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### **Volatonic analysis identifies compounds that can stratify non-alcoholic fatty liver disease**

**Citation for published version:**

Sinha, R, Lockman, KA, Homer, N, Bower, E, Brinkman, P, Knobel, HH, Fallowfield, J, Jaap, AJ, Hayes, P & Plevris, JN 2020, 'Volatonic analysis identifies compounds that can stratify non-alcoholic fatty liver disease', *JHEP Reports*. <https://doi.org/10.1016/j.jhepr.2020.100137>

**Digital Object Identifier (DOI):**

[10.1016/j.jhepr.2020.100137](https://doi.org/10.1016/j.jhepr.2020.100137)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Version created as part of publication process; publisher's layout; not normally made publicly available

**Published In:**

JHEP Reports

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# Journal Pre-proof



A Volatonic Analysis Identifies Compounds for Stratification of Non-Alcoholic Fatty Liver Disease: A Pilot Study

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PII: S2589-5559(20)30071-9

DOI: <https://doi.org/10.1016/j.jhepr.2020.100137>

Reference: JHEPR 100137

To appear in: *JHEP Reports*

Received Date: 11 December 2019

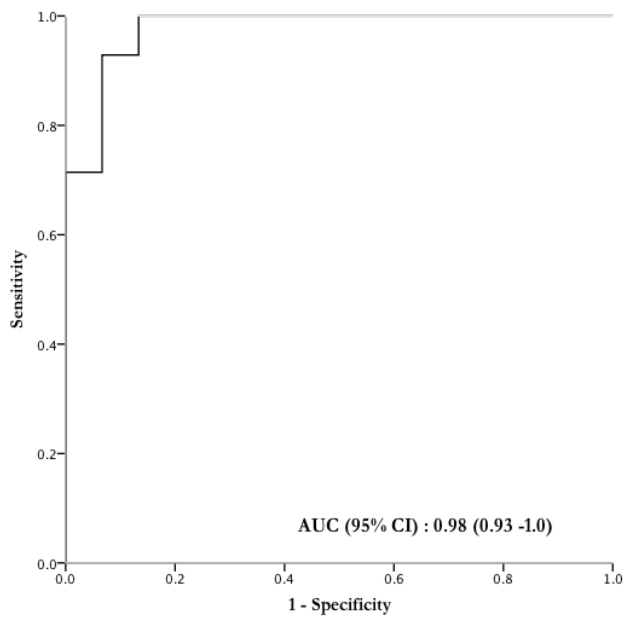
Revised Date: 5 May 2020

Accepted Date: 31 May 2020

Please cite this article as: Sinha R, Lockman K, Homer N, Bower E, Brinkman P, Knobel H, Fallowfield J, Jaap A, Hayes P, Plevris J, A Volatonic Analysis Identifies Compounds for Stratification of Non-Alcoholic Fatty Liver Disease: A Pilot Study, *JHEP Reports* (2020), doi: <https://doi.org/10.1016/j.jhepr.2020.100137>.

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**Graphical abstract**

A combined AUROC classifying patients with NAFLD cirrhosis and healthy control subjects

**Lay Summary**

Breath malodor in failing liver is well known since the ancient Greeks. Analytical chemistry has provided us an insight into ubiquitous volatile organic compounds in liver and other diseases.

This has vastly improved our understanding of mechanistic processes of liver damage. Our study aims to identify volatile organic compounds which are specific to nonalcoholic fatty liver disease which can be exploited for rapid diagnostics.

## A Volatonic Analysis Identifies Compounds for Stratification of Non-Alcoholic Fatty Liver Disease: A Pilot Study

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Conflicts of interest: None.

Authors' contributions: RS, JNP, PCH, study concept and design; RS, EB, NZMH, HH, AL analysis and interpretation of data; RS, AL, JAF, JP drafting of manuscript; RS, AL, NZMH, JAF, HK, PB, AJ, PCH, JP critical revision of manuscript for important intellectual content.

Financial support: The study was funded by the Edinburgh and Lothian Health Foundation - Diabetes Clinical Research Fund

Keywords: Volatile organic compounds, Non-alcoholic fatty liver disease, Volatomics, terpinene, D-limonene, dimethyl sulfide

Total word count: 4438 +467(Supplementary)

Tables: 4

Figures: 4 + 2 (Supplementary)

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**Abstract**

**Background:** Analysis of volatile organic compounds (VOCs) in exhaled breath, ‘volatomics’, provides opportunities for non-invasive biomarker discovery and novel mechanistic insights into a variety of diseases.

**Aim:** The purpose of this pilot study was to compare breath VOCs in an initial cohort of non-alcoholic fatty liver disease (NAFLD) patients and healthy controls.

**Methods:** Breath samples were collected from 15 participants with Child-Pugh Class A NAFLD cirrhosis, 14 with non-cirrhotic NAFLD and 14 healthy volunteers. Exhaled breath samples were collected using an established methodology and VOC profiles were analysed by gas chromatography-mass spectrometry. The levels of 19 VOCs previously associated with cirrhosis were assessed. Peaks of the VOCs were confirmed and integrated using Xcalibur® software, normalized to an internal standard. Receiver Operating Characteristic (ROC) curves were used to determine the diagnostic accuracy of candidate VOCs.

**Results:** Terpinene, dimethyl sulfide (DMS) and D-limonene provided the highest predictive accuracy to discriminate between study groups. Combining DMS with D-limonene led to even better discrimination of NAFLD cirrhosis from healthy volunteers (AUROC 0.98, 95% confidence interval (CI) 0.93 -1.00,  $p<0.001$ ) and NAFLD cirrhosis from non-cirrhotic NAFLD (AUROC 0.91, 95% CI 0.82 – 1.00,  $p<0.001$ ). Breath terpinene concentrations discriminated between non-cirrhotic NAFLD and healthy volunteers (AUROC 0.84, 95% CI 0.68 – 0.99,  $p=0.002$ ).

**Conclusion:** Breath terpinene, dimethyl sulfide and D-limonene are potentially useful volatonic markers for stratifying NAFLD; and a two-stage approach allows differentiation of non-cirrhotic and cirrhotic patients. These observations require validation in a larger NAFLD population. (ClinicalTrials.gov Identifier: NCT02950610)

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver disease worldwide affecting up to 25% of the global population. Its prevalence is expected to escalate in parallel with the inexorable rise of obesity and diabetes. It is estimated that nearly 35% among those with steatosis will progress to non-alcoholic steatohepatitis (NASH) and, in turn, a considerable proportion of those with NASH will advance insidiously to advanced liver disease. The severity of hepatic fibrosis has been shown to correlate with all-cause and disease-specific mortality in NAFLD[1].

Given the potential burden associated with NAFLD, identifying those at high risk of adverse outcomes is crucial. Various imaging techniques and biomarkers have been employed to monitor NAFLD progression, but none can match the sensitivity and specificity achieved with a percutaneous liver biopsy for detection of early stages of liver disease. However, liver biopsy is invasive, prone to sampling error and impractical for disease monitoring[2]. Thus, there is a need to develop a sensitive, specific and non-invasive diagnostic tool that can accurately characterize patients across the entire spectrum of NAFLD.

In recent years, several studies have explored the use of volatile organic compounds (VOCs) in exhaled breath as a non-invasive diagnostic tool in chronic liver disease[3-8]. The underpinning concept is that perturbed metabolic pathways can alter the pattern of breath VOC composition. An example commonly encountered in a clinical setting is *fetor hepaticus* that can occur with metabolic derangements in cirrhosis. Similarly, NAFLD is associated with a myriad of metabolic changes that can influence the composition and concentration of VOCs. Studies have shown changes in VOC composition in obese individuals with NAFLD, however correlations with the different stages of NAFLD have not been established[4]. Alterations in gut microbiota have been shown to contribute to the pathogenesis of NAFLD through metabolic mediators[9]. More

recently, a mechanistic study implicated high-alcohol-producing *Klebsiella pneumoniae* (so called ‘auto-brewery’) as a potential causative factor in some patients with NAFLD[10].

In this pilot study, we examined the pattern of exhaled breath VOCs in patients with cirrhosis and non-cirrhotic NAFLD to identify specific biomarker signals with potential utility for the stratification of NAFLD.

## METHODS

### *Study population*

This study was conducted as a substudy of a larger study: Breath analysis using an electronic nose in non-alcoholic fatty liver disease (‘BEN’; ClinicalTrials.gov Identifier: NCT02950610), a single-centre prospective observational study which aimed to examine exhaled VOC patterns in NAFLD using an electronic nose (eNose). Of the total 90 participants, exhaled breath for gas chromatography mass spectrometry (GC-MS) was collected from the first 45 consecutive participants. This included 30 NAFLD participants with or without cirrhosis (based on histological or clinical criteria) and 15 healthy volunteer controls.

The sample size was based on the following consideration: if molecular compounds are to be used in clinical practice, their association should be considerable. We defined the association to be potentially useful if the correlation coefficient was larger than 0.6/0.7. For correlations to be statistically significant ( $p < 0.05$ ) with 80% power, approximately 15 participants in each group were required. Due to contamination issues, one sample each from the non-cirrhotic NAFLD and healthy volunteer groups was excluded. Male and female adult participants were recruited from the liver outpatient clinics at the Royal Infirmary of Edinburgh (Edinburgh, UK) between March 2016 and February 2017. Exclusion criteria were: known respiratory disease, severe obesity (body mass index (BMI)  $>40$ ), use of antibiotics for preceding 4 weeks, ongoing alcohol

use of more than 21 units for men and 14 units for women, inability to give informed consent, and NAFLD cirrhosis with Child-Pugh score >7.

Participants with NAFLD were divided into: NAFLD without cirrhosis or NAFLD Child-Pugh Class A cirrhosis. This was based on the most recent liver biopsy and/or gastroscopy (performed at least within 1 year of breath test and other investigations); ultrasound, transient elastography (Fibroscan®, Echosens, France), and/or Acoustic Radiation Force Impulse (AFRI) using Siemens ultrasound system (Siemens AG, Erlangen, Germany) and serum hyaluronic acid measured using a radiometric assay (Pharmacia, Uppsala, Sweden), performed within 6 months of breath test.

NAFLD cirrhosis was diagnosed by liver biopsy in two participants, endoscopic features of portal hypertension in 12 participants and radiological features in one participant.

Non-cirrhotic NAFLD was diagnosed by liver biopsy (in five participants) and the remaining nine participants were diagnosed by clinical and radiological features, non-invasive scores – such as Fibrosis -4 (Fib-4) score, aspartate aminotransferase to platelet ratio index (APRI), NAFLD fibrosis score and BARD, serum hyaluronic acid and transient elastography measurements[11-14].

Greater diagnostic weight was given to histological or endoscopic diagnoses (in the case of cirrhosis); and in patients who had a definitive diagnosis of cirrhosis, the non-invasive markers were not considered. The healthy control group consisted of self-declared healthy participants. They were recruited through word-of-mouth and advertising posters in the institution. They were screened using questionnaire, physical examination, reported absence of any medical illness and use of any regular medication.

Measures were taken to mitigate against the effect of dietary, environmental and medication. All participants attended a single study visit at Edinburgh Clinical Research Facility after an



overnight fast. Written informed consent was taken from all participants. Although no strict diet was enforced, both overnight fasting and careful oral hygiene with unchlorinated water preparation was undertaken. Participants refrained from using any perfumes or deodorants on the day of the visit. Smoking and alcohol consumption were restricted for 48 hours. Reconciliation of concomitant medication was ascertained at screening. Drugs were categorised as inducers, inhibitors and substrates[15]. Participants taking enzyme inducing drugs were excluded. A dedicated room was used for breath sample collection. Only the participant and the investigator were allowed to enter the room for the purpose of breath collection. Upon breath collection, the room was again secured. The interval between two consecutive subjects was at least 4 hours.

Anthropometric measurement and venous sample collection were completed prior to the breath sample collection. Central obesity was defined as waist circumference of >94 cm for men and >78 cm for women taken midway between the lowest rib and the iliac crest[16]. Homeostatic Model Assessment (HOMA) was performed in participants without a known diagnosis of diabetes.[17]

#### *Exhaled breath collection*

Participants were given chlorinated water to rinse their mouth before breathing through a mouthpiece with their nose clipped into a 2-way non-rebreathing valve (Hans Rudolph 1410, Hans Rudolph, Kansas City, USA) with an inspiratory VOC filter (A2, North Safety, Middelburg, NL) and an expiratory silica reservoir to dry the expired air. The breathing manoeuvres have been previously described[18]. After 5 minutes of equilibration by tidal breathing with VOC-filtered air, the expiratory port was connected to a 10 L Tedlar sampling bag (SKC Inc., PA, USA). Participants then performed an inspiratory capacity manoeuvre and

exhaled the full expiratory vital capacity into the bag with an expiratory resistance of 20 cmH<sub>2</sub>O to close the soft palate and to obtain an expiratory flow of 0.1 to 0.2 L/s.

The sampling method for VOCs has been described previously[18-21]. This sampling procedure incorporates inspiratory VOC filtering to minimise any external influence on the VOCs[22]. The combination of targeted analyses concerning 19 VOCs and additional stable factor of a dedicated climate-controlled room, reduces the change of a false discovery dramatically. Every conceivable effort was undertaken to minimise contamination and external influence.

We did not capture consecutive breath samples for the same group of participants to monitor variation in VOCs over time, however authors have previously studied variations within a study group and found little variation[23].

#### *Gas Chromatography and Mass Spectrometry (GC-MS) sample analysis*

GC-MS analysis was performed as previously described[22]. Briefly, the content of the Tedlar bags were transferred into stainless-steel adsorption tubes (Gerstel Steel Tenax® GR Sorbent Tubes, closed by Teflon Ferrules and Swagelok Stainless Steel Tube Caps, Philips, Eindhoven, The Netherlands) by a peristaltic pump (flow rate of 200 mL/min) within 30 min of collection. Adsorption tubes were stored in an airtight package at 4°C and transported to Philips Innovation Services (Eindhoven, The Netherlands) for analysis using methodology previously described[20] (Supplementary data).

#### *GC-MS Quality Control (QC)*

The GCMS was performed by an established commercial laboratory (<https://www.philips.com/a-w/research/locations/eindhoven.html>) with a strong reputation in

Europe as a reference measurement centre. Accordingly, our commercial collaborators confirmed through their internal analyses and QC, that the signals had not changed within 28 days of storage. Furthermore, they also confirmed that there was negligible background signal from the Tedlar bags (it can add N,N-dimethylacetamide and phenol to the breath signal). After receipt of the Tenax tubes, the tubes were dried and stored in the fridge until analysis.

### *Chemical Identification*

Owing to the complex nature of untargeted GC-MS data we performed a semi-targeted non-quantitative ubiquity analysis of VOCs in the study groups of 19 VOCs. This set of 19 compounds were selected based on previous supportive literature and biological plausibility in our study population. The studied VOCs and IUPAC (International Union of Pure and Applied Chemistry) are thus listed: butane, 2-butanone, 3-methylpentane, octane, styrene, decane, acetone, isoprene, dimethyl sulfide, cyclopentane, methyl vinyl ketone (3-buten-2-one), dimethyl sulfoxide, benzaldehyde, phenol (hydroxybenzene), D-limonene, acetophenone, undecane (n-undecane), tetradecane and alfa-terpinene (terpinene).

We used Xcalibur version 3.0 (Thermo Fisher Scientific, MA, USA) to assess the data. Files were converted to .raw files. Reconstructed ion chromatograms of each of the 19 compounds were generated. The mass spectra of each discernible peak were assessed using the National Institute of Science and Technology (NIST) library database in the Thermo Library manager section of Xcalibur 3.0. The putative chemical identity was determined by examining representative mass spectral data and m/z ion patterns. A match with a probability greater than 80% according to the NIST library was used to confirm the compound. This was further confirmed using AMDIS freeware (Automated Mass Spectral Deconvolutional & Identification System, <http://www.amdis.net/index.html>).

In our experience general automated peak picking is much faster than manual peak selection and integration, especially for large data sets. Our previous published work has been performed using this technique, where all data analysis was done automatically, but tailored to the specific data[21, 24].

These compounds have been previously shown to be associated with cirrhosis[6][8]. Within the samples we identified these compounds based on the mass spectra and an identifiable peak at consistent retention time. The peak of the extracted ion chromatograms of each compound were integrated using Xcalibur 3.0 (Thermo Fisher Scientific, USA). The peak was recorded as not detected if the ion count was below 500 ion counts at the assigned retention time. All compound peak areas were normalised to the peak area of toluene-d8 in each sample, as this was added to all samples when analysed by GC-MS as an internal standard.

#### *Statistical analysis*

Statistical analyses were performed using IBM SPSS Statistics for Macintosh version 21.0 (IBM Corp., Armonk, NY) and GraphPad Prism version 5.0a (SanDiego, CA, USA). Data were presented as mean (standard deviation (SD)) or median (interquartile range (IQR)) for continuous variables. Categorical variables were presented as frequency and percentage. Data were analysed using ANOVA with Bonferroni correction to minimize false discovery. Multivariable logistic regression analysis was performed to build a model for prediction of cirrhosis and non-cirrhotic liver disease; all breath compounds were considered for inclusion. Discrimination was used for internal model validation; measuring the ability to rank patients by risk of cirrhosis such that patients with a higher predicted risk are more likely to have cirrhosis. Discrimination was measured by the Area Under the Receiver Operating Characteristics curve (AUROC). All individual compounds with AUROCs of 0.80 or above were further assessed to

find the combination of any two that provided the highest AUROC. After choosing the final model, Harrell's method was used to compute the validation metric with over-fitting bias correction through bootstrap resampling[25]. A thousand bootstrap samples ( $B = 1,000$ ) were drawn from the original data set and a new model with the same model settings was built on each bootstrap resample. Prediction on patients that were not chosen in the resample was calculated. An optimism factor was calculated over the 1,000 new models and the bias-corrected validation metric was obtained by subtracting this optimism value from the AUROC directly measured from the original model. Bonferroni correction was applied to decrease the false discovery rate and a  $p$  value  $<0.01$  was considered significant. AUROC curves were used to calculate the performance of diagnostic tests and for calculating the best point of separation between sensitivity and specificity. Given the sample size and to reduce any possibility of beta error, a  $p$ -value lower than 0.01 was considered significant and clinically valuable.

#### *Ethics permission*

The clinical study was conducted according to the ethical principles of the Declaration of Helsinki 2013 and following approval from the East of Scotland Research Ethics Committee (REC reference: 15/ES/0207) and the NHS Lothian Research and Development department (Ref: E151593).

## **RESULTS**

#### *Baseline characteristics*

Baseline characteristics of the study population are summarised in Table 1. Two samples (one non-cirrhotic NAFLD and one healthy control participant) had to be excluded for technical reasons. Participants in the healthy control group were significantly younger (median 39 (IQR

20-59) years) than those with NAFLD without cirrhosis (median 60 (IQR 29-75) years) or with cirrhosis (median 69 (IQR 44 -76) years). Similarly, BMI and waist circumference were lower in healthy controls compared with those with NAFLD.

Among participants with NAFLD cirrhosis, 80% had endoscopic evidence of portal hypertension (six (40%) varices, four (27%) portal hypertensive gastropathy (PHG) and one (6%) each for gastric antral vascular ectasia (GAVE) and dilated azygous vein on endoscopic ultrasound). Of the remaining four (20%) who did not have endoscopic evidence of portal hypertension, two had coarse liver echotexture with splenomegaly on ultrasound and two had coarse liver echotexture alone.

#### *Quantification of exhaled VOCs in the study population*

Of the 19 VOCs studied in the exhaled breath samples, seven compounds (styrene, acetone, isoprene, DMS, D-limonene, acetophenone and terpinene) were significantly different between the groups (Figure 1). There was no correlation between age and VOCs in line with previous findings[7, 8, 26]. However, acetone, isoprene, DMS and D-limonene were correlated with BMI. Given the lower BMI in the control group than those with NAFLD, and because some compounds such as breath acetone have been shown to correlate with body weight, we adjusted each compound for BMI[27]. This was achieved by dividing the intensity of VOC by the corresponding BMI, before the inclusion into the statistical modelling[27]. Significant differences persisted between the cirrhotic and healthy control groups for styrene, acetone, isoprene, DMS, D-limonene, acetophenone and terpinene. Isoprene is a by-product of cholesterol biosynthesis. However, similar to other authors, we found no correlation between the serum cholesterol and isoprene ( $\rho=0.87$  (-0.32, 0.48)  $p=0.654$ )[3].

In our study, D-limonene was inversely correlated with serum albumin (Pearson's  $r=0.7$ ,  $p<0.001$ ). In contrast, APRI score correlated positively with D-limonene (Pearson's  $r=0.6$ ,

$p < 0.01$ ). No significant correlation was found between the detected VOCs and serum hyaluronic acid or transient elastography data (data not shown).

#### *Effect of diabetes on VOCs production*

In the cirrhotic group, 13 participants (87%) had Type 2 diabetes mellitus with HbA1c (IFCC) ranging between 44 mmol/mol to 103 mmol/mol measured within 3 months of breath sampling. Two participants had insulin resistance as evidenced by HOMA-IR score (2.7 and 6.2).

In the non-cirrhotic NAFLD group, eight participants (57%) had Type 2 diabetes mellitus with HbA1c (IFCC) ranging between 45 mmol/mol to 94 mmol/mol and six (43%) participants had insulin resistance with HOMA-IR ranging between 1.7 to 6.9. It is conceivable that insulin resistance and/or Type 2 diabetes had contributed to the high level of acetone, particularly in the cirrhotic group. We also noted that the actual differences in breath acetone were probably bigger than measured here since the concentration of acetone recorded was outside the linear range of the instrument.

#### *Differentiating patients with non-cirrhotic NAFLD from healthy control participants*

Levels of isoprene, acetophenone, and terpinene were significantly lower in non-cirrhotic NAFLD patients than in healthy controls. Of these, terpinene had the highest AUROC for predicting non-cirrhotic NAFLD (0.84 (95% CI 0.68 – 0.99,  $p=0.002$ )) (Table 2 and Figure 2). Combining terpinene with isoprene and/or acetophenone did not improve the diagnostic accuracy.

#### *Differentiating patients with NAFLD cirrhosis from healthy control participants*

Levels of styrene, isoprene, acetophenone and terpinene were significantly lower in participants with NAFLD cirrhosis compared with healthy individuals. In contrast, levels of DMS and D-limonene were significantly higher in patients with NAFLD cirrhosis compared with healthy

controls. DMS and D-limonene had the highest predictive accuracy for predicting NAFLD cirrhosis with AUROCs of 0.94 (95% CI 0.86 – 1.00,  $p < 0.001$ ) and 0.91 (95% CI 0.79 – 1.00,  $p < 0.001$ ), respectively (Table 3). However, combining DMS and D-limonene had even higher predictive accuracy for diagnosing NAFLD cirrhosis with AUROC of 0.98 (95%CI 0.93 – 1.00,  $p < 0.001$ ) (Figure 3).

#### *Differentiating patients with non-cirrhotic NAFLD from NAFLD cirrhosis*

Levels of DMS and D-limonene were significantly higher in patients with NAFLD cirrhosis compared with non-cirrhotic NAFLD. DMS and D-limonene had the highest predictive accuracy for NAFLD cirrhosis with AUROCs of 0.88 (95% CI 0.74 – 1.00,  $p < 0.001$ ) and 0.83 (95% CI 0.68 – 0.98,  $p = < 0.002$ ), respectively (Table 4). However, combining DMS and D-limonene had even higher predictive accuracy for diagnosing NAFLD cirrhosis with AUROC of 0.91 (95%CI 0.82 – 1.00,  $p < 0.001$ ) (Figure 4).

## **DISCUSSION**

In this pilot study, we examined the concentration of VOCs in exhaled breath in adult NAFLD patients with cirrhosis and non-cirrhotic NAFLD. We highlighted three compounds (acetone, DMS and D-limonene) that differentiated between cirrhosis and healthy controls. More importantly, we have shown that DMS and D-limonene can discriminate between NAFLD patients with and without cirrhosis. From a clinical standpoint, the findings are important as these compounds can potentially serve as biomarkers for the stratification of NAFLD.

There have been previous studies that have shown differences in VOC concentrations between chronic liver disease and healthy liver [6, 8]. These differences were identified using GC-MS in patients with liver disease of various aetiologies. In our study, we focused solely on patients with NAFLD. Furthermore, we have adjusted the measured VOC to BMI to provide a more accurate



reflection of the metabolic profile as acetone, breath isoprene, 1-decene, 1-octene, ammonia and hydrogen sulfide have been found to be influenced by body weight[27].

Changes in cellular metabolism, insulin resistance and oxidative stress in NAFLD are dynamic processes. It is possible that there is a distinct VOC profile for the stages of NAFLD and that the pattern is influenced by a complex interaction between oxidative stress, mitochondrial impairment and metabolic pathways. Additionally, the onset of fibrosis can limit the capacity of metabolic and degradation pathways, which can potentially affect VOCs. It is also noteworthy that as NAFLD progresses, the presence of mitochondrial impairment can limit oxidative capacity thus promoting the diversion of acetyl-CoA towards non-oxidative pathways including ketogenesis - hence the higher formation of acetone in cirrhosis[28, 29]. This could also explain lower levels of isoprene in advanced liver disease.

Ketogenesis and levels of acetone can also be induced by fasting. In our study, breath sampling was obtained from all participants after overnight fasting to minimise the known physiological effects of food intake on exhaled VOC concentrations. Although fasting may have influenced the overall levels of acetone, we have shown that the levels of acetone are substantially higher in cirrhotic patients than in healthy controls. Furthermore, our finding is consistent with previous studies[8, 30]

Similarly, a higher concentration of D-limonene in NAFLD cirrhosis could either reflect inefficient metabolism (reduced levels of Cytochrome P450 enzymes - CYP2C9 and CYP2C19) that results in bioaccumulation or represent an adaptation to insulin resistance[6, 7, 26, 31]. D-limonene is a monoterpene that has been shown to have an antidiabetic effect and modulates lipid metabolism[32, 33]. Although elevated D-limonene levels has previously been reported in the context of cirrhosis, our study is first to confirm its presence in exhaled breath in the context of NAFLD cirrhosis[6, 8, 26]<sup>28</sup>. D-limonene is also a major constituent in citrus essential oil,

which is used in various foods as a flavouring agent. Although food diary was not recorded in the present study, we applied strict environmental and participant preparation measures to minimise dietary influence or any other contamination. Additionally, previous work did not find any correlation between diet and breath limonene concentration[26]. Therefore, taken together, this suggests that our findings are specific for liver disease.

It has long been considered that methyl-mercaptans and DMS are responsible for *fetor hepaticus*. DMS, being a derivative of methanethiol, has also been implicated in hepatic encephalopathy[34]. DMS breath levels are elevated in cirrhosis and correlate with the degree of porto-systemic shunting[34-36]. Our findings support this notion as 80% of the participants with NAFLD cirrhosis in our study had signs of portal hypertension on gastroscopy. All of the cirrhotic participants were Child-Pugh Class A without clinical signs of encephalopathy. We speculate that a probable source of DMS is microbiota as previous studies have linked this to oral as well as gut dysbiosis[37, 38]. Methanethiol may be converted to hydrogen sulfide and oxidized to sulphate for detoxification which can be utilised by sulphate-reducing bacteria; a process predominantly occurring in caecal tissue[39]. Pyrosequencing or next-generation sequencing studies to establish an association of VOCs with microbiota would require a higher number of well-characterised participants with a specific preparation. This is beyond the scope of our present feasibility study.

Another abundant isomeric monoterpene detected in the exhaled breath in our study was terpinene. Consistent with previous studies, terpinene was able to discriminate cirrhosis and non-cirrhotic NAFLD from healthy controls [7]. There is very little in the published literature about terpinenes and their role in the NAFLD pathophysiology is unknown. Terpinenes have been implicated in the oxidative stress pathways[40]. Thus, it is possible that the differences in the

levels of alpha-terpinene between healthy and NAFLD participants reflect the presence of oxidative stress in NAFLD.

Whilst we cannot totally exclude the possibility that medications may have influenced the breath concentrations of terpenes, we mitigated this through medication reconciliation at screening. We speculate from the outset that Cytochrome (CYP) activity would be impaired and drug metabolism (and interactions) would be complex. Therefore, attempts were made to maintain homogeneity of medication use in our participants by careful selection. Drugs were categorised either as 'inducers', 'inhibitors' or 'substrates' as previously described. None of the participants were on enzyme inducers or inhibitors. Therefore, it is unlikely that any of concomitant medications used by the participants could have influenced the terpenes, either directly or indirectly.

There were some limitations to our study. Firstly, the participants were primarily selected based on clinical characterisation rather than exclusively on histological criteria, as this was a pragmatic observational pilot study. As such, it is likely that the non-cirrhotic NAFLD group consisted of participants with a variable degree of disease activity and fibrosis. NAFLD is a dynamic process and compensated advanced chronic liver disease (cACLD) is a continuum. The mean annual fibrosis progression rate in patients with NASH is 0.14 stages, compared with 0.07 stages in patients with NAFLD[41]. Therefore, although our study groups were as well characterised as possible, it is feasible that there was some overlap between participants with Childs A cirrhosis and participants with advanced fibrosis in the non-cirrhotic group. Similarly, healthy controls in our study did not undergo clinical phenotyping. It is conceivable that some of the participants in this group may have hepatic steatosis or even NASH [42]. The small sample size and case selection limitations mean that the findings may not be generalisable to the wider NAFLD population.

Secondly, although the participants underwent an overnight fast, the potential impact of diet and dietary preferences on the breath VOCs cannot be completely excluded. Thirdly, our data are cross-sectional and derived from a single-centre. Our findings will require further validation in external (longitudinal) cohorts of NAFLD patients. Finally, we have performed semi-targeted analysis rather than untargeted, thus, selection bias cannot be completely excluded. However, there is a risk in 'omics data that the lowest responders are lost as the sensitivity of untargeted analysis is not the strength of the technique. Our approach therefore was to principally focus on specific compounds, and we interrogated 19 different compounds that have previously been described in the literature as having an association with liver disease. However, as we studied a semi-targeted analysis of VOCs in the context of NAFLD, it is possible that some VOCs with a pathogenic role in the NAFLD may have been missed in our analysis.

We intentionally did not study alcoholic liver disease as the pathogenesis and the difference in the impact of metabolic dysregulation would have introduced a substantial heterogeneity. However, it would be interesting to compare the VOC profiles of the two aetiologies to identify novel (and possibly shared) pathogenetic mechanisms, as indicated by intriguing observations in a murine NAFLD model[10].

In conclusion, our study shows that breath VOCs can be a potential non-invasive diagnostic tool in NAFLD. We have shown that VOCs such as DMS and D-limonene can differentiate NAFLD cirrhosis from healthy liver. Furthermore, isoprene and terpinene concentrations can distinguish between NAFLD patients with and without cirrhosis. Correlation of VOCs with histological stages of liver disease is now required, which can facilitate non-invasive stratification of NAFLD. It will also be of interest to perform a larger study to determine the contribution of portal hypertension to VOCs production. Through our pilot study, we highlight potential biomarkers which now require further external validation in larger populations of NAFLD patients with

histologically defined disease. In addition, a parallel study of VOCs and gut microbiome profiling can provide further mechanistic insights. The emerging artificial intelligence methodology with sophisticated algorithms may also generate new stage-specific breath signatures for diagnosis and monitoring of disease progression in NAFLD.

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Characteristics	NAFLD cirrhosis (n=15)	Non-cirrhotic NAFLD (n=14)	Healthy controls (n=14)	p-value
Women (%)	4 (27)	5 (36)	9 (64)	0.70
Age	69 (44 -76)	60 (29 -75)	39 (20-59)	<0.001***
Height (meters)	1.73 (1.56 -1.81)	1.72 (1.50 -1.87)	1.72 (1.54 -1.84)	0.93
Weight (kilograms)	96.4 (76 -118)	103.8 (73.4 -126.8)	61.45 (48.7 -74.7)	<0.001***
BMI (kg/m <sup>2</sup> )	34.2 (25.4 – 37.8)	35 (30.9 – 39.8)	21.6 (18.6 – 24.7)	<0.001***
Waist circumference (cm)				<0.001***
Women	110 (100 – 119)	106 (95 – 126)	74 (64.5 – 80)	
Men	110 (96 -130)	110 (106 -136)	80 (73 – 85)	
Smoking status (%)				0.16
Current smoker	1 (7)	2 (14)	1 (7)	
Ex-smoker	5 (33)	2 (14)	0 (0)	
Non-smoker	9 (60)	10 (72)	13 (93)	
Alcohol (units/week)	0 (0 – 10)	1 (0-7)	2.5 (0-10)	0.20
Coffee consumption(cups/day)	3 (0-7)	2 (0 -7)	2 (0-4)	0.15
Modality of diagnosis				0.16
Histology	2 (13)	5 (35)		
Non-invasive	13 (87)	9 (65)		
Elastography				
TE	17 (8.6) †	8.9 (5.1)	-	<0.001***
ARFI	3.2 (1.3) ‡	-	-	
Hyaluronic acid (µg/L)	200 (241) ±	40 (30)	-	<0.001***
Fibrosis score				
APRI score	0.42 (0.33)	0.24 (0.15)		0.007**
NAFLD fibrosis score	1.54 (1.17)	-0.38 (1.66)		0.017**
Fib-4 score	2.31 (1.15)	1.39 (0.77)		0.004**
BARD Score	4 (1)	3 (3)		0.082



## Laboratory parameters

HbA1c (IFCC)	54 (36 – 103)	49 (32 - 94)	-	1.00
HOMA	4.45 (2.7 -6.2) ^	5.35 (1.7 -6.9) ^^	-	0.91
GGT (U/L)	147 (44 -843)	61 (22 -387)	-	0.07
Albumin (g/L)	36 (31 - 40)	39 (35 - 44)	-	0.02**
AST (U/L)	32 (25 -71)	27 (21 -78)	-	0.27
ALT (U/L)	55 (14 – 69)	44 (16 -115)	-	0.14
Platelets (10 <sup>9</sup> /L)	210 (61- 271)	229 (108 -289)	-	0.14

**Table 1:** Baseline characteristics of study participants. Data presented as median and IQR or frequency (*n*) and percentage where appropriate. NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; TE, transient elastography; ARFI, acoustic radiation force impulse; IFCC, International Federation of Clinical Chemistry); HOMA, Homeostatic Model Assessment; GGT, gamma-glutamyl transpeptidase; AST, aspartate aminotransferase; ALT, alanine aminotransaminase, † (n=10); ‡(n=3); ±(n=13), ^ (n=2); ^^ (n=6)

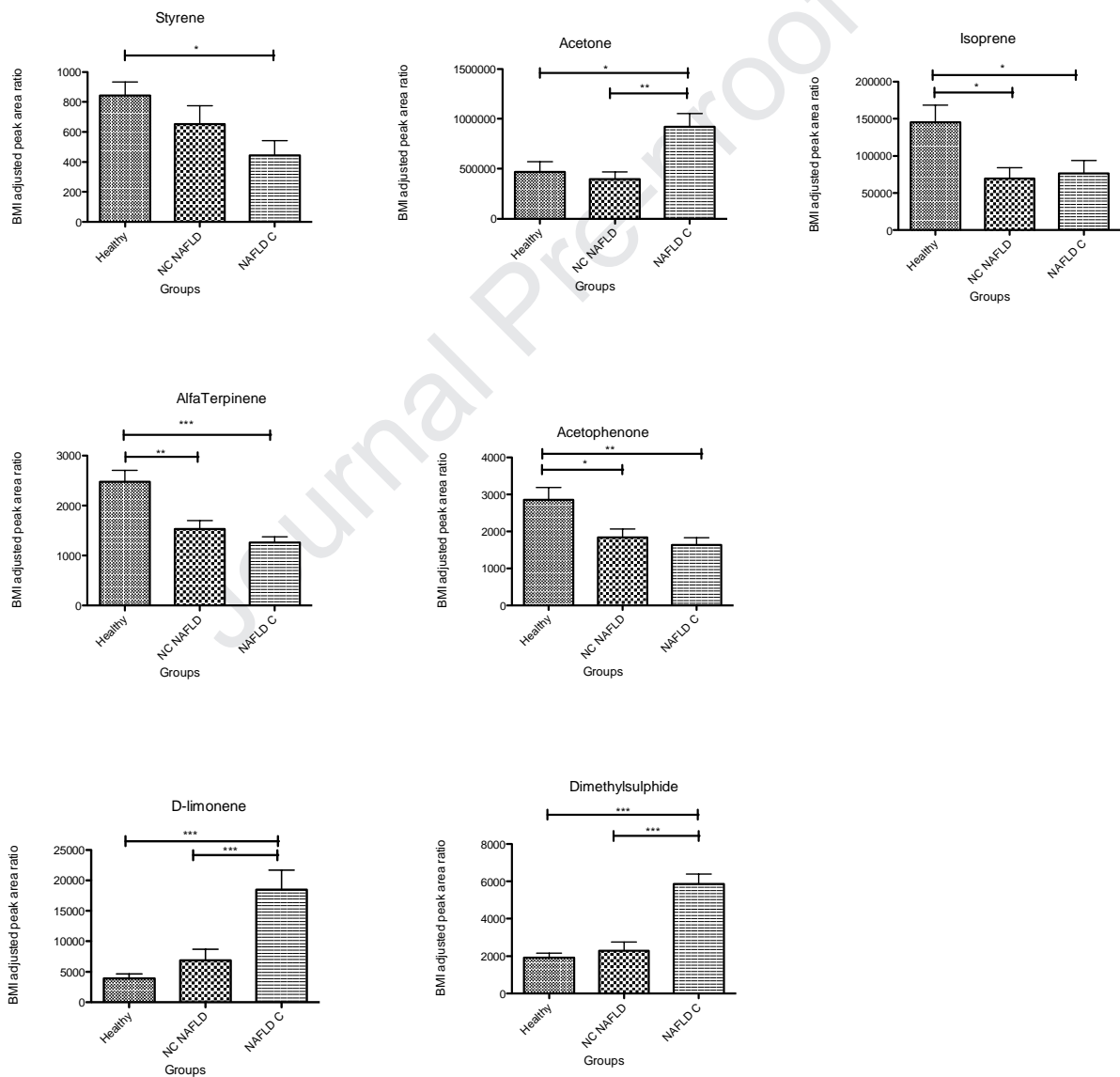


Figure 1: Levels of volatile organic compounds in exhaled breath in study participants. Healthy (healthy controls; *n*=14), non-cirrhotic NAFLD (NC NAFLD; *n*=14) and NAFLD cirrhosis (NAFLD C; *n*=15). Levels are adjusted for a unit toluene-d8 (internal standard reference) and body-mass index (BMI). Data expressed as mean with

standard error of mean. One-way analysis of variance (ANOVA) is performed with post-test Bonferroni correction. Significance is denoted as  $p < 0.05$  \*,  $p < 0.01$  \*\*,  $p < 0.001$  \*\*\*

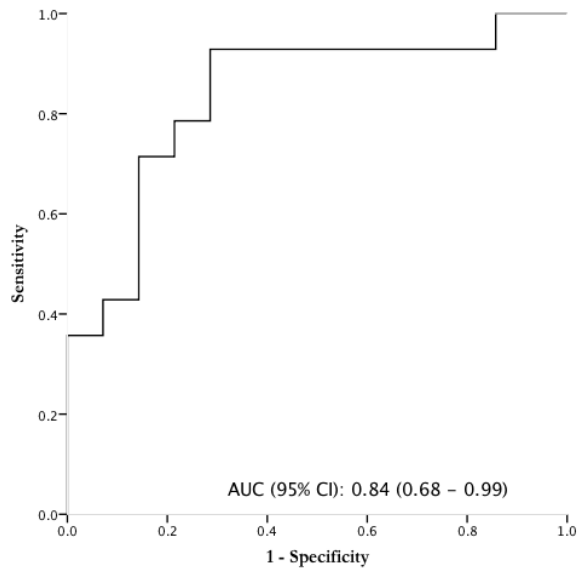


Figure 2. Receiver operating characteristic (ROC) curve for breath terpinene in classifying patients with non-cirrhotic NAFLD versus healthy participants. AUC, area under the ROC curve; NAFLD, non-alcoholic fatty liver disease; CI, confidence interval.

Volatile organic compounds	AUROC (95% confidence interval)	$p$ -value
isoprene	0.75 (0.57 – 0.94)	0.022
acetophenone	0.80 (0.63 – 0.97)	0.007
terpinene	0.84 (0.68 -0.99)	0.002**

Table 2: Area under receiver operating curves for different volatile organic compounds in classifying patients with non-cirrhotic NAFLD versus healthy participants. AUROC, area under receiver operating characteristic.

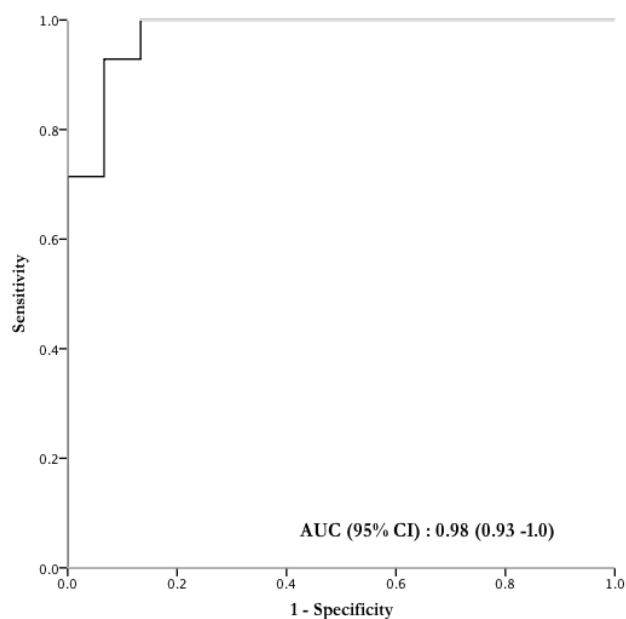


Figure 3. Receiver operating characteristic curve for a combination of D-limonene and dimethyl sulfide in classifying patients with NAFLD cirrhosis ( $n=15$ ) versus control subjects ( $n=14$ ). AUC, area under the ROC curve; NAFLD, non-alcoholic fatty liver disease; CI, confidence interval.

Volatile organic compounds	AUROC (95% confidence interval)	$p$ -value
styrene	0.37 (0.16 -0.58)	0.239
acetone	0.77 (0.58 -0.95)	0.015
isoprene	0.49 (0.27 – 0.72)	0.965
Dimethyl sulfide	0.94 (0.86 -1.00)	<0.001***
D-limonene	0.91 (0.79 -1.00)	0.002***
acetophenone	0.40 (0.12 – 0.61)	0.36
terpinene	0.34 (0.13 -0.54)	0.13

Table 3: Area under receiver operating curves for different volatile organic compounds to in classifying patients with NAFLD cirrhosis versus control subjects. AUROC, area under receiver operating characteristic.

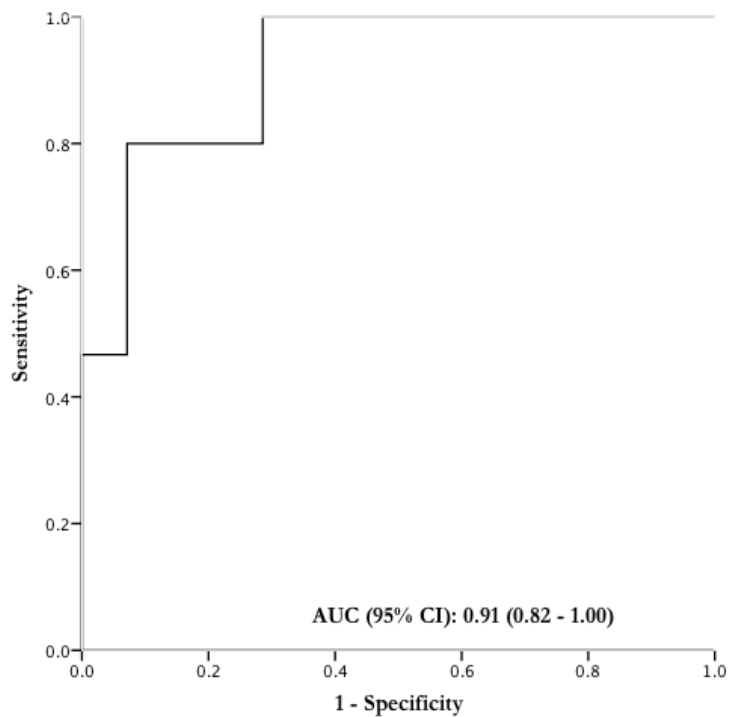


Figure 4: Receiver operating characteristic curve for a combination of D-limonene and dimethyl sulfide in classifying patients with NAFLD cirrhosis versus non-cirrhotic NAFLD. AUC, area under the ROC curve; NAFLD, non-alcoholic fatty liver disease; CI, confidence interval.

Volatile organic compounds	AUROC (95% confidence interval)	<i>p</i> -value
Dimethyl sulfide	0.87 (0.74 -1.00)	<0.001***
D-limonene	0.83 (0.68 -0.98)	0.002**
acetone	0.81 (0.63 -0.99)	0.005

Table 4: Area under receiver operating curves for different volatile organic compounds for classifying patients with NAFLD cirrhosis versus differentiate NAFLD cirrhosis from non-cirrhotic NAFLD. AUROC, area under receiver operating characteristic.

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## Highlights

- Metabolic dysfunction in liver disease is reflected in the bio-composition of exhaled breath
- Specific volatile organic compounds can be measured in breath samples (volatomics) and have diagnostic potential in chronic liver disease
- Levels of alfa-terpinene, dimethyl sulfide and D-limonene in exhaled breath may be used to stratify patients with non-alcoholic fatty liver disease