Targeted Compound Analysis with Breath Biopsy

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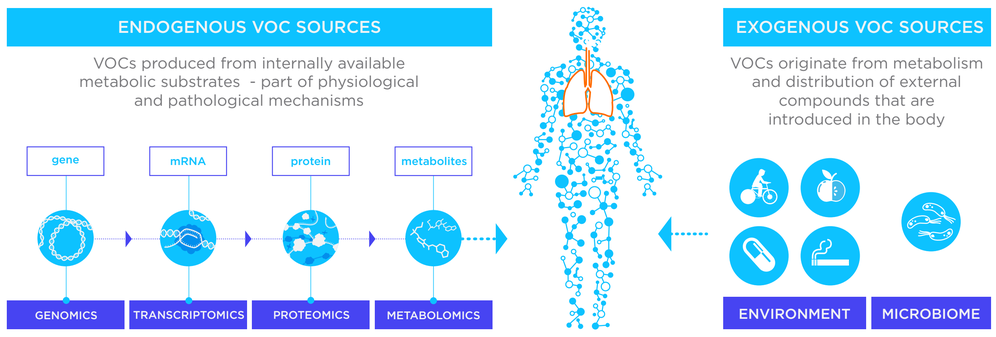
Introduction

- After five decades of breath research focussing on the identification of endogenous VOCs as disease biomarkers, **no breath test using VOCs is routinely used in clinic.**
- Hypothesis-driven administration of exogenous compounds could provide a means to investigate the analysis of selected compounds in breath.
- **We propose a novel approach that uses exogenous VOC (EVOC) probes to measure the activity of metabolic enzymes *in vivo*, as well as the function of organs, through breath analysis.**

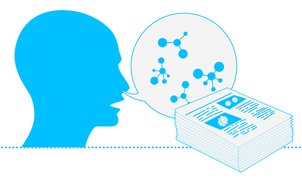
Origins of Endogenous and Exogenous VOCs in Breath

VOCs are low molecular weight metabolites that are excreted in breath as a result of metabolic processes in the body, and can be of endogenous and/or exogenous origin.



Historical Challenges in Breath Research

Almost fifty years after the first studies in breath research [1], while many biomarkers have been proposed in proof of concept studies, there are no VOC-based tests routinely applied in the clinic. *Why?*



- 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

- Study design and size - small patient numbers in pilot studies and lack of blinded validation studies

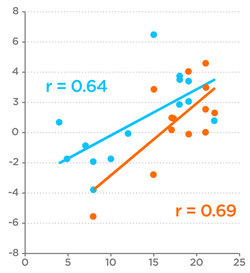
- High end, expensive spectrometer vs. low performance eNose
- Different analytical techniques required in biomarker discovery and clinical translation

WITHOUT SOLVING THESE YOU CAN'T HAVE CONFIDENCE IN BIOMARKER DISCOVERY AND VALIDATION

LIMITATIONS OF 'OMICS STUDIES

- Typically small-size cross-sectional case-control studies screening as many potential biomarkers as possible
- A small ratio of subjects to biomarkers (typically 1:5 - 1:10)
- High risk of overfitting, resulting in falsely identified **'voodoo' correlations** [2]
- Risk of obscuring true biomarkers

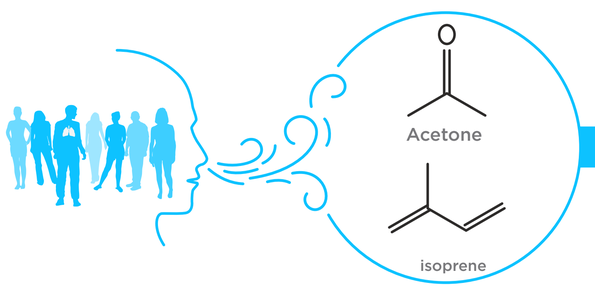
$$\frac{\text{Number of subjects}}{\text{Number of features}} < 1$$



A 'MEASURE-ALL' UNTARGETED APPROACH CAN BE CHALLENGING WHEN SUBJECT NUMBERS ARE RELATIVELY SMALL COMPARED TO THE MEASURED FEATURES

These challenges cause research results to gravitate to the exhaled VOCs occurring in the widest range of subjects at the highest concentrations.

Consequently, certain abundant VOCs, e.g. acetone and isoprene, are overrepresented in the literature, and are reported to be associated with a wide range of health states.

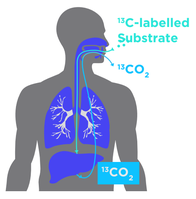


Found to be associated with many unrelated diseases
- Lung cancer
- Cystic fibrosis
- Asthma
- Malaria
- Pneumonia
- Influenza
- Renal disease
- Liver disease
- etc.....

COMMON ENDOGENOUS VOCS LIKELY TO BE HIGHLY UNSPECIFIC BIOMARKERS

Stable Isotope Probes – Proven to Enable Highly Accurate Breath Tests

- Stable isotope tests are based on administration of ^{13}C labelled compounds, biotransformation of the substrate to generate $^{13}CO_2$, followed by isotope enrichment analysis in breath
- A powerful strategy for assessing metabolic phenotypes and organ functions *in vivo*
- All probes lead to breath secretion of $^{13}CO_2$ - can only assess one target at a time
- Only a handful of products approved for commercialization – perhaps because if **invasive** intravenous administration of the probe is required, this impacts on regulatory approval



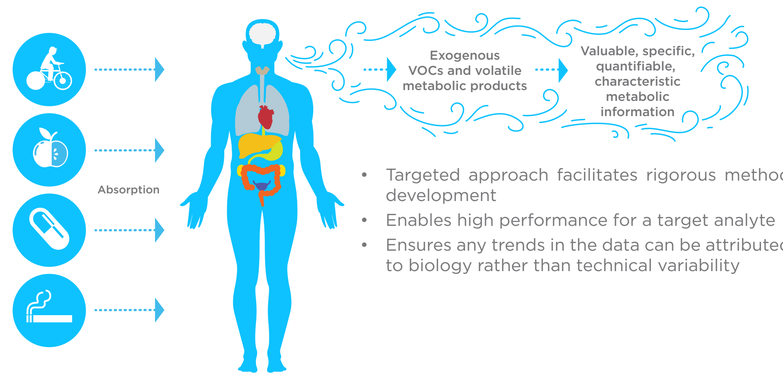
References

[1] Pauling L, Robinson A B, Teranishi R and Cary P 1971 Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography Proc. Natl. Acad. Sci. U. S. A. 68 2374–6.

[2] Broadhurst D I and Kell D B 2006 Statistical strategies for avoiding false discoveries in metabolomics and related experiments Metabolomics 2 171–96.

Targeted Breath Analysis using EVOC Probes

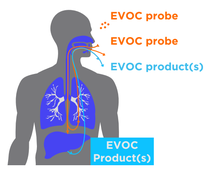
- EVOC probes are volatile compounds that, administered to a subject through various routes, undergo metabolism and distribution in the body and are excreted via breath.
- Virtually any exogenous VOC that is metabolized by the human body can offer a readout of metabolic enzymes/organs.



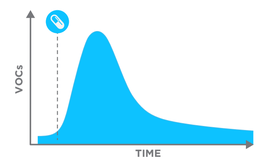
- Targeted approach facilitates rigorous method development
- Enables high performance for a target analyte
- Ensures any trends in the data can be attributed to biology rather than technical variability

EVOC Probes for *In Vivo* Metabolic Phenotyping

The kinetics of metabolism and subsequent breath excretion of the EVOC probe, or of its products, could enable *in vivo* assessment of the activity of specific metabolic enzymes and organ function.



- Breath clearance of the EVOC probe can be monitored in breath, as well as multiple products that can derive from metabolism of the EVOC probe by specific enzymes
- Can administer a cocktail of probes – test multiple targets
- Completely **non-invasive**
- Targeted hypothesis-driven approach



Terpenes as EVOC Probes

- Ingestion of an EVOC probe resulted in a marked increase in concentration of terpenes in breath after 30 minutes, compared to baseline levels (Figure 1).
- No increase was observed for endogenous compounds not contained in the EVOC probe, such as acetone and isoprene.

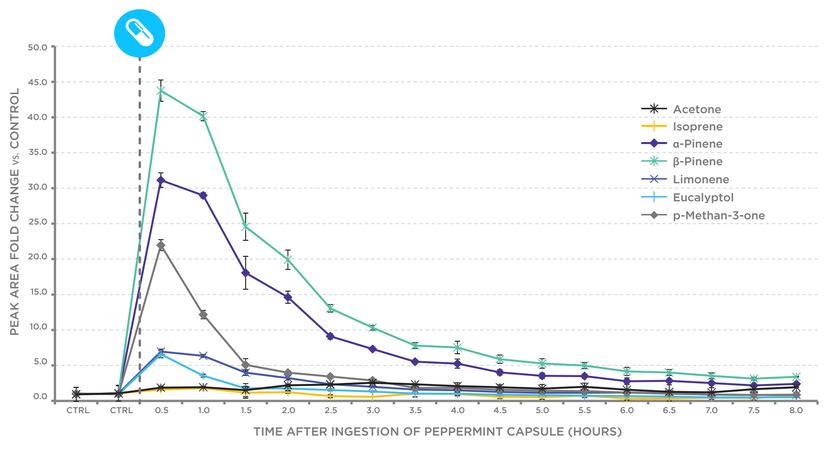


Figure 1: Washout curves of acetone, isoprene, and different terpenes/terpenoids from one healthy subject at baseline (Ctrl) and after ingestion of the EVOC probe (peppermint oil capsule). Data were normalized on average baseline levels, error bars represents SD of 2 breath samples obtained simultaneously by the ReCIVA Breath Sampler at each time point.

High Reproducibility in Breath Measurement of EVOC Probes

Reproducible fold changes were observed in longitudinal study over 5 weeks (Figure 2).

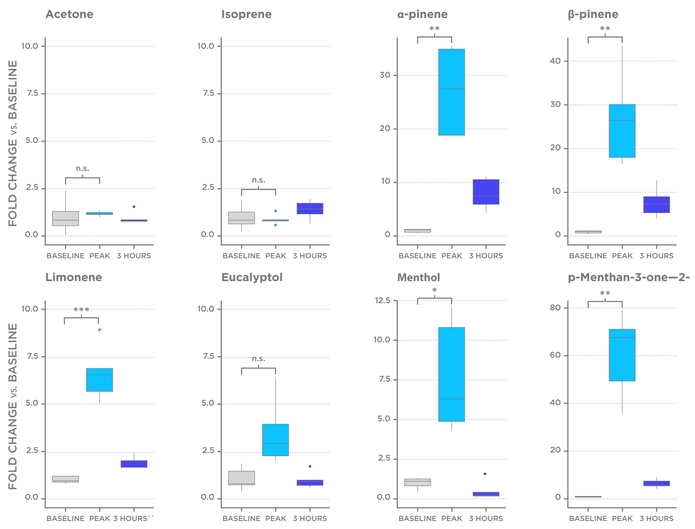
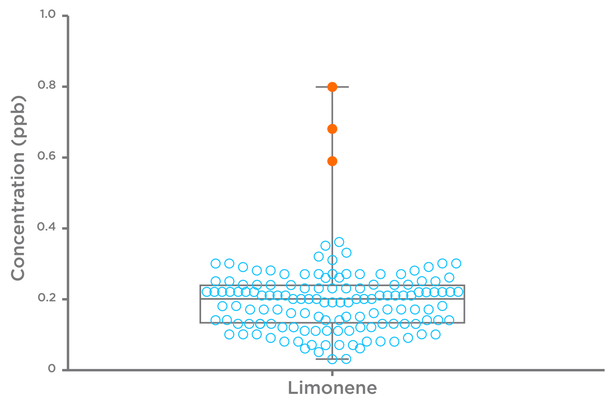


*Figure 2: Boxplots of breath levels of acetone, isoprene and different terpenes at baseline (before EVOC), peak (45 min after EVOC) and 3 hours after EVOC, acquired across 5 weeks in one healthy subject. *, **, and *** represent paired t-student p-value < 0.05, 0.01, 0.001, respectively. n.s. = not significant.*

EVOC Probes Overcome Challenges Associated with Biological Variability



EVOC probes resulted in a significant increase in limonene levels, generating a separated distribution of breath limonene concentrations (Figure 3).

Figure 3: Comparison of background levels of limonene and EVOC-induced limonene changes. Breath concentrations of limonene were measured in 136 subjects (blue circles) and compared with limonene levels after EVOC probe administration (orange dots).

Conclusions

- **EVOC Probes hold great potential for development of specific disease biomarkers** – by building on understanding of biological pathways, specific metabolic targets can be identified and exploited to reveal the presence of disease.
- EVOC Probe strategies could **increase signal-to-noise ratio**, and help to overcome challenges associated with **intra- and inter-individual variation** in biomarker measurements.
- EVOC Probes could **herald a new wave of substrate-based breath tests, translating breath biopsy to clinical applications.**