Breath Limonene and Liver Disease – Using EVOC® Probes to Assess Metabolic Pathways

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Metabolomics Scientist

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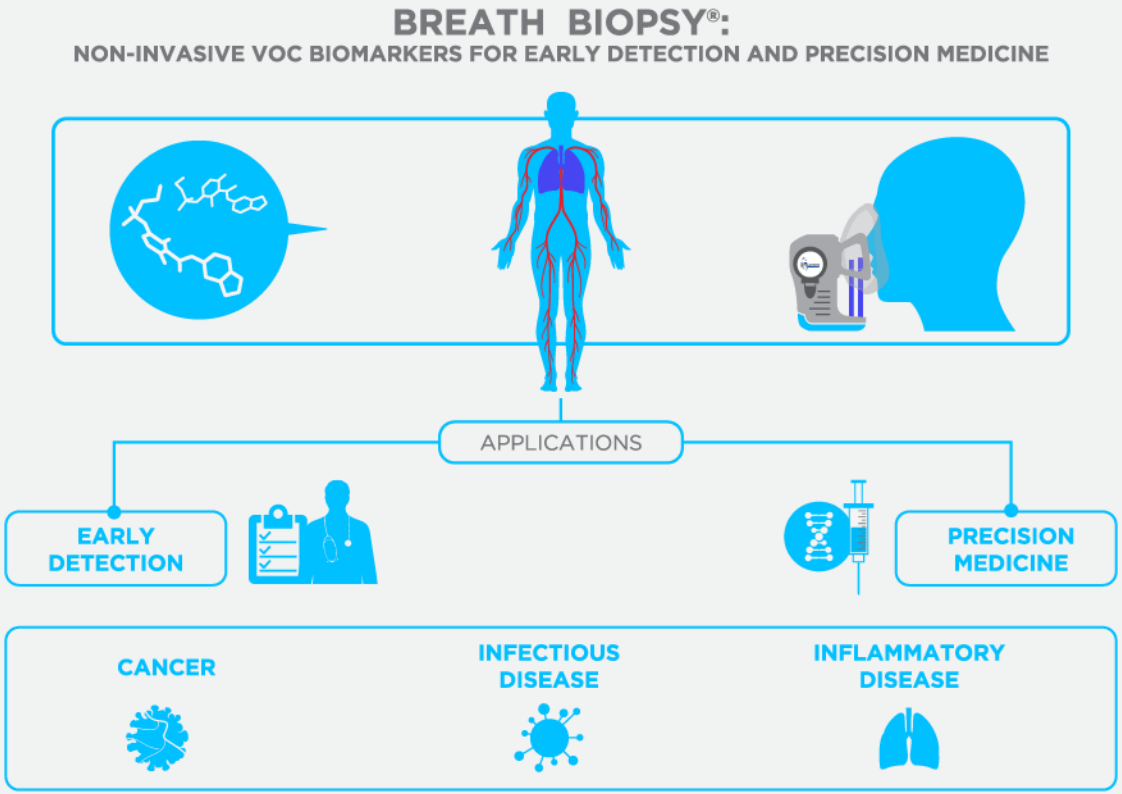
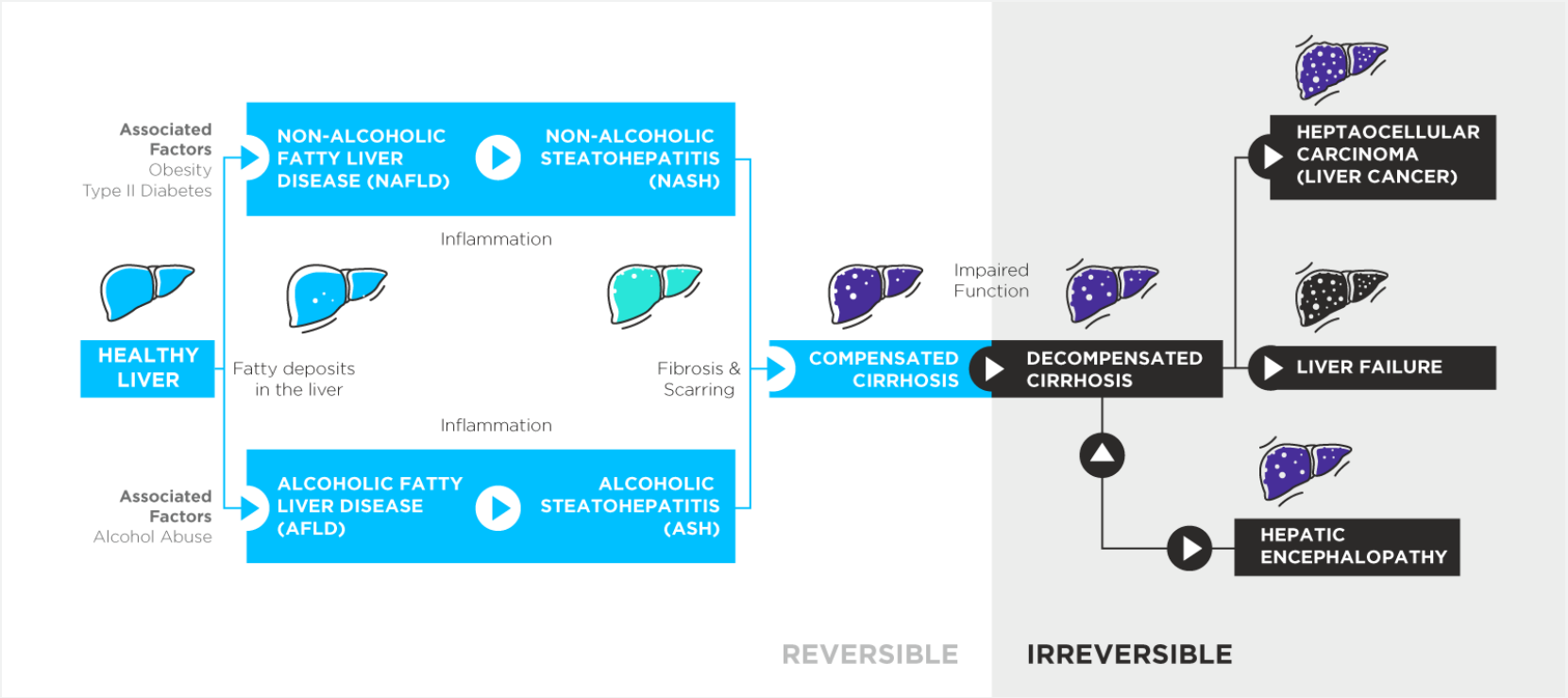
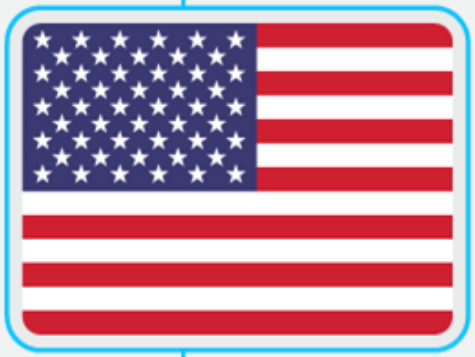
Targeting two significant markets in early detection and precision medicine



Stages of liver disease progression



Unmet need - economic burden



"Nonalcoholic fatty liver disease, or NAFLD, which affects roughly 100 million Americans, costs the United States healthcare system $32 billion annually […]"

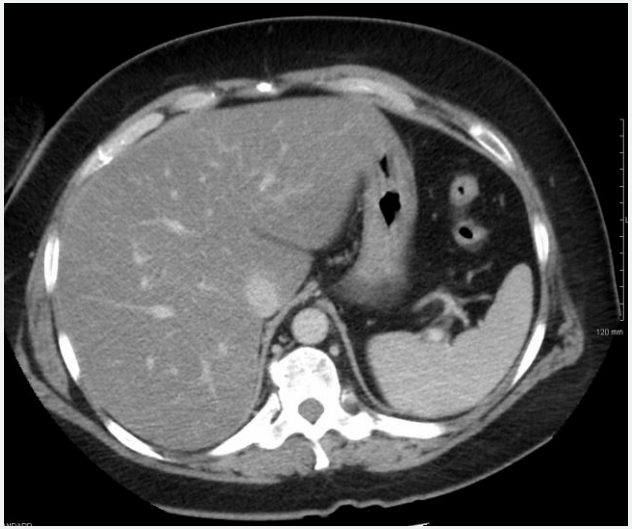
Intermountain Medical Center. "Economic burden of fatty liver disease in US is $32 billion annually, new study finds." ScienceDaily. ScienceDaily, 3 July 2018. <www.sciencedaily.com/releases/2018/07/180703105956.htm>

State-of-the-art diagnosis

- Liver enzymes
- Ultrasound and CT
- Liver biopsy
- Vibration-Controlled Transient Elastography (VCTE)

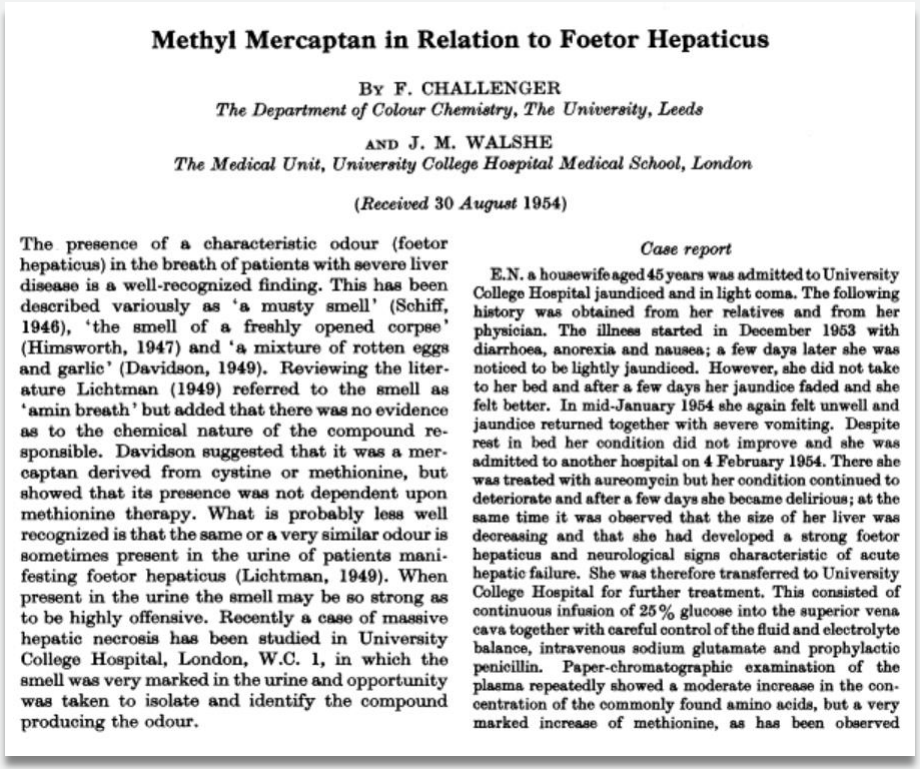
State-of-the-art diagnosis

- Liver enzymes
  - Measure liver damage
- Ultrasound and CT
  - Indirect method
- Liver biopsy
  - Gold standard, but localized and very invasive
- Vibration-Controlled Transient Elastography (VCTE)
  - Indirect method



No single test for function of the liver

Fetor hepaticus and advanced liver failure



Methyl Mercaptan in Relation to Foetor Hepaticus

By F. CHALLENGER
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AND J. M. WALSHE
The Medical Unit, University College Hospital Medical School, London

(*Received* 30 *August* 1954)

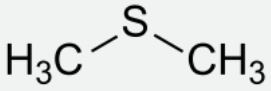
The presence of a characteristic odour (foetor hepaticus) in the breath of patients with severe liver disease is a well-recognized finding. This has been described variously as 'a musty smell' (Schiff, 1946), 'the smell of a freshly opened corpse' (Himsworth, 1947) and 'a mixture of rotten eggs and garlic' (Davidson, 1949). Reviewing the literature Lichtman (1949) referred to the smell as 'amin breath' but added that there was no evidence as to the chemical nature of the compound responsible. Davidson suggested that it was a mercaptan derived from cystine or methionine, but showed that its presence was not dependent upon methionine therapy. What is probably less well recognized is that the same or a very similar odour is sometimes present in the urine of patients manifesting foetor hepaticus (Lichtman, 1949). When present in the urine the smell may be so strong as to be highly offensive. Recently a case of massive hepatic necrosis has been studied in University College Hospital, London, W.C. 1, in which the smell was very marked in the urine and opportunity was taken to isolate and identify the compound producing the odour.

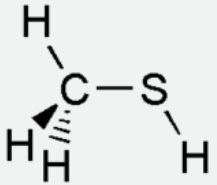
Case report

E.N. a housewife aged 45 years was admitted to University College Hospital jaundiced and in light coma. The following history was obtained from her relatives and from her physician. The illness started in December 1953 with diarrhoea, anorexia and nausea; a few days later she was noticed to be lightly jaundiced. However, she did not take to her bed and after a few days her jaundice faded and she felt better. In mid-January 1954 she again felt unwell and jaundice returned together with severe vomiting. Despite rest in bed her condition did not improve and she was admitted to another hospital on 4 February 1954. There she was treated with aureomycin but her condition continued to deteriorate and after a few days she became delirious; at the same time it was observed that the size of her liver was decreasing and that she had developed a strong foetor hepaticus and neurological signs characteristic of acute hepatic failure. She was therefore transferred to University College Hospital for further treatment. This consisted of continuous infusion of 25% glucose into the superior vena cava together with careful control of the fluid and electrolyte balance, intravenous sodium glutamate and prophylactic penicillin. Paper-chromatographic examination of the plasma repeatedly showed a moderate increase in the concentration of the commonly found amino acids, but a very marked increase of methionine, as has been observed

Biochem J. 1955 Mar; 59(3): 372–375.

- "breath of the dead" strong musty smell (Schiff, 1946)
- sign of advanced liver failure
- caused by thiols passing directly into the lungs
  - in portal hypertension (portosystemic shunting)
- responsible compounds likely dimethyl sulfide, methanethiol





High concentration of exogenous VOC limonene associated with liver disease

EBioMedicine 2 (2015) 1243–1250

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Research Paper

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

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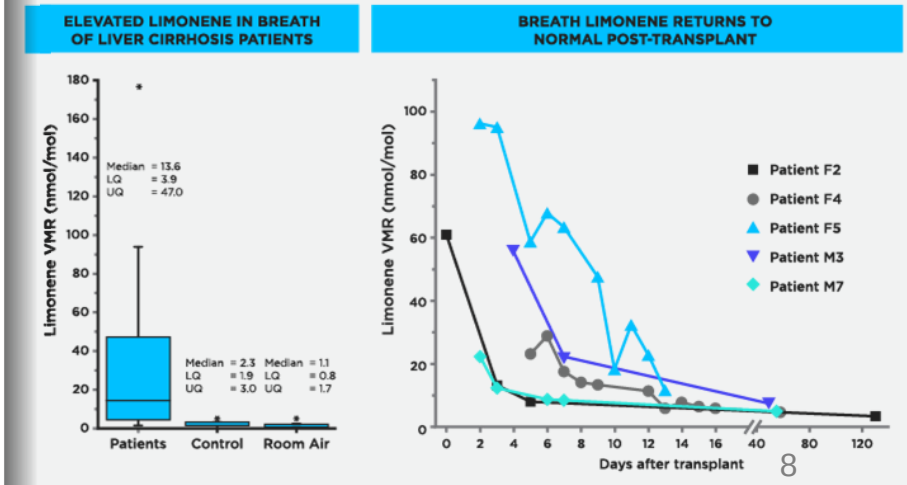
ABSTRACT

Background: The burden of liver disease in the UK has risen dramatically and there is a need for improved diagnostics.
Aims: To determine which breath volatiles are associated with the cirrhotic liver and hence diagnostically useful.
Methods: A two-stage biomarker discovery procedure was used. Alveolar breath samples of 31 patients with cirrhosis and 30 healthy controls were mass spectrometrically analysed and compared (stage 1). 12 of these patients had their breath analysed 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-butanone and carbon disulfide. On an individual basis limonene has the best diagnostic capability (the area under a receiver operating characteristic curve (AUROC) is 0.91), but this is improved by combining methanol, 2-pentanone and limonene (AUROC curve 0.95). Following transplant, limonene shows wash-out characteristics.
Conclusions: Limonene, methanol 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.

1. Introduction

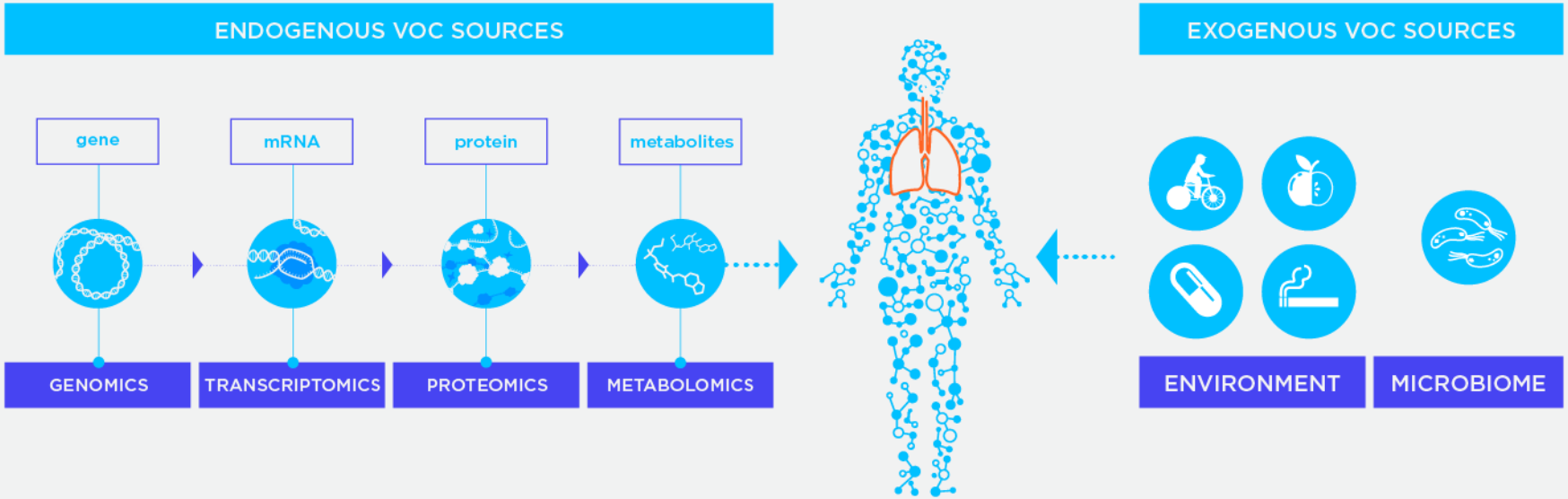
The publication of the 2014 Lancet Commission on liver disease has highlighted how the burden of liver disease in the UK has risen sharply over the past few decades and that it poses a major public health issue for 83% of deaths (Davies, 2012). It is the third biggest cause of premature mortality, with 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 with liver cirrhosis have raised levels of limonene in their breath due to failure of the liver to produce metabolic enzymes
- After liver transplant, limonene levels in exhaled breath return to normal as metabolism is restored
- VOCs in breath can be used to monitor a patient's response to therapeutic intervention

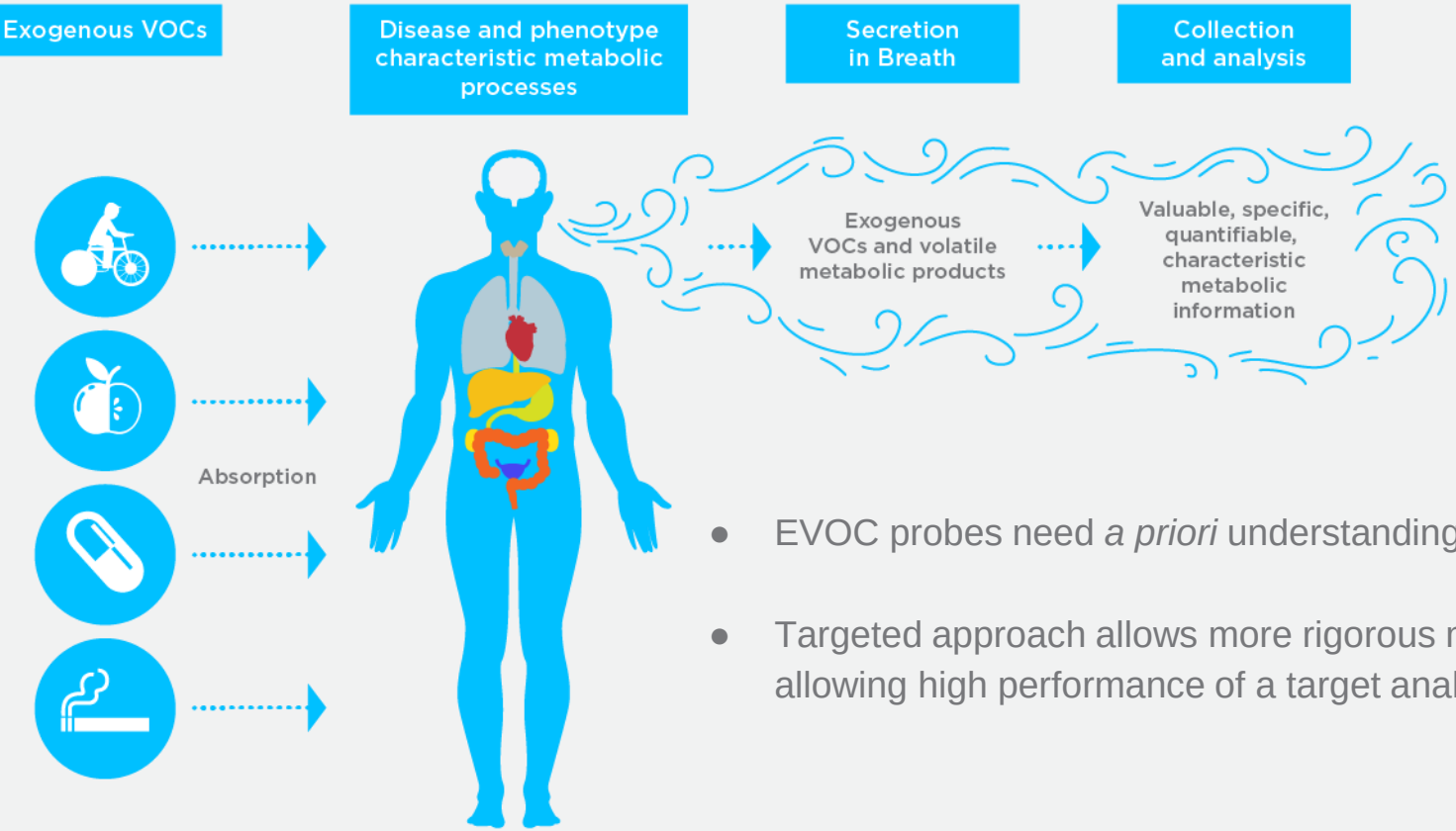


Fernández del Río R et al., EBioMedicine (2015); 2(9); 1243–1250

Endogenous vs exogenous VOCs

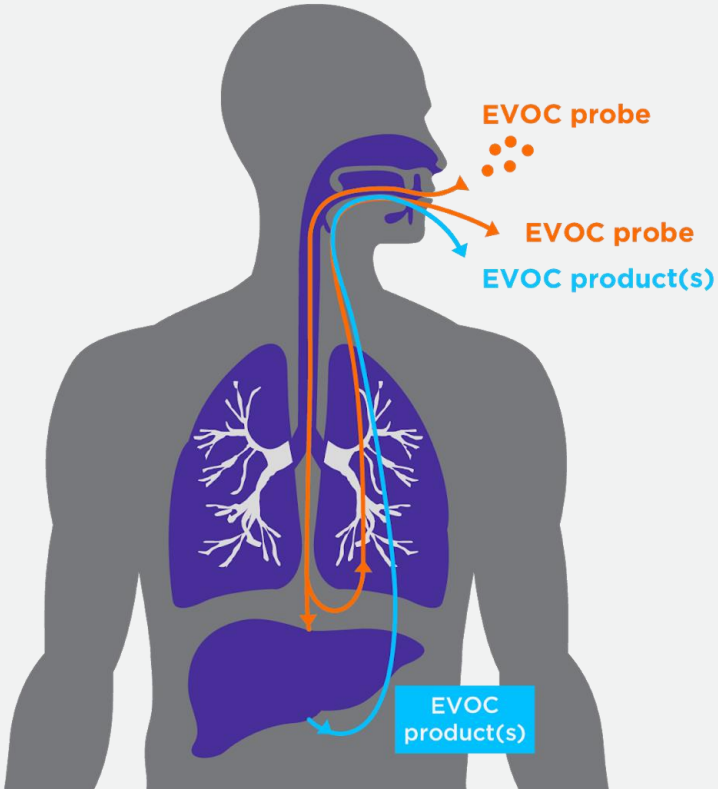


Exogenous VOC (EVOC®) probe administration



- EVOC probes need *a priori* understanding of disease mechanisms.
- Targeted approach allows more rigorous method development allowing high performance of a target analyte.

EVOC® probes - key features



- Exogenous VOCs can be used to assess metabolic function *in vivo*
- Enzymatic activity assessed by monitoring EVOC Probe clearance **and** the secretion of metabolic product(s).

- **Completely non-invasive**
- **Can administer cocktail of probes** to test multiple targets
- **Safe probes simplify regulatory requirements**
- **EVOC Probe substrates are very low cost**

Pilot study aims

- Independent validation of the effect of liver disease on exhaled limonene levels previously reported by Fernandez del Rio *et al.*
- Identify prospective breath biomarkers for differentiating healthy controls, cirrhosis, and hepatocellular carcinoma (HCC)
- Explore changing breath biomarker profiles relative to liver disease severity

Pilot study design:

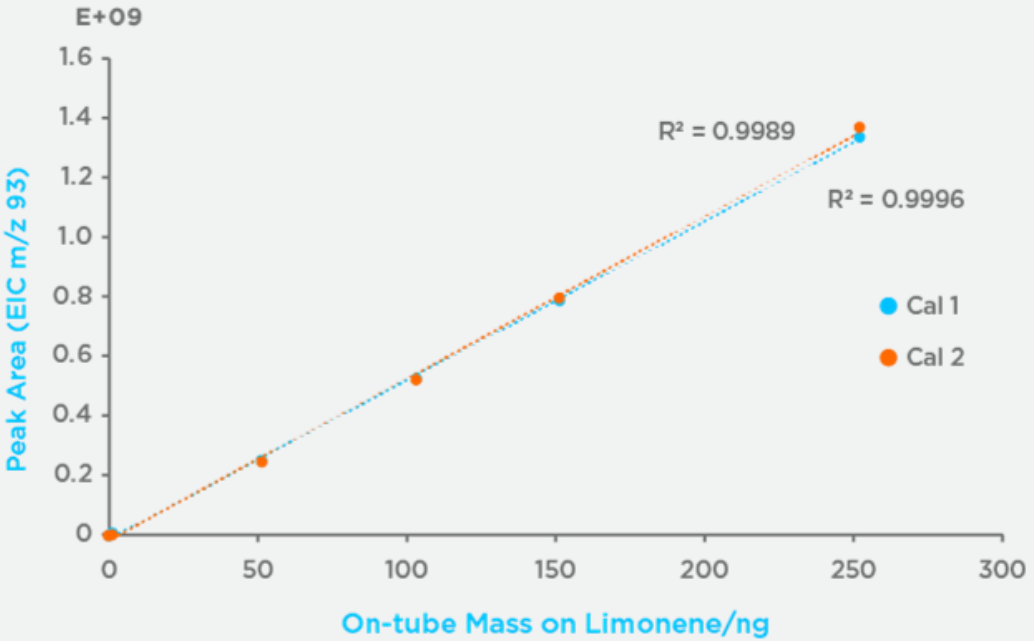
- Three equal (n=15) study groups: Cirrhosis, HCC and Healthy Controls
- All participants aged 30+
- Cirrhosis and HCC groups matched for age, gender and known risk-factors
- Initial comparison of Unhealthy (Cirrhosis & HCC) vs. Healthy
- HCC patients with severe liver damage excluded to aid identification of HCC biomarkers

Discovery or targeted analysis?

- Discovery is necessary where biomarkers attributed to a disease are unknown
- Where promising biomarkers have been discovered, a targeted analysis is possible
  - analytical method + data analysis method optimized for target
  - quantification of target compounds

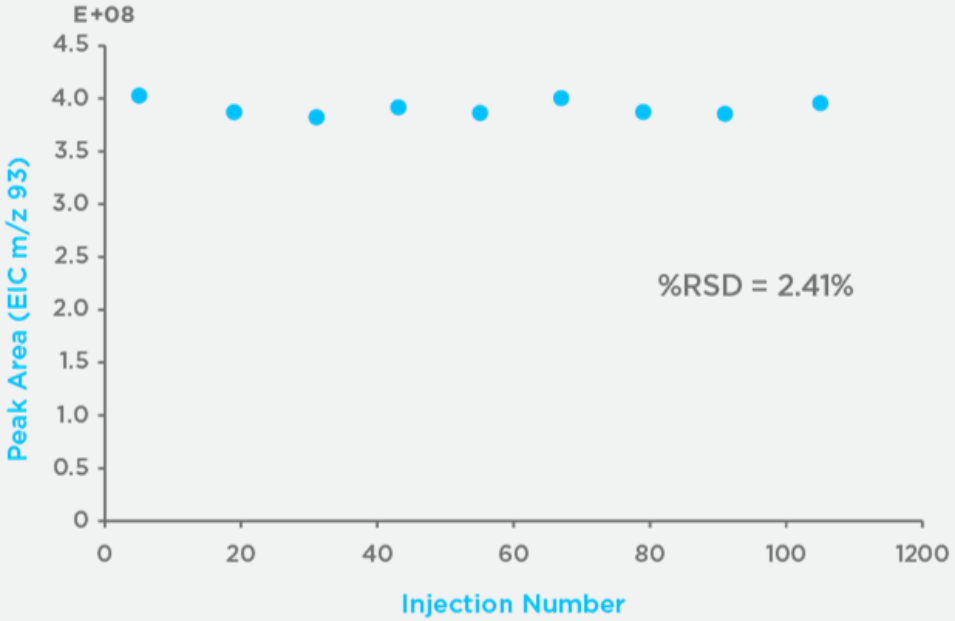
- Thermo Orbitrap Q Exactive has been selected for the work discussed here
  - Higher linear dynamic range
  - Compound identification (NIST + accurate mass)
  - A wealth of high resolution information for retrospective analysis

Quantification of limonene



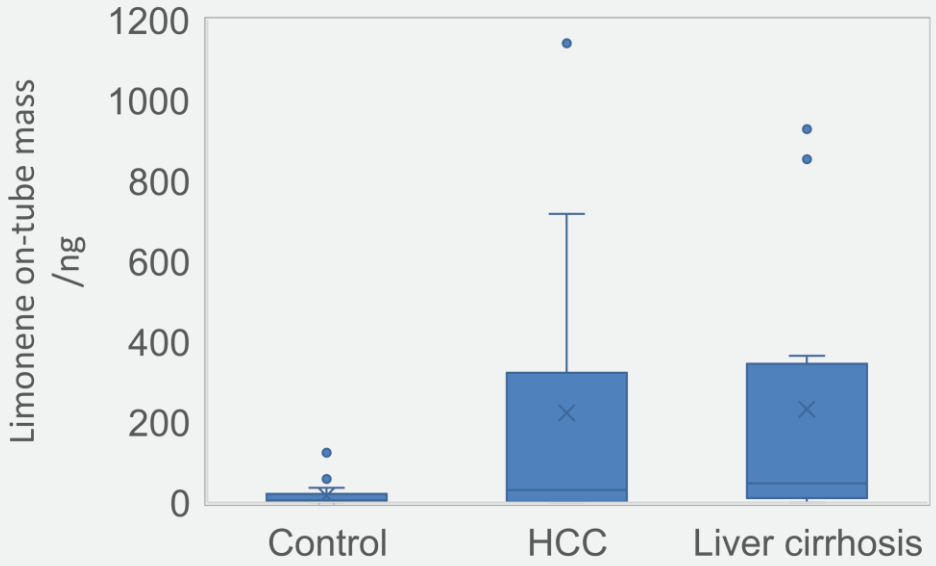
- Calibration curve at start and end of sequence
- Linearity up to 250ng on-tube

Quantification of limonene in breath



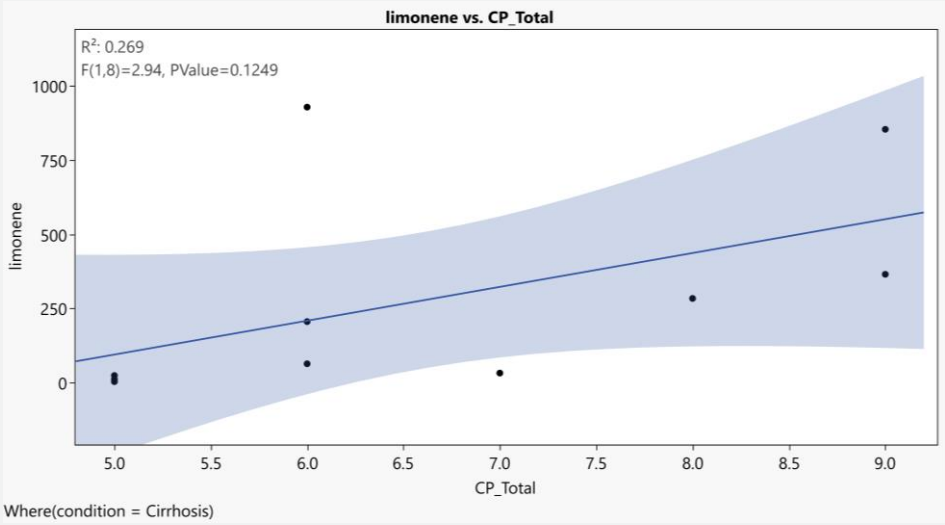
- Good precision across sequence ensures analytical variability is low
- Average %accuracy of QC is 91.6%, giving confidence in standard preparation
- QC independently prepared from calibrants

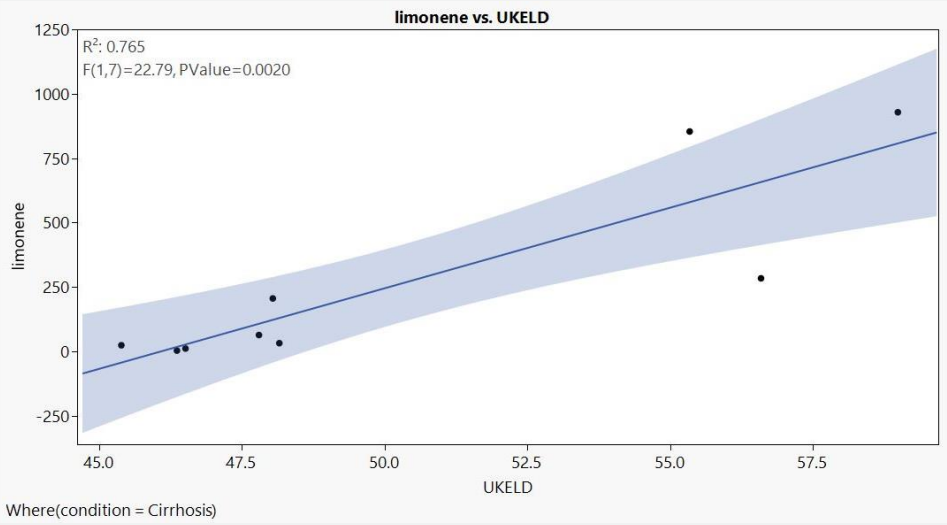
Limonene levels increased in liver disease



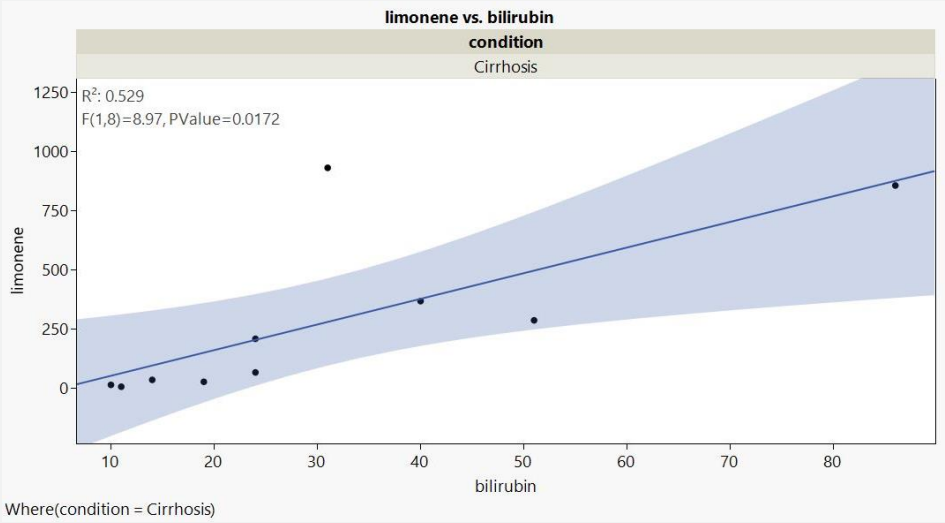
- 21 controls
- 13 HCC
- 12 Liver cirrhosis

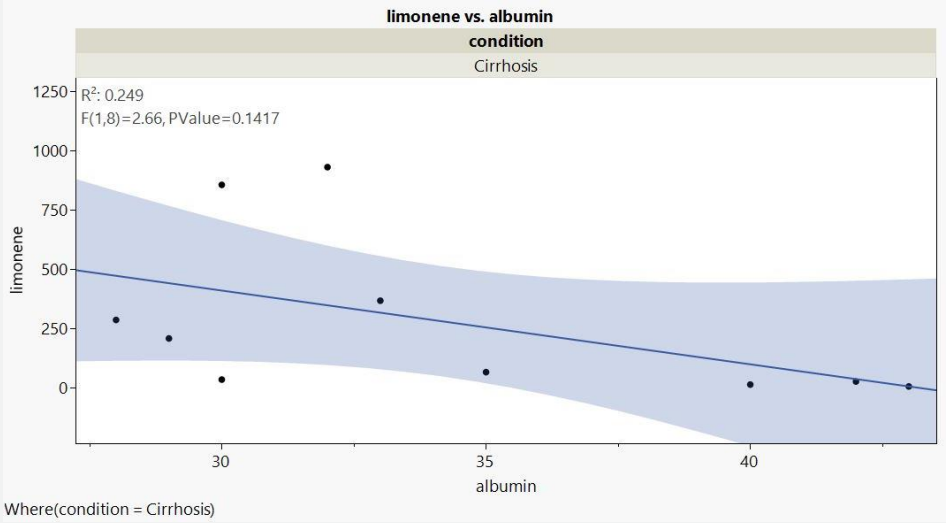
Focus on liver cirrhosis



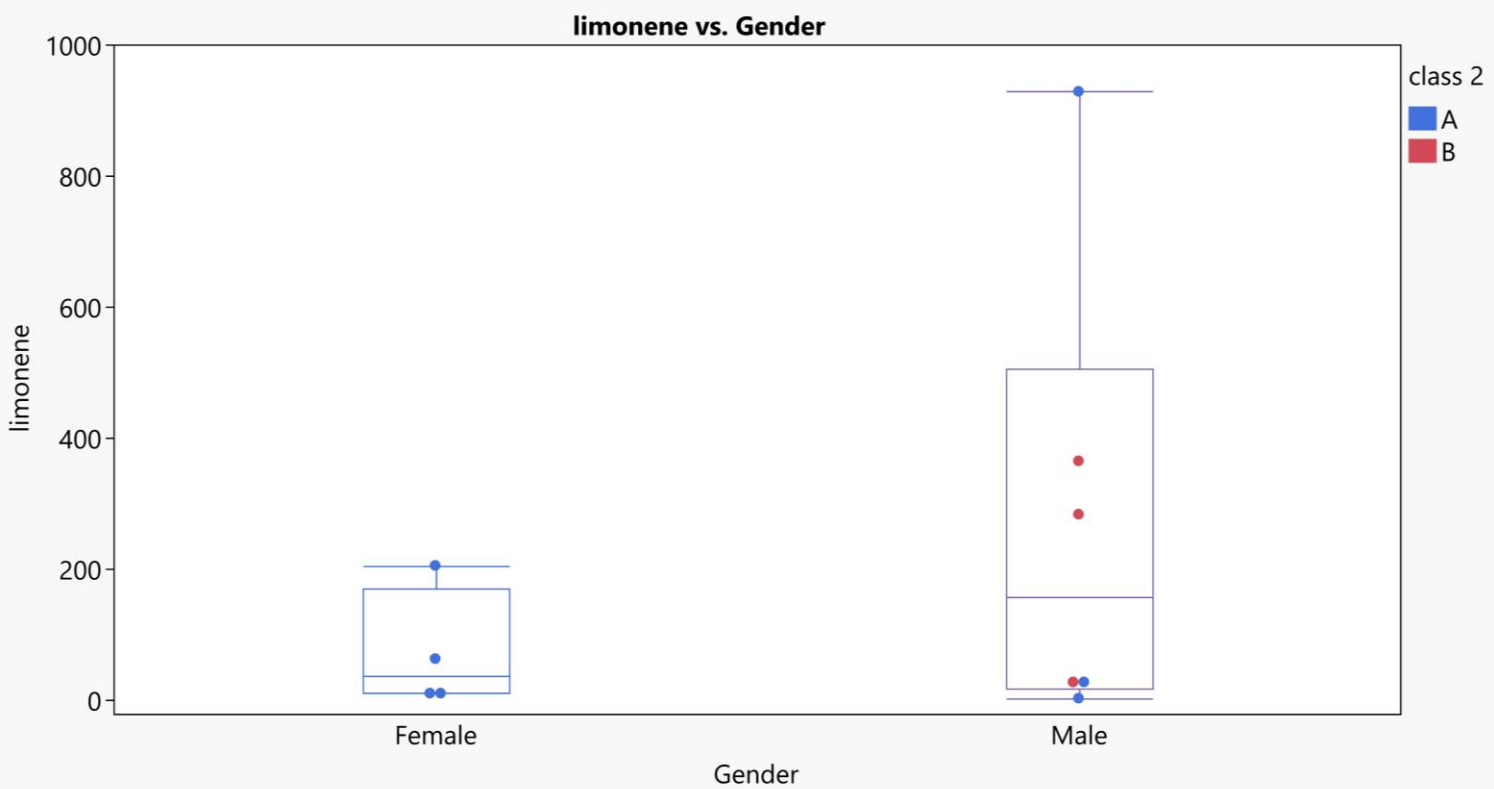


Focus on liver cirrhosis

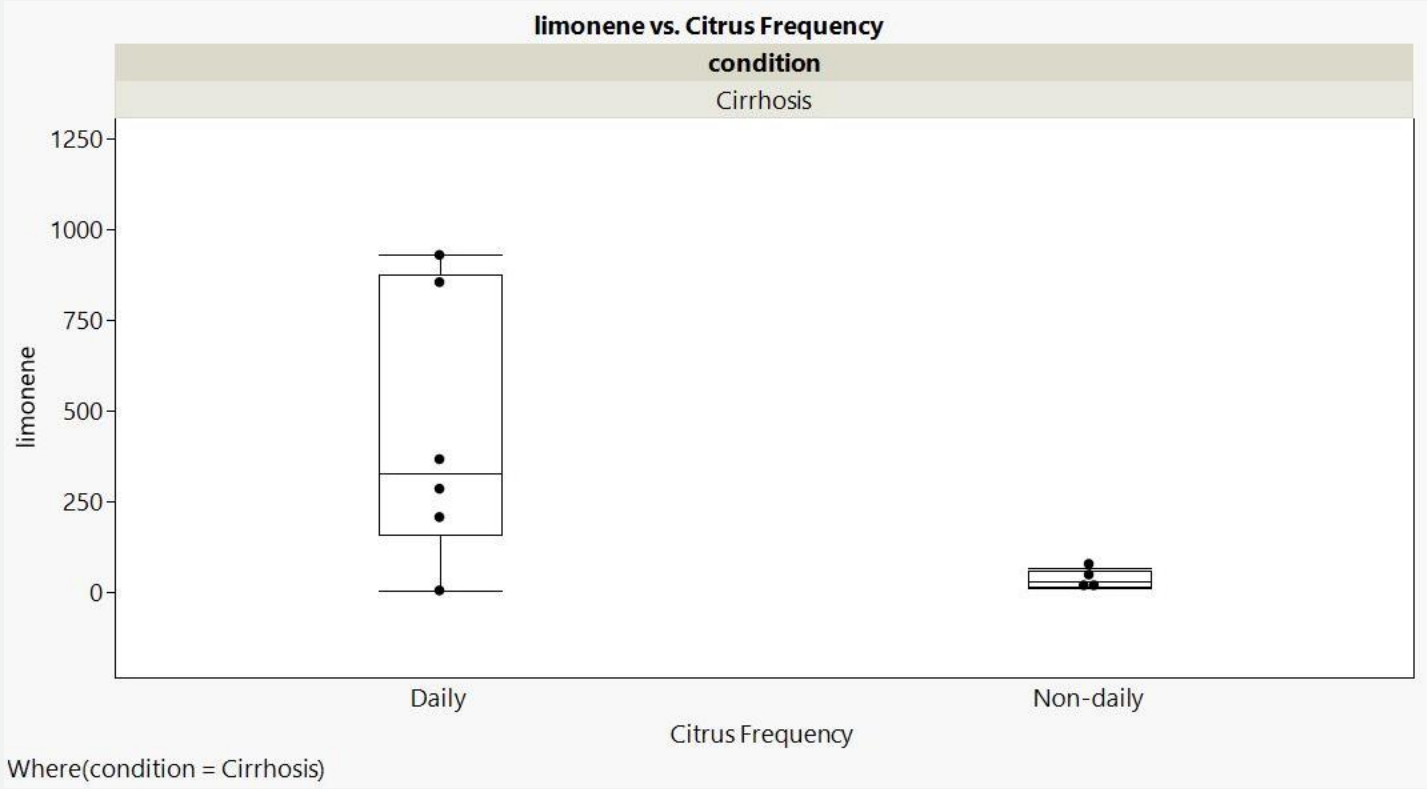




Contributing factors



Contributing factors



Next steps

- Continue recruitment
- Expand analysis to other compounds/terpenes → discovery
- Challenge liver with limonene EVOC probe
  - Overcomes problems with different exposure through diet
  - Could enable us to see differences in earlier disease stages (NASH)

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