

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

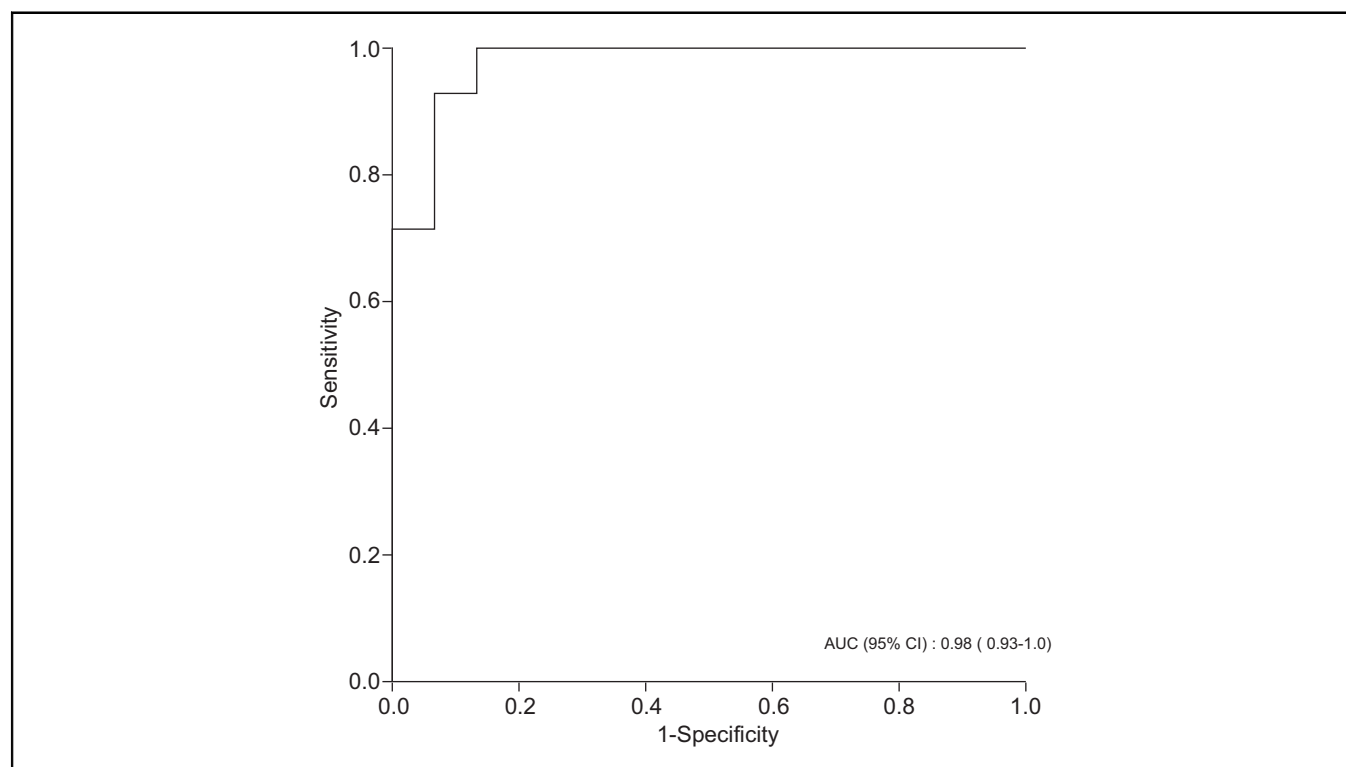
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Graphical abstract



Highlights

- Metabolic dysfunction in liver disease is reflected in the biocomposition 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 can be used to stratify patients with non-alcoholic fatty liver disease.

Lay summary

Breath malodour has been associated with a failing liver since the ancient Greeks. Analytical chemistry has provided us an insight into ubiquitous volatile organic compounds associated with liver (and other) diseases. This has vastly improved our understanding of the mechanistic processes of liver damage. Our study aims to identify volatile organic compounds which are specific to non-alcoholic fatty liver disease and that can be exploited for rapid diagnostics.

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



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JHEP Reports 2020. <https://doi.org/10.1016/j.jhepr.2020.100137>

Background & aims: 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. The purpose of this pilot study was to compare breath VOCs in an initial cohort of patients with non-alcoholic fatty liver disease (NAFLD) 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, normalised to an internal standard. Receiver-operating characteristic (ROC) curves were used to determine the diagnostic accuracy of the candidate VOCs.

Results: Terpinene, dimethyl sulfide, and D-limonene provided the highest predictive accuracy to discriminate between study groups. Combining dimethyl sulfide with D-limonene led to even better discrimination of patients with NAFLD cirrhosis from healthy volunteers (AUROC 0.98; 95% CI 0.93–1.00; $p < 0.001$) and patients with NAFLD cirrhosis from those with non-cirrhotic NAFLD (AUROC 0.91; 95% CI 0.82–1.00; $p < 0.001$). Breath terpinene concentrations discriminated between patients with 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; in addition, a 2-stage approach enables the differentiation of patients with cirrhosis from those without. However, these observations require validation in a larger NAFLD population. (ClinicalTrials.gov Identifier: NCT02950610).

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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 continue 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 used 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 characterise 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*, which can occur with metabolic derangements in cirrhosis. Similarly, NAFLD is associated with myriad metabolic changes that can influence the composition and concentration of VOCs. Although studies have shown changes in VOC composition in

Keywords: Volatile organic compounds; Non-alcoholic fatty liver disease; Volatomics; Terpinene; D-limonene; Dimethyl sulfide.

Received 11 December 2019; received in revised form 5 May 2020; accepted 31 May 2020; available online 15 June 2020

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obese individuals with NAFLD, 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](https://clinicaltrials.gov/ct2/show/study/NCT02950610) Identifier: NCT02950610), a single-centre prospective observational study that 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 participants with NAFLD 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 $>0.6/0.7$. For correlations to be statistically significant ($p < 0.05$) with 80% power, ~15 participants in each group were required. Given 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 as follows: known respiratory disease; severe obesity [body mass index (BMI) >40]; use of antibiotics for preceding 4 weeks; ongoing alcohol use of >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 the Siemens ultrasound system (Siemens AG, Erlangen, Germany), and serum hyaluronic acid (HA) measured using a radiometric assay (Pharmacia, Uppsala, Sweden), performed within 6 months of breath test.

NAFLD cirrhosis was diagnosed by liver biopsy in 2 participants, endoscopic features of portal hypertension in 12 participants, and radiological features in one participant.

Non-cirrhotic NAFLD was diagnosed by liver biopsy (in 5 participants) and the remaining 9 participants were diagnosed by clinical and radiological features, non-invasive scores, such as the Fibrosis-4 (Fib-4) score, aspartate aminotransferase:platelet ratio index (APRI), NAFLD fibrosis and BARD scores, serum HA, and transient elastography measurements.^{11–14}

Greater diagnostic weight was given to histological or endoscopic diagnoses (in the case of cirrhosis); in patients who

had a definitive diagnosis of cirrhosis, the non-invasive markers were not considered. The healthy control group comprised self-declared healthy participants. They were recruited through word-of-mouth and advertising posters in the institution. They were screened using questionnaire, physical examination, and reported absence of any medical illness and use of any regular medication.

Measures were taken to mitigate against the effect of diet, environmental factors, 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 were undertaken. Participants refrained from using any perfumes or deodorants on the day of their visit. Smoking and alcohol consumption were restricted for 48 h preceding the visit. 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 2 consecutive participants was at least 4 h.

Anthropometric measurement and venous sample collection were completed before breath sample collection. Central obesity was defined as a waist circumference >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 unchlorinated 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, KS, USA) with an inspiratory VOC filter (A2, North Safety, Middelburg, NL, USA) and an expiratory silica reservoir to dry the expired air. The breathing manoeuvres have been previously described.¹⁸ After 5 min of equilibration by tidal breathing with VOC-filtered air, the expiratory port was connected to a 10 L Tedlar sampling bag (SKC Inc., Eighty Four, 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 cmH₂O to close the soft palate and to obtain an expiratory flow of 0.1–0.2 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 an additional stable factor of a dedicated climate-controlled room, reduces dramatically the change of a false discovery. Every conceivable effort was undertaken to minimise contamination and external influence.

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

GC-MS sample analysis

GC-MS analysis was performed as previously described.²² Briefly, the content of the Tedlar bags was 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 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 described²⁰ (see the [supplementary data online](#)).

GC-MS QC

GC-MS was performed by an established commercial laboratory (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 quality control (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 (which 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 semitargeted 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 as listed by the International Union of Pure and Applied Chemistry (IUPAC) were: 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 alpha-terpinene (terpinene).

We used Xcalibur version 3.0 (Thermo Fisher Scientific, Waltham, 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 >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, www.amdis.net/index.html).

In our experience, general automated peak picking is faster than manual peak selection and integration, especially for large data sets. Our previous published work was 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. The peak was recorded as not detected if the ion count was <500 ion counts at the assigned retention time. All compound peak areas were normalised to the peak area of

toluene-d8 in each sample, because 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, USA) and GraphPad Prism version 5.0a (San Diego, 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 minimise false discovery. Multivariable logistic regression analysis was performed to build a model for the prediction of cirrhosis and non-cirrhotic liver disease; all breath compounds were considered for inclusion. Discrimination was used for internal model validation; that is, measuring the ability to rank patients by risk of cirrhosis, such that patients with a higher predicted risk were 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 2 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 based on patients who 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 <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 National Health Service (NHS) Lothian Research and Development Department (Ref: E151593).

Results

Baseline characteristics

Baseline characteristics of the study population are summarised in [Table 1](#). Two samples (1 patient with non-cirrhotic NAFLD and 1 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 [6 (40%) varices, 4 (27%) portal hypertensive gastropathy (PHG), and 1 (6%) each for gastric antral vascular ectasia (GAVE) and dilated azygous vein on

Table 1. Baseline characteristics of study participants.

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 (years)	69 (44–76)	60 (29–75)	39 (20–59)	<0.001
Height (m)	1.73 (1.56–1.81)	1.72 (1.50–1.87)	1.72 (1.54–1.84)	0.93
Weight (kg)	96.4 (76–118)	103.8 (73.4–126.8)	61.45 (48.7–74.7)	<0.001
BMI (kg/m ²)	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 ⁹ /L)	210 (61–271)	229 (108–289)	–	0.14

Data presented as median and IQR or frequency (n) and percentage where appropriate. Student's *t* test or Mann-Whitney *U* test were used to compare normally distributed continuous variables and non-parametrically distributed data, respectively.

ALT, alanine aminotransaminase; ARFI, acoustic radiation force impulse; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyltransferase; HOMA, homeostatic model assessment; NAFLD, non-alcoholic fatty liver disease; TE, transient elastography.

[†] n = 10.

[‡] n = 3.

[‡] n = 13.

[~] n = 2.

^{^^} n = 6.

endoscopic ultrasound]. Of the remaining 4 (20%) who did not have endoscopic evidence of portal hypertension, 2 had coarse liver echotexture with splenomegaly on ultrasound and 2 had coarse liver echotexture alone.

Quantification of exhaled VOCs in the study population

Of the 19 VOCs studied in the exhaled breath samples, 7 compounds (styrene, acetone, isoprene, DMS, D-limonene, acetophenone, and terpinene) were significantly different between the groups (Fig. 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 in 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 inclusion into the statistical modelling.²⁷ Significant differences persisted between the group with cirrhosis and the healthy control group 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 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 $r = 0.7$, $p < 0.001$). By 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 HA or transient elastography data (data not shown).

Effect of diabetes on VOC production

In the group with cirrhosis, 13 participants (87%) had type 2 diabetes mellitus with HbA1c International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) ranging from 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, 8 participants (57%) had type 2 diabetes mellitus (T2DM) with HbA1c (IFCC) ranging from 45 mmol/mol to 94 mmol/mol and 6 (43%) participants had insulin resistance with HOMA of Insulin Resistance (HOMA-IR) ranging from 1.7 to 6.9. It is conceivable that insulin resistance and/or T2DM had contributed to the high level of acetone, particularly in the group with cirrhosis. We also noted that the actual differences in breath acetone were probably bigger than measured here because the concentration of acetone recorded was outside the linear range of the instrument.

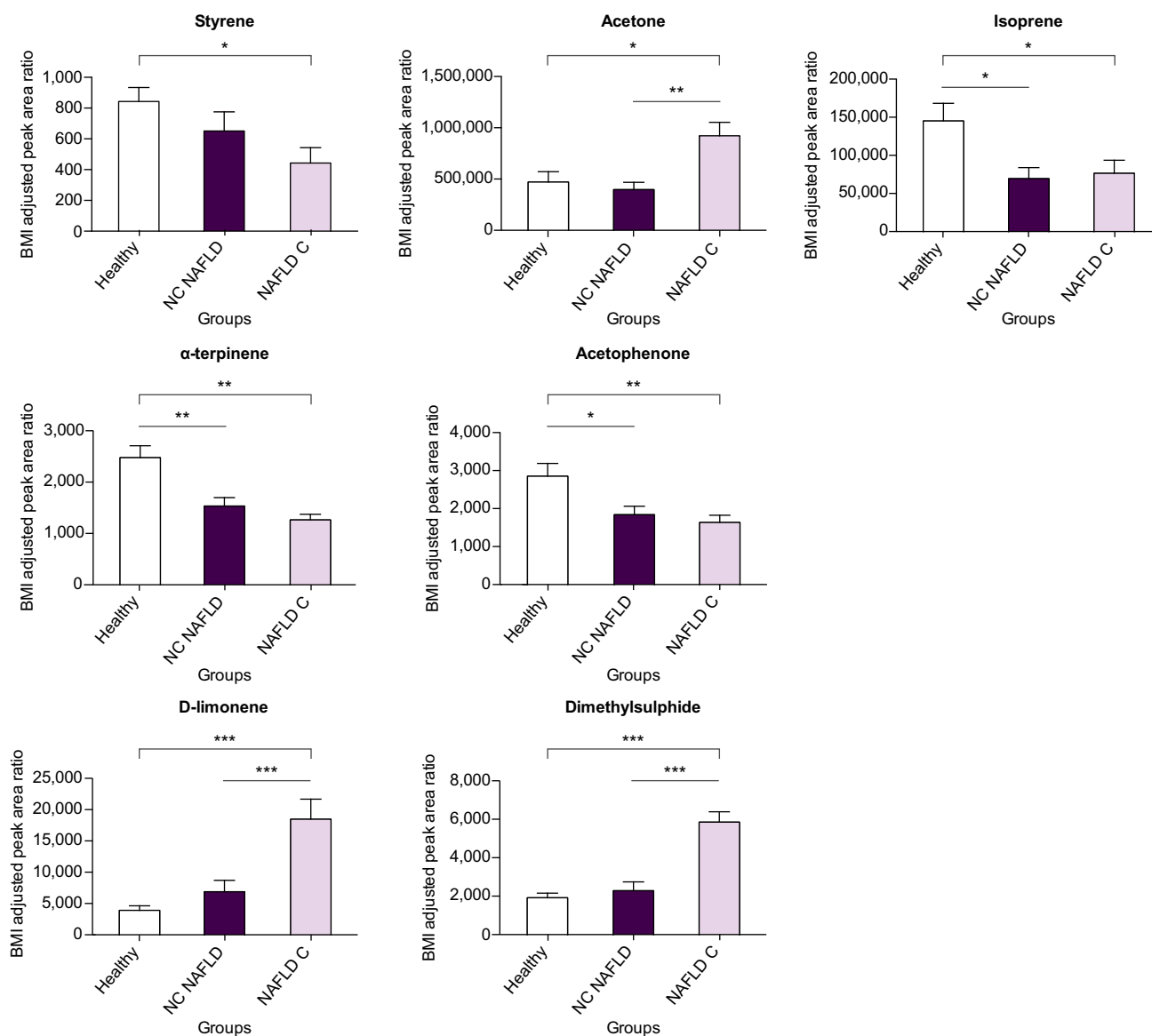


Fig. 1. Levels of volatile organic compounds in exhaled breath in study participants. Healthy (healthy controls; n = 14), NC NAFLD (n = 14) and NAFLD C (n = 15). Levels were adjusted for a unit toluene-d8 (internal standard reference) and BMI. Data expressed as mean with standard error of mean. A one-way ANOVA was performed with post-test Bonferroni correction. Significance is denoted as **p* <0.05, ***p* <0.01, and ****p* <0.001. BMI, body mass index; NAFLD C, non-alcoholic fatty liver disease with cirrhosis; NC NAFLD, non-cirrhotic non-alcoholic fatty liver disease.

Differentiating patients with non-cirrhotic NAFLD from healthy control participants

Levels of isoprene, acetophenone, and terpinene were significantly lower in patients with non-cirrhotic NAFLD 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 Fig. 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. By contrast, levels of DMS and D-limonene were significantly higher in patients with NAFLD cirrhosis compared with healthy controls. DMS and

Table 2. AUROC for different volatile organic compounds for classifying patients with non-cirrhotic NAFLD vs. healthy participants.

Volatile organic compound	AUROC (95% CI)	<i>p</i> 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

Discrimination used for internal model validation - ranking patient with non-cirrhotic NAFLD was measured by AUROC. AUROC, area under receiver operating characteristic; NAFLD, non-alcoholic fatty liver disease.

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) (Fig. 3).

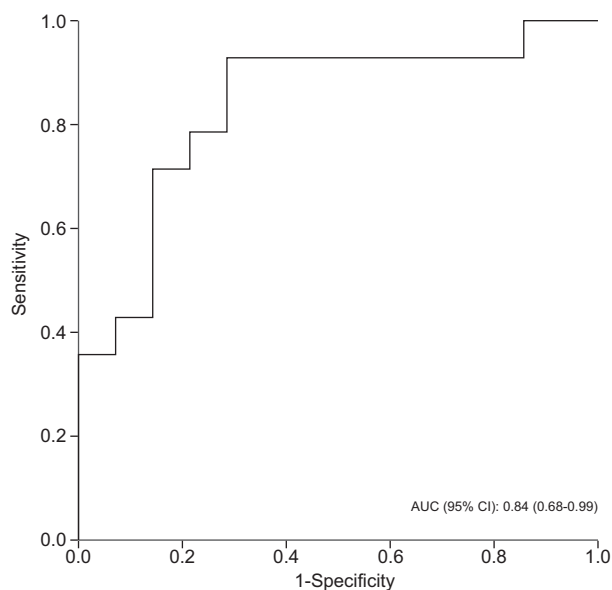


Fig. 2. AUROC curve for breath terpinene in classifying patients with non-cirrhotic NAFLD ($n = 14$) vs. healthy participants ($n = 14$). Discrimination used for internal model validation - ranking patient with non-cirrhotic NAFLD was measured by AUROC. AUROC, area under the receiver operating curve; NAFLD, non-alcoholic fatty liver disease.

Table 3. AUROC for different volatile organic compounds for classifying patients with NAFLD cirrhosis vs. healthy participants.

Volatile organic compound	AUROC (95% CI)	<i>p</i> 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

Discrimination used for internal model validation - ranking patient with NAFLD cirrhosis was measured by AUROC. AUROC, area under receiver operating characteristic; NAFLD, non-alcoholic fatty liver disease.

Differentiating patients with non-cirrhotic NAFLD from those with NAFLD cirrhosis

Levels of DMS and D-limonene were significantly higher in patients with NAFLD cirrhosis compared with those with non-cirrhotic NAFLD. DMS and D-limonene had the highest predictive accuracy for NAFLD cirrhosis, with AUROCs of 0.87 (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, $p < 0.001$) (Fig. 4).

Discussion

In this pilot study, we examined the concentration of VOCs in exhaled breath in adult patients with NAFLD with cirrhosis and those with non-cirrhotic NAFLD. We highlighted 3 compounds (acetone, DMS, and D-limonene) that differentiated between patients with cirrhosis and healthy controls. More importantly, we showed that DMS and D-limonene can discriminate between patients with NAFLD and with or without cirrhosis. From a clinical standpoint, the findings are important because these compounds could serve as biomarkers for the stratification of NAFLD.

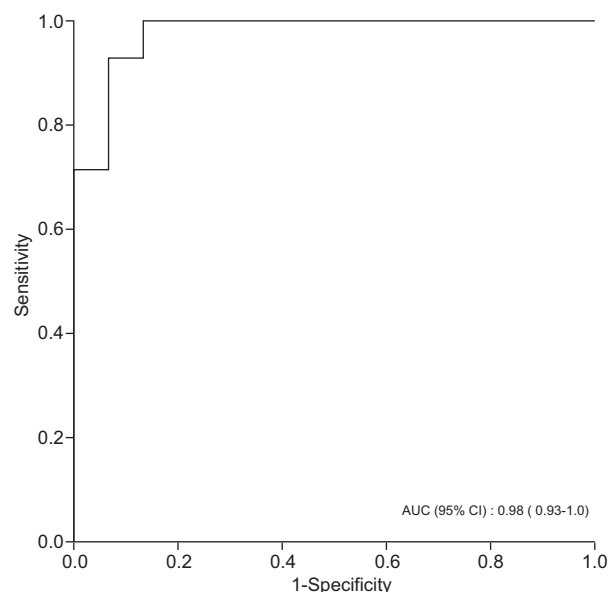


Fig. 3. AUROC curve for a combination of D-limonene and dimethyl sulfide in classifying patients with NAFLD cirrhosis ($n = 15$) vs. healthy participants ($n = 14$). Discrimination used for internal model validation - ranking patient with NAFLD cirrhosis was measured by AUROC. AUROC, area under the receiver operating curve; NAFLD, non-alcoholic fatty liver disease.

Table 4. AUROC for different volatile organic compounds for classifying patients with NAFLD cirrhosis vs. those with non-cirrhotic NAFLD.

Volatile organic compounds	AUROC (95% CI)	<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

Discrimination used for internal model validation - ranking patient with NAFLD cirrhosis was measured by AUROC. AUROC, area under receiver operating characteristic; NAFLD, non-alcoholic fatty liver disease.

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 focussed solely on patients with NAFLD. Furthermore, we adjusted the measured VOC to BMI to provide a more accurate reflection of the metabolic profile because 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 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

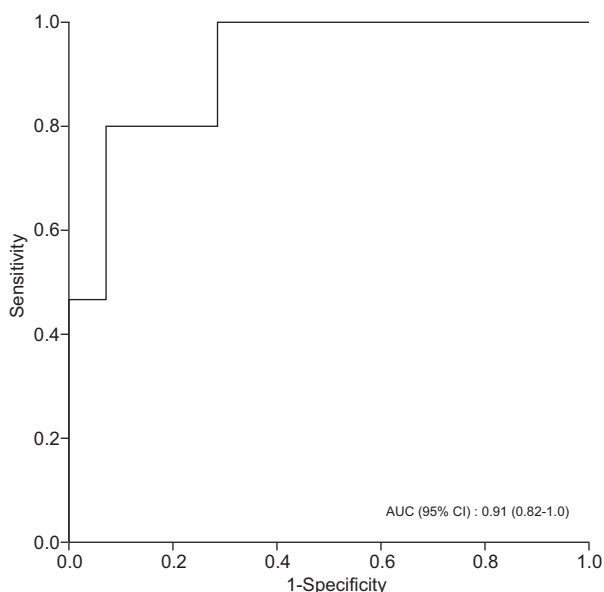


Fig. 4. AUROC curve for a combination of D-limonene and dimethyl sulfide in classifying patients with NAFLD cirrhosis (n = 15) vs. non-cirrhotic NAFLD (n = 14). AUROC, area under the receiver operating curve; NAFLD, non-alcoholic fatty liver disease.

concentrations. Although fasting might have influenced the overall levels of acetone, we showed that the levels of acetone were substantially higher in patients with cirrhosis 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 reflect either inefficient metabolism (reduced levels of Cytochrome P450 enzymes; CYP2C9 and CYP2C19) that results in bioaccumulation or 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 have previously been reported in the context of cirrhosis, our study is the first to confirm its presence in exhaled breath in the context of NAFLD cirrhosis.^{6,8,26,28} D-limonene is also a major constituent in citrus essential oil, which is used in various foods as a flavouring agent. Although a 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 portosystemic shunting.³⁴⁻³⁶ Our findings support this notion because 80% of the participants with NAFLD cirrhosis in our study had signs of portal hypertension on gastroscopy. All of the participants with cirrhosis were Child-Pugh class A without clinical signs of encephalopathy. We speculate that a probable source of DMS is the microbiota, given that previous studies have linked this to oral as well as gut dysbiosis.^{37,38} Methanethiol can be converted to hydrogen

sulfide and oxidised to sulfate for detoxification, which can be utilised by sulfate-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 patients with cirrhosis and NAFLD or with non-cirrhotic NAFLD from healthy controls.⁷ There is little in the published literature about terpinenes, and their role in NAFLD pathophysiology is unknown. However, they have been implicated in oxidative stress pathways.⁴⁰ Thus, it is possible that the differences in the levels of alpha-terpinene between healthy controls and participants with NAFLD reflect the presence of oxidative stress in NAFLD.

Although we cannot totally exclude the possibility that medication might have influenced the breath concentrations of terpinenes, we mitigated this through medication reconciliation at screening. We speculated from the outset that CYP activity would be impaired and drug metabolism (and interactions) would be complex. Therefore, attempts were made to maintain the 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 taking enzyme inducers or inhibitors. Therefore, it is unlikely that any of the concomitant medication used by the participants could have influenced the terpenes, either directly or indirectly.

There were some limitations to our study. First, the participants were primarily selected based on clinical characterisation rather than exclusively on histological criteria, because this was a pragmatic observational pilot study. As such, it is likely that the non-cirrhotic NAFLD group comprised 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 Child-Pugh 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 might have had hepatic steatosis or even NASH.⁴² The small sample size and case selection limitations mean that the findings might not be generalisable to the wider population with NAFLD.

Second, although the participants underwent an overnight fast, the potential impact of diet and dietary preferences on the breath VOCs cannot be completely excluded. Third, our data were cross-sectional and derived from a single-centre. Our findings will require further validation in external (longitudinal) cohorts of patients with NAFLD. Finally, we performed semitargeted rather than untargeted analysis; thus, selection bias cannot be completely excluded. However, there is a risk in 'omics data that the lowest responders are lost because the sensitivity of untargeted analysis is not the strength of the technique. Therefore, our approach 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, because we studied a semitargeted analysis of VOCs in the context of NAFLD, it is possible that some VOCs with a pathogenic role in the NAFLD were missed in our analysis.

We intentionally did not study alcoholic liver disease because the pathogenesis and 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 2 aetiologies to identify novel (and possibly shared) pathogenetic mechanisms, as indicated by intriguing observations in a murine NAFLD model.¹⁰

Conclusion

In conclusion, our study revealed breath VOCs as a potential non-invasive diagnostic tool in NAFLD. We showed that VOCs, such as DMS and D-limonene, can differentiate NAFLD

cirrhosis from healthy liver. Furthermore, isoprene and terpinene concentrations can distinguish between patients with NAFLD 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 have highlighted potential biomarkers that now require further external validation in larger populations of patients with NAFLD with histologically defined disease. In addition, a parallel study of VOCs and gut microbiome profiling would provide further mechanistic insights. The emerging artificial intelligence methodology with sophisticated algorithms could also generate new stage-specific breath signatures for the diagnosis and monitoring of disease progression in NAFLD.

Abbreviations

ALT, alanine aminotransaminase; APRI, aminotransferase:platelet ratio index; ARFI, acoustic radiation force impulse; AST, aspartate aminotransferase; AUROC, area under the receiver-operating characteristics curve; BMI, body mass index; GAVE, gastric antral vascular ectasia; GC-MS, gas chromatography mass spectrometry; GGT, gamma-glutamyltransferase; HA, hyaluronic acid; HOMA, homeostatic model assessment; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PHG, portal hypertensive gastropathy; QC, quality control; T2DM, type 2 diabetes mellitus; TE, transient elastography; VOCs, volatile organic compounds.

Financial support

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

Conflicts of interest

The authors have no conflicts of interest pertaining to this study to declare.

Please refer to the accompanying ICMJE disclosure forms for further details.

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.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2020.100137>.

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Author names in bold designate shared co-first authorship

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