK

A B S T R A C T

Background: The burden of liver disease in the UK has risen dramatically and there is a need for improved diagnostics. Aim: To determine which breath volatiles are associated with the cirrhotic liver and hence diagnostically useful. Methods: A two-stage biomarker discovery procedure was used. A total of breath samples of 31 patients with cirrhosis and 30 healthy controls were mass spectrometrically analyzed and compared (stage 1). 13 of these patients had their breath analyzed after liver transplant (stage 2). Five patients were followed longitudinally, as in-patients in the post-transplant period.

Results: Seven volatiles were elevated in the breath of patients versus controls. Of these, five showed statistically significant decrease post-transplant: limonene, methanol, 2-pentanone, 2-butane and carbon dioxide. On an individual basis limonene has the best diagnostic capability (the area under the receiver operating characteristic curve (AUROC) is 0.84), but this is improved by combining methanol, 2-pentanone and limonene (AUROC curve 0.90). Following transplant, limonene shows wash-out characteristics.

Conclusions: Limonene, methane and 2-pentanone are breath markers for a cirrhotic liver. This study raises the potential to investigate these volatiles as markers for early-stage liver disease. By monitoring the wash-out of limonene following transplant, graft liver function can be non-invasively assessed.

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1. Introduction

The publication of the 2014 Lancet Commission on liver disease has highlighted how the burden of liver disease in the UK has risen sharply over the past few decades and that it poses a major public health issue for 83% of deaths (Davies, 2012). It is the third biggest cause of premature mortality, with three quarters of liver deaths due to alcohol (Williams et al., 2014). Liver disease has a widespread effect not only to the patient, encompassing physical and psychological morbidity and mortality, but also incurring significant societal costs. One of the

- Patients suffering from liver cirrhosis have raised levels of limonene in their breath due to the liver failing to produce enzymes for metabolism.
- After liver transplant, limonene levels in exhaled breath returned to normal as the metabolism resumed.
- Shows VOCs in breath can be used to monitor a patient’s response to therapeutic intervention.
Administer Exogenous VOC (EVOC) Probes

- Exogenous VOC (EVOC) probes need *a priori* understanding of disease molecular mechanisms.
- Targeted approach allows more rigorous method development allowing high performance of a target analyte.
- EVOC probes allow optimisation of all aspects from sampling to data analysis.
- Ensure trends in data attributable to biology rather than technical variability.
- Already proven to enable the construction of highly accurate tests.

**Exogenous VOCs**

- Absorption

**Disease and phenotype characteristic metabolic processes**

- Secretion in Breath

**Collection and analysis**

- Valuable, specific, quantifiable, characteristic metabolic information

- Exogenous VOCs and volatile metabolic products
Breath Biopsy EVOC Probes to Determine Metabolizer Phenotype: Matching the Right Patient to the Right Drug and Dose

The majority of small molecule drugs are metabolized by a class of enzymes called CYP450. These drugs include anticoagulants, antidepressants, pain medication, statins, and oncology drugs with a combined total of billions of prescriptions per year.

70% of drugs are metabolized by only four enzymes:

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Main Therapeutic Indication</th>
<th>Metabolised by</th>
<th>Prescriptions (%) US, 2018</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipitor</td>
<td>Cardiovascular</td>
<td>3A4/5</td>
<td>13.5</td>
</tr>
<tr>
<td>Zoloft</td>
<td>Mental Health</td>
<td>2D6, 2C9, 2D6, 2C9, 3A4/5</td>
<td>38.9</td>
</tr>
<tr>
<td>Prozac</td>
<td>Mental Health</td>
<td>2D6</td>
<td>20.3</td>
</tr>
<tr>
<td>Tranquil</td>
<td>Pain</td>
<td>2B6, 2D6, 3A4/5</td>
<td>24.9</td>
</tr>
<tr>
<td>Crestor</td>
<td>Cardiovascular</td>
<td>2C9</td>
<td>22.7</td>
</tr>
<tr>
<td>Warfarin</td>
<td>Cardiovascular</td>
<td>1A2, 3A4/5</td>
<td>20.8</td>
</tr>
<tr>
<td>Nexium</td>
<td>Gastrointestinal</td>
<td>3A4/5, 2C9</td>
<td>13.0</td>
</tr>
<tr>
<td>Spleva Handihaler</td>
<td>Respiratory</td>
<td>3A4/5, 2D6</td>
<td>9.4</td>
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<tr>
<td>Januvia</td>
<td>Diabetes</td>
<td>3A4/5</td>
<td>6.9</td>
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<td>Eliquis</td>
<td>Cardiovascular</td>
<td>3A4/5, 2C19</td>
<td>3.3</td>
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<tr>
<td>Tamoxifen</td>
<td>Oncology</td>
<td>2D6, 3A4/5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Pharmacogenomic tests can determine metaboliser genotype, but actual phenotype can be changed by a number of factors including other drugs and diet.

Changes in metaboliser phenotype can lead to drug toxicity or inefficacy.

In 2018, over 2.2 million Adverse Drug Reactions and more than 230 thousand deaths are expected in the United States alone.

Breath Biopsy EVOC Probes to Determine Metabolizer Phenotype: Matching the Right Patient to the Right Drug and Dose

Exogenous VOC (EVOC) probes, comprised of GRAS (generally recognised as safe) compounds, are metabolised by the same CYP450 enzymes as drugs. By measuring the kinetics of a mix of EVOC probe substrates and metabolites on breath, we can determine metaboliser phenotype.

<table>
<thead>
<tr>
<th>Example EVOC probe</th>
<th>CYP450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limonene</td>
<td>2C9,2C19</td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>3A4</td>
</tr>
<tr>
<td>Linalool</td>
<td>2C19,2D6</td>
</tr>
</tbody>
</table>

Concentration of EVOC probe compounds measured with Breath Biopsy platform

Over $1.1b global pharmacogenomics diagnostics market\(^\d\) by 2021, growing at >20%

\(^\d\) Companion Diagnostics: Technologies and Markets. BCC Research Report code BIO077C March 2017
Terpene EVOC probe washout curve experiment

Repeated breath collects from one individual over 8 hour time period (16 timepoints)

Four replicate VOC sample tubes collected and analyzed at each timepoint

2 pre-ingestion controls
Terpene EVOC probe washout curve - fold change vs control

- Concentration of EVOC probe in breath rises rapidly after administration.
- Rate of decrease of probe concentration in breath as it is metabolized relates directly to metabolizer phenotype.
EVOC probe fold change for multiple substrates
Repeat sampling over 5 weeks

Washout experiment repeated 9 times on the same individual over 5 weeks

Two replicate VOC sample tubes analyzed at each timepoint
Baseline, peak and post 3hr EVOC probe concentrations

**Acetone**

- Baseline: 2.5, Peak: 2.5, 3 Hours: 2.5
- FOLD CHANGE vs. BASELINE: n.s.

**Isoprene**

- Baseline: 10.0, Peak: 10.0, 3 Hours: 10.0
- FOLD CHANGE vs. BASELINE: n.s.

**α-pinene**

- Baseline: 30.0, Peak: 30.0, 3 Hours: 30.0
- **Significant Difference (**

**β-pinene**

- Baseline: 40.0, Peak: 40.0, 3 Hours: 40.0
- **Significant Difference (**
Baseline, peak and post 3hr EVOC probe concentrations

**Limonene**

**Eucalyptol**

**Menthol**

**p-Menthan-3-one—2-**
Limonene breath concentration after EVOC administration

- Background level of EVOC probe compound limonene measured in 136 people (blue circles).
- After administration of EVOC probe breath concentration of limonene (orange circles) increased sharply.
Volunteer A - Eucalyptol washout before and after GFJ CYP3A4 inhibitor
Advantages of EVOC Probes

- Exogenous VOCs can also be used to assess metabolic function *in vivo*
- Probe and any volatile metabolic products are rapidly secreted in breath
- Assess enzymatic activity by monitoring clearance of the EVOC probe from the system and the secretion of metabolic product(s) generated.

+ Completely non-invasive
+ Can administer cocktail of probes - test multiple targets
+ Safe probes simplify regulatory requirements
+ EVOC probe substrates are very low cost
NAFLD and NASH

Risk factors for Progression: diabetes, weight gain, hypertension, menopause, genetic polymorphisms

NAFLD
- Diabetes
- Age
- Obesity

25% over 3y
44% over 6y

NASH

5% - 18%
(rapid progression)

NASH with fibrosis

20-30% over 3y
42% over 6y

NAFLD related cirrhosis

Up to 38%

HCC

2.4% - 12.8%
3.2-7.2y

Unknown

Adapted from
Bertot & Adams, 2016
Global Prevalence of NAFLD

NAFLD Prevalence
- 30%
- 20.0-29.9%
- 10.0-19.9%
- <10%
- Data not available
Economic Burden / Cost of Disease

### Direct Medical Cost
Annual hospitalisation costs for NAFLD (without cirrhosis), applied to all non-cirrhosis states

### Societal Costs
Monetary value assigned to QALYs lost due to NAFLD

- **Approximately 52 million people with NAFLD**
  - Estimated annual direct medical costs of approx. €35bn (from €354 to €4,421 per patient)
  - Estimated annual societal costs of approx. €200bn (from -€2,500 to €4,421 per patient)
  - Totalling -€235bn per year (from -€2,975 to €5,460 per patient)

- **Highest total costs in patients age 45-65 due to increased prevalence in this group**
- **Highest individual patient costs in group aged 65+, reflecting individual in more advance state of disease**
- **This is likely to increase as the incidence of NAFLD rises**

- **Over 64 million people projected to have NAFLD**
- **Estimated annual direct medical costs of $103bn ($1,613 per patient)**
- **Estimated annual societal costs of $189bn ($2,947 per patient)**
- **Totalling $292bn per year ($4,560 per patient)**
CYP3A4 Activity and Expression in Nonalcoholic Fatty Liver Disease- Woolsey et al

**CYP3A4 activity and expression in NAFLD.** (A) Plasma MDZ concentrations 3 hours after oral MDZ microdose (100 mg) in healthy control (n = 20) and biopsy proven NAFLD subjects (SS, n = 1; NASH, n = 9). Shown as Tukey box plots with median (line), 25 to 75 percentiles (box), and minimum/maximum values (whiskers). Statistical analysis by two-tailed t test (control versus NASH). (B) Fasting, plasma 4b-OHC concentrations in control (n = 20) and NAFLD subjects (SS, n = 7; NASH, n = 23). Statistical analysis by one-way ANOVA followed by the Dunnett test. (C) Plasma 4b-OHC concentrations in healthy controls (n = 20) and NAFLD subjects according to histologic assessment of fibrosis (no fibrosis, n = 6; fibrosis, n = 24). Statistical analysis by one-way ANOVA followed by the Dunnett test. (D) CYP3A4 mRNA expression in archived normal liver tissue (n = 9) and NAFLD liver biopsy samples (SS, n = 3; NASH, n = 14) compared using one-way ANOVA followed by the Dunnett test. Bars represent means with S.E.M. Gene expression was normalized to a commercial normal pooled human liver RNA sample. **P , 0.001; ***P , 0.0001. ANOVA, analysis of variance.
Opportunities for Breath Biopsy Test

Each green star represents an opportunity for a reliable and frequent non-invasive test to be conducted, reducing the likelihood of progression.

Although costs would increase at each of these timepoints, cost burden could be reduced through:
- Earlier intervention
- Less progression to severe disease
- Less need for transplant
- Less death from disease
- Less survivors with poor QoL

*It should be noted that these opportunities are related to the development of an effective treatment, but the pipeline in this area is rich.

Proposed use of Owlstone Breath Biopsy® is to monitor patients in the NASH stage of disease progression. Administered once every 24 months (in line with NICE guidance for use of the ELF test)
Designing EVOC probes

1. **Optimise pairing of substrate to enzyme(s):** different enzymes have different substrate-specificity, and this might also be affected by disease conditions. Moreover, it is unlikely that an EVOC probe is metabolised by a single enzyme, as usually multiple enzymes contribute to metabolism of exogenous compounds. While this aspect can be harnessed for the development of multiplex approaches aimed at assessing multiple enzyme activities at once, this is a critical issue when building assays for specific enzymes. Screening of different EVOC probes, and analysis of the specificity of different enzymes for the same probe, can finally lead to an optimised match between EVOC probe and enzyme of interest.

2. **Identify viable route of administration:** several routes of administration (oral, intravenous, sublingual, inhalation, transdermal, etc) can be envisioned based on the enzyme activity of interest. Organ/tissue distribution of the target enzyme will dictate the choice of the route of administration. For instance, if the target enzyme is present in the gastrointestinal tract, oral administration is preferable, while in case of hepatic expression either oral or intravenous administration could constitute viable options. Note, the route of administration can drastically affect assay kinetics, with oral administration usually being slower than intravenous injection due to first-pass metabolism.

3. **Distribution kinetics:** distribution of different compounds in the body is affected by route of administration, metabolism kinetics, as well as by physicochemical properties of the EVOC probe itself. For instance, lipophilic compounds will be retained in fat deposits, and excreted via breath, for longer time than hydrophilic compounds. These considerations have to be taken into account when selecting EVOC probes.

4. **Likelihood of secretion in breath:** this aspect will depend on the EVOC probes, or derived metabolites, and is based on volatility of the compounds of interest. This depends on physical properties of the compounds, such as boiling point and water/air partition coefficient. Selection of EVOC probes that, not only are metabolised by the enzyme of interest, but also are secreted in breath at high proportions, is fundamental for the development of EVOC probe strategies.

5. **Dosage of EVOC probes:** the amounts of EVOC probe that reach the enzyme of interest will determine the ability of the assay to reveal differences in enzymatic activity. Indeed, evaluation of enzymatic activity is usually measured as a function of substrate concentration [85]. Defined ranges of substrate concentrations are needed to assess differences in enzyme Vmax (the maximal catalytic rate with saturating concentrations of substrate) or Km (enzyme affinity for the substrate). Appropriate dosage of EVOC probe will change according to the enzyme of interest.

6. **Kinetics of metabolism and breath excretion of the EVOC probe** itself, and/or of product metabolites, in healthy subjects have to be determined in order to measure intra- and inter-individual variability, as well as to assess contribution of potential confounding factors such as diet, lifestyle, age, gender, current medication, etc. Breath values from healthy subjects can
Owlstone Medical Cancer Clinical Trials

- Oesophageal
- Pancreatic
- Kidney
- Prostate
- Bladder
- Stomach

- Lung
- Colon

- University of Cambridge
- Cancer Research UK

LUCID
InTERCEPT
NHS
LuCID: Lung Cancer Indicator Detection

- Funded by the NHS
- Largest breath biomarker trial ever undertaken in the world recruiting up to 4,000 patients
- Evaluating utility of breath biomarker detection in detection of lung cancer

Chief Investigator
- Dr Robert Rintoul, Papworth Hospital NHS Foundation Trust Cancer Research UK Cambridge Institute
Understanding of endogenous VOC pathways

**A. LIPID PEROXIDATION**

- Unsaturated lipid → Lipid peroxy radical → Lipid hydroperoxide → ROS
- ROS reacts with Fe-S to form Fenton reaction

**B. MEVALONATE PATHWAY**

1. Glucose → Fatty acids → Amino acids
2. Glutamine → HMG-CoA → Acetate
3. Statins → HMGCR
4. Neopentyl diphosphate (IPP) → Mevalonate
5. Isoprenyl diphosphate (IPP) → Farnesyl diphosphate (FPP)
6. Squalene → Geranyl-geranyl diphosphate (GGPP)
7. Ubiquinone

**C. KETONES**

- Starvation
- Diabetes
- OAA → Citrate → TCA cycle → Acetate
- HMG-CoA → Acetoacetate
- BH4 → Acetone
- β-Hydroxybutyrate → Liver

**Further Reading**

- Extrahepatic tissues
  - OXCT1
  - Succinyll-CoA → TCA cycle
  - Citrate
  - OAA → Acetone
  - Acetoacetate
  - Acetoacetyl-CoA → BH4
Exogenous probes are the basis of PET scans

FDG IN NORMAL CELLS

FDG IN CANCER CELLS
Aldo-keto reductases (AKRs) control lipid peroxidation in lung cancer.
AKR1B10-B15 are not normally expressed in the lungs
Assessing presence of cancer through altered metabolic ACTIVITY

HEALTHY PATIENT

LUNG CANCER PATIENT
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