Original Article

Breath Biopsy® to Identify Exhaled Volatile Organic Compounds Biomarkers for Liver Cirrhosis Detection

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Abstract

Background and Aims: The prevalence of chronic liver disease in adults exceeds 30% in some countries and there is significant interest in developing tests and treatments to help control disease progression and reduce healthcare burden. Breath is a rich sampling matrix that offers non-invasive solutions suitable for early-stage detection and disease monitoring. Having previously investigated targeted analysis of a single biomarker, here we investigated a multiparametric approach to breath testing that would provide more robust and reliable results for clinical use. ***Methods:*** To identify candidate biomarkers we compared 46 breath samples from cirrhosis patients and 42 from controls. Collection and analysis used Breath Biopsy OMNI™, maximizing signal and contrast to background to provide high confidence biomarker detection based upon gas chromatography mass spectrometry (GC-MS). Blank samples were also analyzed to provide detailed information on background volatile organic compounds (VOCs) levels. ***Results:*** A set of 29 breath VOCs differed significantly between cirrhosis and controls. A classification model based on these VOCs had an area under the curve (AUC) of 0.95±0.04 in cross-validated test sets. The seven best performing VOCs were sufficient to maximize classification performance. A subset of 11 VOCs was correlated with blood metrics of liver function (bilirubin, albumin, prothrombin time) and separated patients by cirrhosis severity using principal component analysis. ***Conclusions:*** A set of seven VOCs consisting of previously reported and novel candidates show promise as a panel for liver disease detection and monitoring, showing correlation to disease severity and serum biomarkers at late stage.

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Introduction

Progression of chronic liver diseases to cirrhosis is often asymptomatic, with more than 50% of the cases diagnosed at advanced stages with clinical decompensation, when therapeutic interventions are often ineffective for preserving liver function.[1,2] Early detection is still limited by available diagnostic tests, which are inadequate for population screening or lack the required sensitivity or specificity. The gold standard remains liver biopsy despite the proliferation of non-invasive surrogate imaging techniques and serological markers.[3] These tests also rely on anatomical alterations rather than hepatic function and are mainly effective at advanced stages.[2–4] Additionally, absence of non-invasive functional tests represents a hurdle for clinical outcome evaluation of therapeutic interventions.

Analysis of volatile organic compounds (VOCs) in exhaled breath represents an emerging diagnostic approach with the potential to develop functional, non-invasive tests for early detection based on the reduced hepatic function associated with chronic liver diseases, especially clearance and protein synthesis capacity.[5–8] Impaired hepatic function shifts the spectrum of compounds detoxified by the liver. A subset of these can be measured in breath as VOCs and are potential biomarkers.[8–13] In our previous study,[8] limonene, an exogenous

Table 1. Subject characteristics

Characteristic	Control	Cirrhosis	*p*-values
Patients, *n*	42	46 (14 with HCC)	
Age in years, median (range)	62 (34–81)	58.5 (35–79)	0.61
Male/female sex	21/21	29/17	
Height median, cm (range)	170 (157–191)	173 (150–197)	0.67
Weight median, kg (range)	76 (51–149)	85 (48–165)	0.03
BMI median, kg/m^2 (range)	25.5 (18.9–46.0)	29.7 (18.0–43.4)	0.01
Child-Pugh class A/B/C/na*	–	30/12/1/3	
MELD median (range)	–	8 (7–19)	
UKELD median (range)	–	48.1 (44.7–60.5)	
Total bilirubin median, µmol/L (range)	–	17 (7–86)	
Serum albumin median, g/L (range)	–	35 (24–45)	
INR median, % (range)	–	1.07 (0.82–1.78)	
ALT median, IU/L (range)	–	27 (14–105)	
ALP median, IU/L (range)	–	100 (40–440)	
Creatinine, µmol/L	–	67 (38–147)	
Sodium, mM	–	139 (126–144)	

*Some blood metrics were not available for three patients. HCC, hepatocellular carcinoma; BMI, body mass index; MELD, model of end-stage liver disease; UKELD, the United Kingdom model for end-stage liver disease; INR, international normalized ratio; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

VOC taken-up mainly through the diet, was elevated in the breath of patients with cirrhosis and had diagnostic potential in agreement with previous exploratory studies.[10,14] This compound is metabolized in the liver by the enzymes CYP2C9 and CYP2C19 to trans-carveol and perillyl alcohol.[15] In the cirrhotic liver, reduced activity of these enzymes impairs hepatic clearance, resulting in extended limonene half-life in the bloodstream, which in turn, raises its abundance in the breath.[10] We additionally showed that limonene correlated with blood metrics that reflect hepatic clearance and protein synthesis capacity, indicating that a limonene breath test could be applied as functional means for chronic liver disease detection.[8]

Nonetheless, the complexity of hepatic metabolic pathways does not allow a comprehensive evaluation of liver function from a single biomarker. Thus, combination of multiple VOCs generated by alterations of different metabolic pathways provides a more exhaustive picture of the liver condition, improving diagnostic performances of a breath test and providing the potential to detect early-stage liver disease. To these purposes, we used Breath Biopsy OMNI global VOC analysis to discover differentially abundant VOCs in patients with cirrhosis, compared to controls in order to identify potential disease-related biomarkers that could be used to diagnose patients with progressive liver disease, from early to end stage, in large-scale cohorts.

Methods

Study design and subjects

This cross-sectional case-control study was part of the Owlstone Medical (Cambridge, UK) and Cancer Research UK (CRUK) funded PAN-study (NCT03756597, US National Library of Medicine), and was approved by the ethics committee of the East of England–Cambridge East Research Ethics Committee (REC reference: 18/EE/0041. IRAS ID: 237560). All participants provided written informed consent. A total of 46 subjects with cirrhosis, and 42 controls were enrolled with random recruitment. Subjects >30 years of age were recruited from the clinical research facility at Addenbrooke's Hospital (Cambridge) or through the Cambridge BioResource. Patients had an established histological or radiological diagnosis of cirrhosis according to European Association for the Study of the Liver and American Association for the Study of Liver Diseases guidelines.[16,17] Disease severity was classified using the Child-Pugh (CP) scoring system.[18] Per-protocol only patients with CP class A or B were eligible for the study regardless of disease etiology. Patients who developed hepatocellular carcinoma (HCC) in the context of cirrhosis were not receiving any anticancer treatment at the time of sampling. Control subjects had no known liver disease and were excluded if they were under medical investigation or had a history of non-skin malignancy in the last 2 years. No dietary restrictions were applied to any participant. Data about the participants are reported in Table 1.

Breath sampling

Acquisition of Breath Biopsy samples was performed by using the ReCIVA® Breath Sampler (Owlstone Medical) as previously reported.[8,19] Detailed methods are available in the Supplementary File 1.

Analytical measurements

Breath samples were analyzed using Breath Biopsy OMNI global VOC analysis as previously described.[8] Detailed methods are available in the Supplementary File 1.

Feature extraction

Raw data files, collected in profile mode, were centroided using the peak picking vendor algorithm of the ProteoWizard-MSConvert application.[20] The centroid data were imported into MZmine 2.53 to proceed with feature extraction workflow.[21] ADAP chromatogram builder module was used to de-

tect the peak with the a minimum highest intensity of 1.0E5 and m/z tolerance of 5 PPM.[22] Detected features were deconvoluted using the Wavelet ADAP algorithm with the parameters S/N of 3, S/N estimator wavelet coefficient. SN; coefficient/area threshold of 100, peak duration range of 0–1.0 m, and RT wavelet range of 0–0.15 m. Hierarchical clustering was used to combine peaks into analytes and construct fragmentation spectra for each analyte as described by Smirnov, *et al*.[23] ADAP Aligner (GC) was used for retention time alignment, with a minimum confidence of 0.1, an RT tolerance of 0.5 m, an m/z tolerance of 5 ppm, score threshold of 0.75, and a score weight of 0.1. Retention time similarity was calculated using the retention time difference (fast) method. Compounds were tentatively identified using the National Institute of Standards and Technology (NIST) library and in-house high resolution accurate mass (HRAM) library (https://www.owlstonemedical.com/about/blog/2022/may/30/biomarker-analysis-breath-hram-library/). Database hits were filtered according to their structure relevance to standard compounds. Identity of compounds was validated by standards injection (R)-(+)-Limonene,[9–12,14,24,25] (183164, (Sigma Aldrich, Gillingham, UK), 2-pentanone,[9,10,24,26] (471194; Sigma Aldrich), dimethyl selenide,[9,26] (41572; Sigma Aldrich), Indole (I3408; Sigma Aldrich), and eucalyptol (29210; Sigma Aldrich).

Data handling and statistical analysis

Data were analyzed by the Python and R programming languages (Python Software Foundation, Python Language Reference, version 2.7. http://www.python.org and R Core Team, 2021, R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org). Data visualization was performed with matplotlib,[27] seaborn,[28] and ggplot2,[29] libraries.

Intensity of VOCs (count/m), median, and range were compared between groups using the Mann-Whitney U-test for non-parametric data, and *p*-values <0.05 were considered statistically significant unless stated otherwise. A total of 11 VOCs with a Benjamin-Hochberg adjusted *p*<0.1 advanced to investigation for correlation with blood metrics of liver function. Data was pre-processed as described below and breath-sample assay results were averaged. Correction for instrument fluctuation was obtained by probabilistic quotient normalization.[30] Log transformation was performed to bring VOC intensity closer to a normal distribution.

To build a classification model, first dimensionality reduction was performed by considering as candidate biomarkers only features identified as statistically significant in the univariate analysis (*p*<0.05) between cirrhosis and controls. This selection reduced the feature space that any model needs to search, and thus the likelihood of unstable results, or overfitting.[31] The top VOCs for classification were identified by stability selection and LASSO logistic regression.[32] Model performance was validated by five-fold cross validation, with VOC selection performed within each fold training set. Only features above a certain stability score were selected in the model. The importance of each feature for classification was expressed as the resulting average stability score across the five-fold cross validation.

Stability selection chooses the features that behave consistently through permutations of a dataset. In the context of a clinical test, it may be important to reduce the number of features/VOCs required for further measurement. To this end, an analysis was conducted to observe how few features with similar AUCs could be obtained compared with the stable model. Step-forward feature selection,[33] with a simple five-fold cross validation was used to obtain the best performing models with one or two features, or so on, minimizing the number of VOCs necessary for a potential breath test.

Starting from a feature set size of one, classification performance of combinations of candidate biomarkers expressed as AUCs was measured by adding one new VOC at a time to the model.

Within the cirrhosis group, the relationship between blood bilirubin, albumin and international normalized ratio (INR), and breath metrics was assessed by canonical correlation analysis (CCA).[34] CCA finds the relationship between two multivariate sets of variables measured for the same set of samples and was considered as the extension of bivariate correlations. The identified new bases (new directions) for each data set were a linear combination of the original parameters. The new bases (i.e., canonical variates) represented maximized correlations of the original parameters in two datasets (i.e., VOCs and blood metrics). The resulting CCA score plot was generated using statistically significant canonical variates. In the score plot, each point corresponded to the combined information from breath and blood samples collected from the same patient. The shape of the projected data point indicates the correlation between two blocks of data, which were VOCs in breath and blood metrics. The contribution of the original parameters to the correlation between the two blocks of variables was estimated by calculating the canonical loadings, which expressed the correlation between the original variable and the canonical variate. Variance of selected features in relation to severity of cirrhosis was assessed by principal component analysis (PCA).

Results

Subject characteristics

Breath samples were collected from 42 controls, mean 62 (range 34–81) years of age, 21 men and 21 women, and 46 patients with cirrhosis (14 complicated by HCC, mean 58.5 (range 35–79) years of age, 29 men and 17 women. Subject characteristics are shown in Table 1, and in Ferrandino *et al*.[8] An unbalanced sex ratio was observed in the cirrhosis group, consistent with the known male preponderance of liver disease. However, no sex-linked differences in exhaled VOCs were identified (Supplementary Fig. 1) or have been previously described.[8,25,35] Significant differences in body weight (*p*=0.03) and body mass index (BMI, *p*=0.01) were observed between the study groups.

Generation of the discovery dataset

Automated feature extraction performed on a total of 478 ion chromatograms, 176 breath samples measured in duplicate, 123 calibration standards, 109 quality checks, and 70 blanks, resulted in a data frame of 2,593 molecular features (MFs). Of these, 768 had <50% missing values. Discriminatory potential between study groups of environmental contaminants may originate from spurious correlations between variables generated by data normalization.[36] To exclude those contaminants, we compared the intensity of each MF with those measured in blanks. A total of 196 MFs referred to as VOCs were significantly elevated in breath compared with blanks (*p*<0.1, Mann-Whitney U-test, and positive fold change in breath) and were used to generate the discovery dataset. An initial quality control based on these VOCs was constructed by PCA and separated breath samples from blanks over the first two principal components, and explained 24.1% and 14.4 % of data variance, respectively (Supplementary Fig. 2)

Identification of VOCs associated with cirrhosis

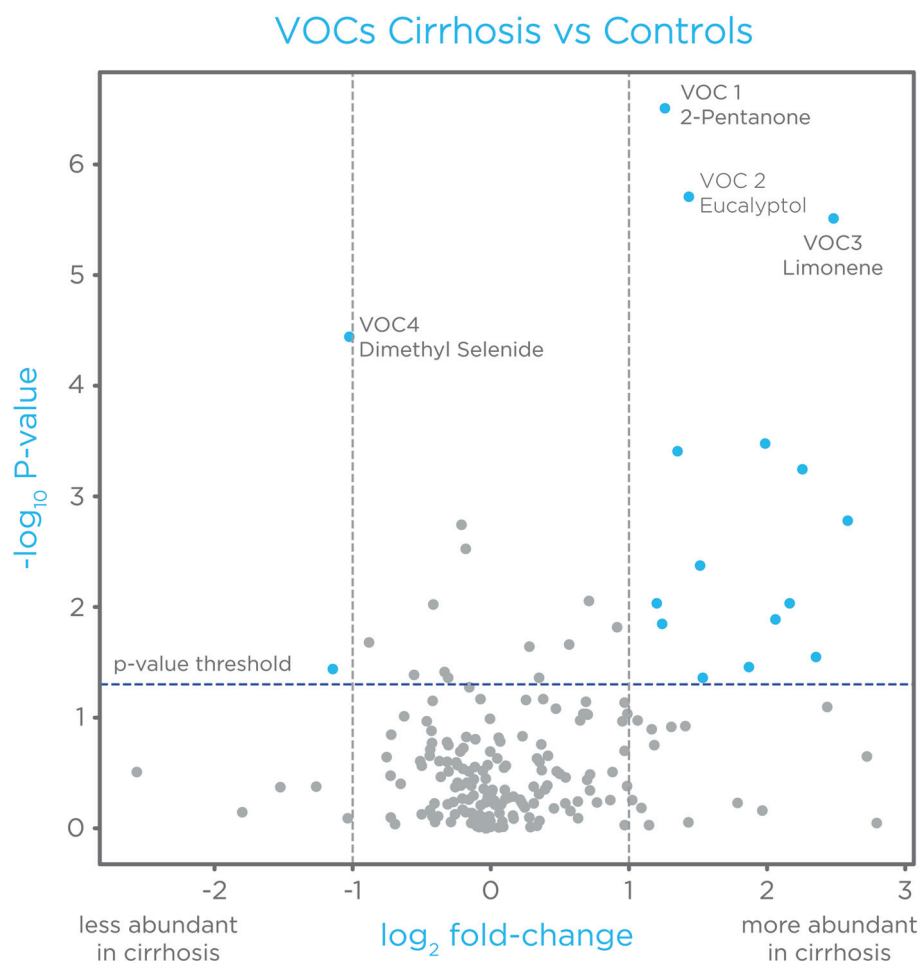An exploratory, cirrhosis-associated VOC profile generated

VOCs Cirrhosis vs Controls

Fig. 1. Volcano plot of exhaled VOCs. The X-axis represents the $\log_2$ mean ratio fold-change of the relative abundance of each VOC between cirrhosis and controls. The Y-axis represents the *p*-value of each VOC. Compounds with fold-change >2 and *p*<0.05 are highlighted in blue. Limonene and 2-pentanone were elevated in the breath of patients with cirrhosis and dimethyl selenide was reduced, as expected. VOC, volatile organic compound.

by univariate analysis with uncorrected *p*-values of <0.05 (Mann-Whitney U-test) and log fold-change ≥ 2, resulted in 15 upregulated and 2 downregulated VOCs in the breath of cirrhosis patients compared with controls (Fig. 1). As expected, limonene (*m/z* 91.05, RT ~11 m), and 2-pentanone (*m/z* 71.04, RT ~5 m) were upregulated, and dimethyl selenide (m/z 109.96, RT ~3 min), was downregulated in the breath of patients with cirrhosis (Fig. 1) as previously reported.[9–12,14,24–26]

Estimation of classification performance

A total of 29 on-breath VOCs were differentially abundant (unadjusted *p*-value <0.05, Mann-Whitney U-test) between controls and cirrhosis groups (Fig. 2) and were first used to generate individual receiver operating characteristic (ROC) plots to estimate individual diagnostic performance predicting the presence of cirrhosis. The top four compounds with AUC of 0.82, 0.80, 0.79, and 0.76 are shown in Figure 3. Subsequently, the performance of combinations of VOCs was explored. A stratified five-fold cross validation train/test split of 70%/30% was performed to build a classification model. In the training sets, the model returned an average AUC of 0.99±0.00 (Fig. 4A), and in the test sets, an average AUC of 0.95±0.04 (Fig. 4B). The corresponding confusion matrix

generated by cross-validated predictions (Fig. 4C) had three false positives (7%) that were misclassified because of elevated levels of limonene or 2-pentanone. Of the eight false negatives (17%), six were CP class A, two were CP class B, and three had HCC with CP class A.

To estimate a trade-off between the number of features to measure, and classification performance in a potential breath test, we used step-forward feature selection. Average AUC plateaus after the first seven features (Table 2) were added to the model (Fig. 4D). Identity of these top performing VOCs is reported in Table 2 and their detailed fragmentation pattern is reported in Supplementary Table 1. Additionally, potential effects of cirrhosis comorbidities, namely, obesity (BMI>30), type 2 diabetes, HCC, and portal hypertension on these seven top performing VOCs were explored. Significantly increased limonene and 2-pentanone were found in patients with portal hypertension (*n*=7), and significantly increased indole with obesity (*n*=23, Supplementary Table 2). No significant differences were found for the other comorbidities.

Identification of breath compounds correlated with hepatic function in subjects with cirrhosis

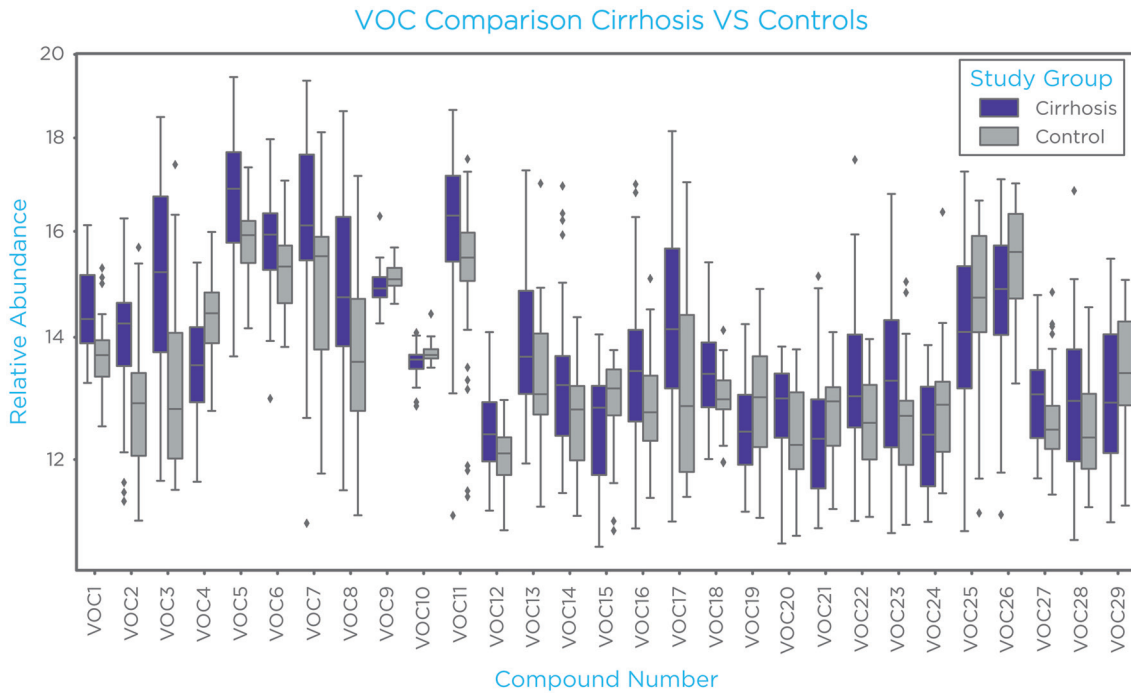A correction for multiple testing using the Benjamini-Hoch-

Fig. 2. Box plots of discriminatory VOCs between cirrhosis and controls. A total of 29 on-breath VOCs were found significantly different ($p<0.05$, Mann-Whitney U-test) between control and cirrhosis groups. VOCs, volatile organic compounds.

berg (BH) method returned a subset of 11 VOCs with an adjusted $p<0.1$. Correlations between these selected breath compounds and blood metrics of liver function within the cirrhosis group, namely bilirubin, albumin, and prothrombin time expressed as INR, were first investigated by generating a Pearson correlation matrix visualized in Figure 5A and Table 3. As expected, limonene (VOC3) had a positive correlation with bilirubin and INR and a negative correlation with albumin, as previously reported.[8] Other MFs with retention time of ~11 m had a similar pattern. Consistently, dimethyl selenide (VOC4) and VOC10 that were downregulated in the breath of patients with cirrhosis (Fig. 2), had a positive correlation with albumin. No correlations were observed between VOCs and markers of hepatic inflammation, such as alanine aminotransferase (ALT), or markers of advanced portal hypertension, such as serum sodium, and creatinine (Supplementary Fig. 3). Interestingly, none of the compounds identified as background contaminants were correlated with blood metrics (data not shown).

Collective correlations between breath and blood metrics of subjects with cirrhosis were further investigated by using the CCA.[34] The resulting CCA score plot of the first canonical variates showed that the set of variables measured in blood significantly correlated with the set of variables measured in breath ($R^2=0.842$, Fig. 5B). Inter-relation between individual exhaled VOCs and blood metrics of liver function was investigated by computing the canonical loadings. These parameters represent the correlation between each variable and its resulting canonical variate, reflecting the contribution of the variable to the overall correlation. Among the set of blood metrics, albumin had a correlation that was the reverse of bilirubin and INR, as expected. Albumin contributed the most to the overall correlation, while INR had the smallest contribution (Fig. 6A). Among the set of breath metrics, eight upregulated VOCs, including limonene (VOC3) and 2-pentanone (VOC1), showed positive loading, while dimethyl

selenide (VOC4) and VOC10, which were found downregulated (Fig. 2) had a negative loading, and VOC9 had a loading near zero (Fig. 6B).

Effect of cirrhosis severity on exhaled VOCs

Given that bilirubin, albumin, and INR are used to calculate the CP score, as a readout of hepatic function impairment associated with cirrhosis,[18] we investigated the relation of the identified VOCs and this scoring system. The subset of 11 VOCs reported in Table 2 were used to perform PCA and projected data points for each patient were colored by CP score
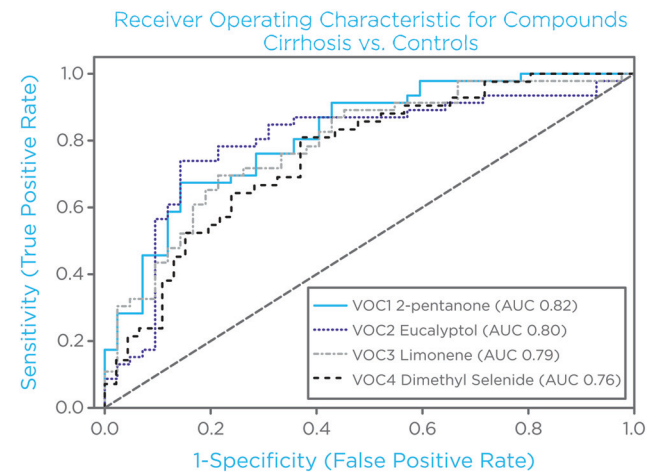

Fig. 3. Receiver operating characteristic plots of the four top single VOCs comparing cirrhosis vs. controls. The top 4 ROC plots for on-breath VOCs were calculated to explore their discriminatory performance. 2-pentanone, limonene, and dimethyl selenide were found among them. ROC, receiver operating characteristic; VOCs, volatile organic compounds.
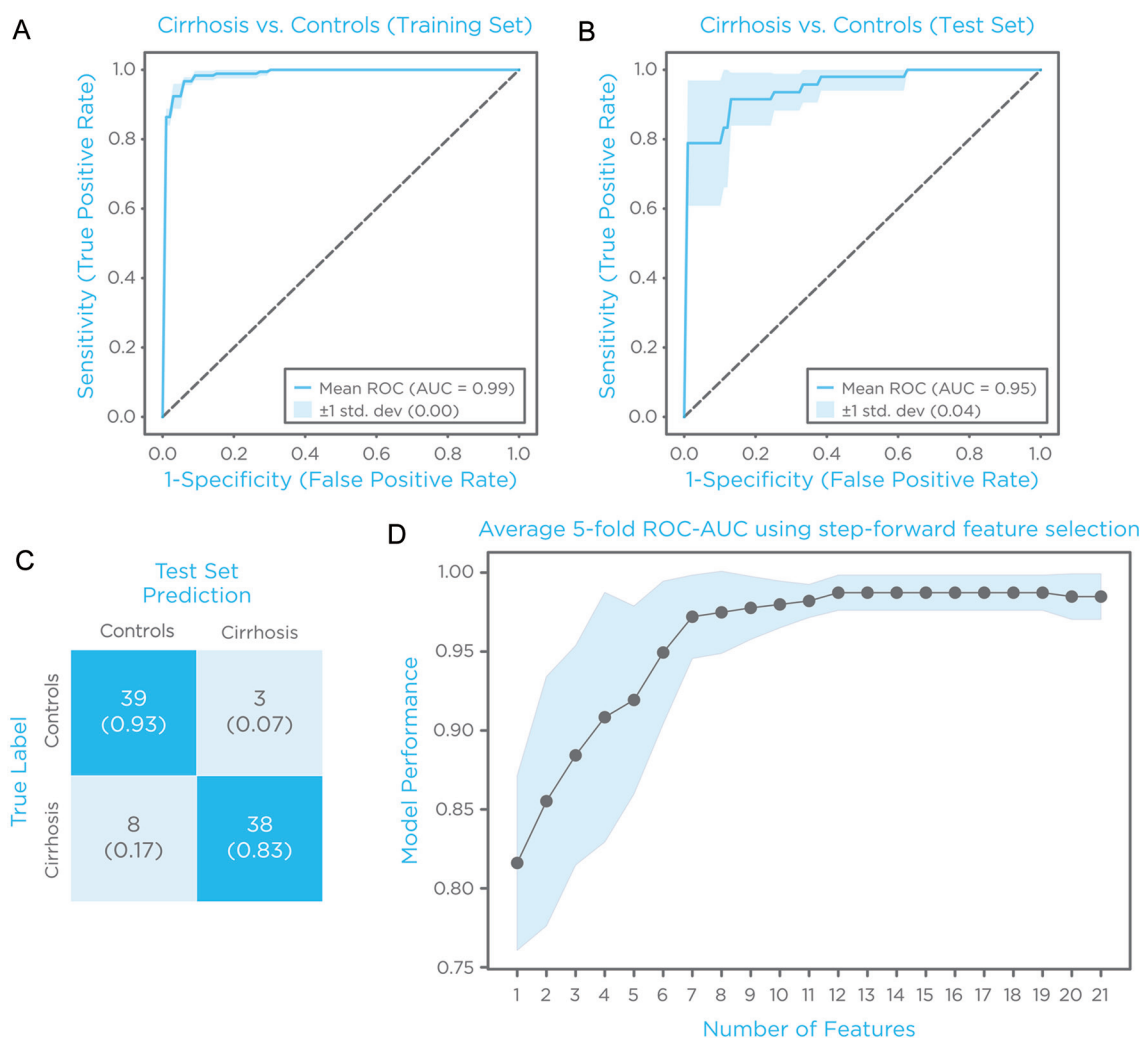
Fig. 4. Classification performance of combined VOCs. (A) ROC plot and confidence interval obtained for the training set. (B) ROC plot and confidence interval obtained for the test set. (C) Corresponding confusion matrix generated using the Youden index as threshold. (D) Improvements of classification performance by addition of VOCs to the model. ROC, receiver operating characteristic; VOCs, volatile organic compounds.

(Fig. 7). PC1 explained 10.1%, and PC2 explained 4.4% of variance and distinguished patients with CP scores >5 from controls. Conversely, ~50% of the patients with CP score of five overlapped with controls. Two patients with a CP score of >5 overlapped with controls (PC1<0 and PC2<1, Fig. 7). Of those, one had a score of seven points due to the presence

of moderate ascites, and the other had an albumin of <35 g/L (34 g/L).

Discussion

This pilot study demonstrated that a panel of exhaled VOCs

Table 2. Best performing VOCs

VOC	Compound ID	RT	*m/z*	Average stability score	Identification
VOC1	2-Pentanone	5.30598	71.04919	0.916	HRAM, Standard
VOC12	1-pentene, 4-methyl, or 1-hexene	7.62411	42.03163	0.890	NIST
VOC17	Indole	17.13762	116.90607	0.886	HRAM, Standard
VOC4	Dimethyl selenide	3.30723	109.963	0.762	HRAM, Standard
VOC3	Limonene	11.00585	91.05427	0.734	HRAM, Standard
VOC2	Eucalyptol	11.34312	139.11181	0.732	HRAM, Standard
VOC29	Benzene, (1-propylnonyl)-	20.9004	133.19707	0.6	NIST

HRAM, breath biopsy high resolution accurate mass library; VOC, volatile organic compound; RT, retention time.
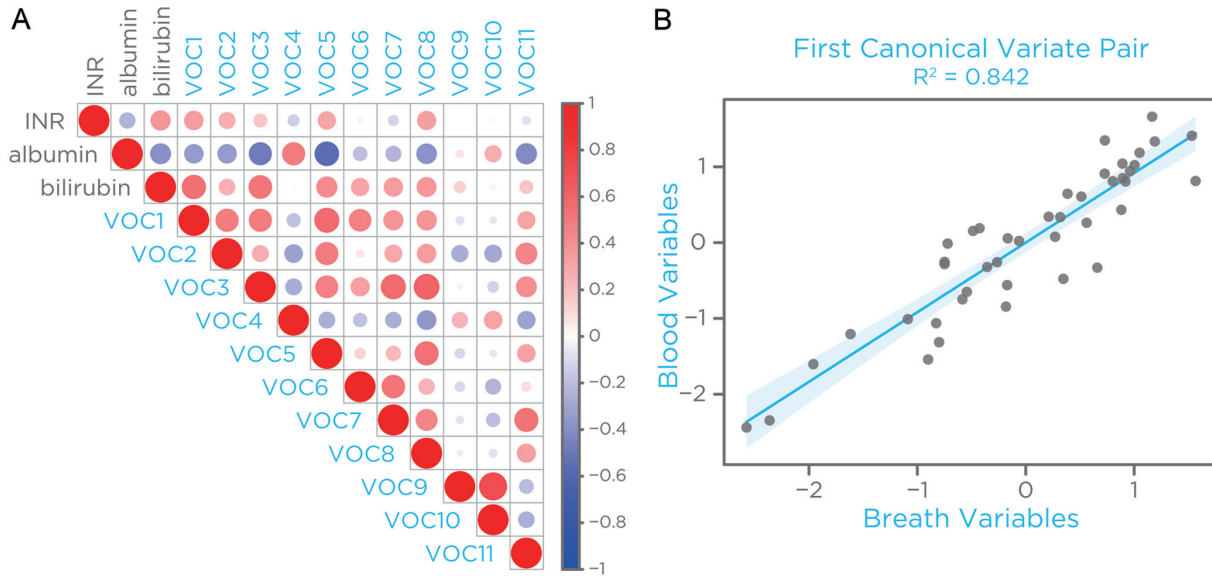
Fig. 5. Correlation of breath VOCs with blood metrics of liver function in cirrhosis subjects. (A) Correlation plot of identified VOCs and serum bilirubin, albumin, and INR. Blue indicates a negative and red a positive correlation. Circle size and color intensity show the magnitude of the correlation. (B) CCA score plot using the first canonical variates of selected sets of VOCs and blood metrics of liver function. Each projected data point represents the combined information of breath VOCs and blood metrics of one cirrhotic patient. The CCA analysis revealed significant correlations, with R²=0.842. CCA, canonical correlation analysis; INR, international normalized ratio; VOCs, volatile organic compounds.

discriminated cirrhosis patients from controls with no known liver diseases. The AUC of the test set was 0.95, which was better than the AUC of 0.78 measured when using limonene alone.[8] The spectrum of alterations of the exhaled VOCs reflects disease-associated hepatic impairment as demonstrated by correlations with blood biomarkers of clearance and the protein synthesis capacity of the liver. Our data point toward the use of these VOCs as potential biomarkers for functional, non-invasive detection of chronic liver disease. Several studies have identified breath VOCs associated with different stages of liver disease.[4,37,38] However, only few of them attempted to explore correlations of VOCs with blood biomarkers of liver function.[8,11,14] In this study, comprehensive analysis projected the entire set of discriminant exhaled VOCs into the set of blood metrics of liver function, measured

in subjects with cirrhosis, demonstrating a significant collective correlation between the two sets of variables and quantifying the contribution of each single variable to the overall correlation. Additionally, lower-dimensional data, obtained by projection of discriminatory features onto the first two components, revealed a separation of subjects with more advanced cirrhosis estimated by CP score. This innovation in the data analysis workflow contributed one additional layer of confidence to the validity of the identified discriminatory features and demonstrated that alterations of VOCs in breath depend on the extent of disease-associated liver dysfunction. The results expand our previous finding that limonene was correlated with albumin, bilirubin, and INR.[8]

Taken together, the correlation data provide a novel mechanistic understanding. Alteration of serum bilirubin, albumin,

Table 3. Area under the curve (AUC) and correlation of VOCs with blood metrics

VOC	Identity	AUC	Correlation (coefficient *r*, *p*-value)		
			Bilirubin	**Albumin**	**PT-INR**
VOC1	2-pentanone	0.82	0.548, 0.01	−0.346, 0.014	0.347, 0.013
VOC2	Eucalyptol	0.80	0.258, 0.07	−0.348, 0.013	0.264, 0.064
VOC3	Limonene	0.79	0.516, 0.01	−0.487, 0.01	0.178, 0.216
VOC4	Dimethyl selenide	0.76	−0.012, 0.936	0.492, 0.01	−0.135, 0.352
VOC5	Terpene	0.72	0.415, 0.003	−0.552, 0.01	0.309, 0.029
VOC6	2-Pentanone (*m/z* 43.02369)	0.72	0.308, 0.03	−0.196, 0.173	−0.022, 0.881
VOC7	Terpene	0.71	0.344, 0.014	−0.236, 0.098	−0.112, 0.441
VOC8	Terpene	0.70	0.368, 0.009	−0.388, 0.005	0.338, 0.016
VOC9	Unknown	0.69	0.136, 0.346	0.067, 0.642	0.0, 1.0
VOC10	Unknown	0.68	0.028, 0.845	0.267, 0.061	−0.014, 0.921
VOC11	Terpene	0.67	0.177, 0.22	−0.412, 0.003	−0.077, 0.594

VOC, volatile organic compound; AUC, area under the curve; PT-INR, prothrombin time-international normalized ratio.
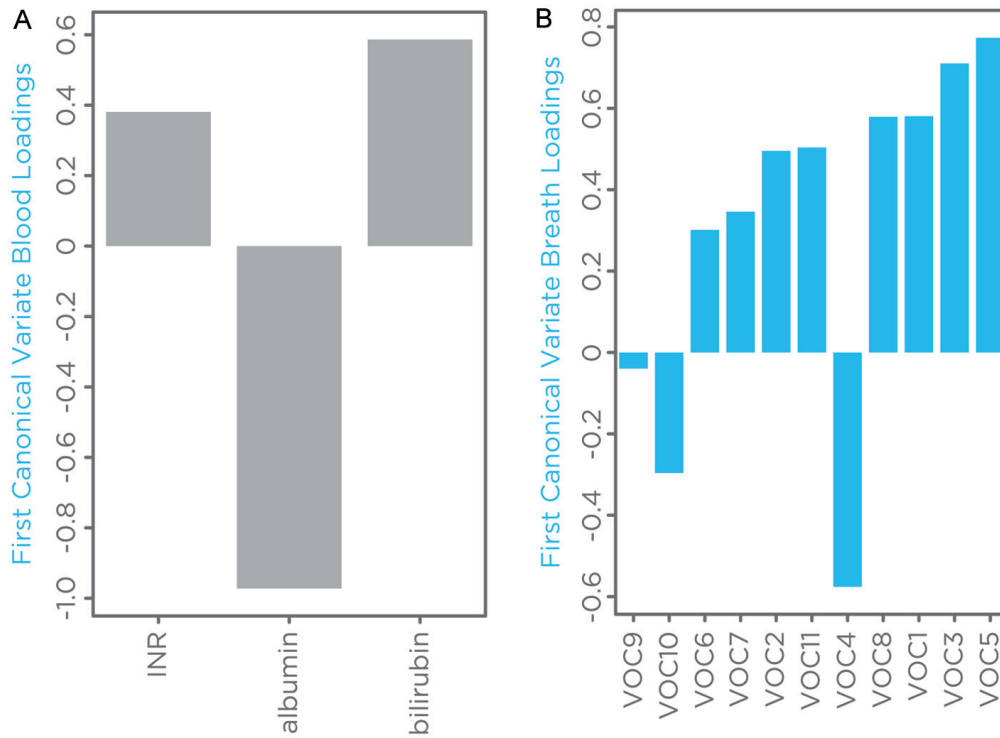
Fig. 6. Canonical loadings of set of variables. Canonical loadings represent the correlation between a variable and its canonical variate and express the contribution of each variable to the overall correlation. (A) Loadings for blood variables, albumin has opposite correlation than bilirubin and INR, as expected. (B) Loadings for breath VOCs, limonene and 2-pentanone had a positive contribution. Dimethyl selenide (VOC4), which was downregulated in the breath of patients with cirrhosis, had a negative correlation. INR, international normalized ratio; VOCs, volatile organic compounds.
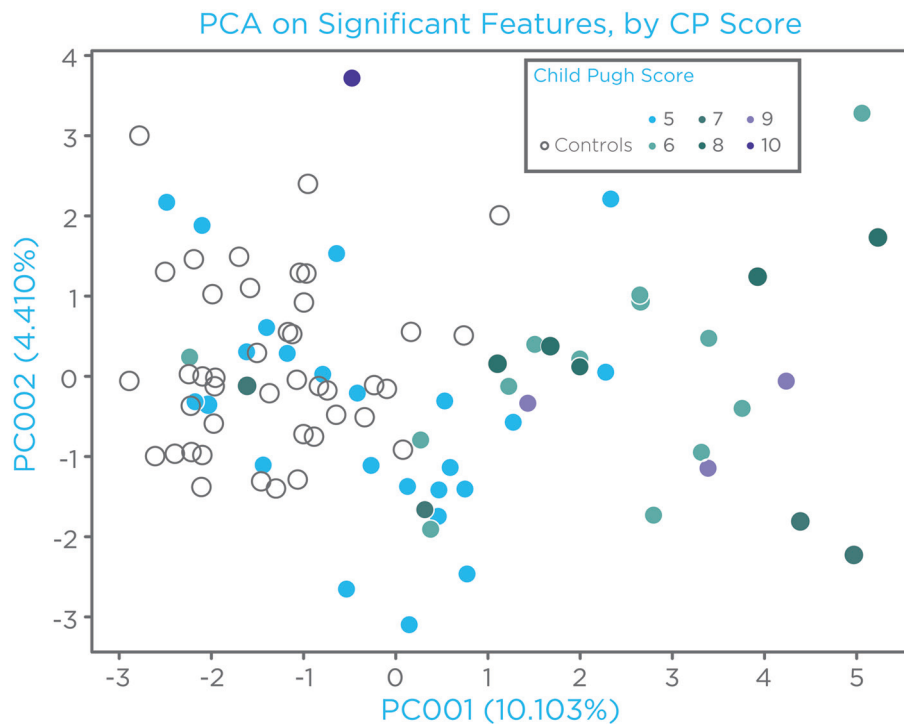

Fig. 7. VOC alterations in relation to cirrhosis severity. Projected data points of the first two components of a PCA calculated using breath variables with a BH adjusted $p < 0.1$. PC1 explains 10.1% and PC2 4.4% of variance and shows that separation of cirrhotic patients based on their CP score. About 50% of the patients with a CP=5 cluster with controls. BH, Benjamin-Hochberg; CP, Child-Pugh; PCA, principal component analysis; VOC, volatile organic compound.

and INR are considered a consequence of reduced clearance and protein synthesis by the liver. Therefore, we speculate that alterations of the identified VOCs are consequence of impaired liver function and can be used to monitor the progression of chronic liver disease. VOC9 was significantly elevated in the breath of patients with cirrhosis and had a poor correlation with blood metrics, indicating that it may be correlated with other alterations associated with chronic liver disease such as inflammation and/or fibrosis. In validation studies, potential correlations of these VOCs with liver biopsy histopathology would pave the way to replacing invasive procedures by breath collection for prognosis following therapeutic interventions.

The results reported here are consistent with existing literature. For example, Dadamio *et al*. 2011,[9] Fernandez Del Rio *et al*. 2015,[10] and Pijls *et al*. 2016,[12] also found a spectrum of cirrhosis-associated compounds in breath. As in those studies, we observed elevated signals of limonene,[9–12,14,24,25] 2-pentanone,[9,10,24,26] and an alkene (1-pentene, 4-methyl or 1-hexene),[9] and reduced signals of dimethyl selenide[9,26] in the breath of patients with cirrhosis. In contrast, we found an elevated indole signal, which Dadamio *et al*. 2011 found to be reduced in the breath of patients with cirrhosis.[9] In that study, eucalyptol was detected but with no significant differences in the study groups. We found it was elevated in the breath of subjects with cirrhosis. The discrepancies may be explained by different dietary habits that determine the ingestion of foods from which the VOCs originated.

A study strength is the procedures used for breath collection, which are a substantial advance over prior studies. Firstly, we ensured the inhaled air was purified to minimize environmental contribution to the signal. Secondly, the volume of sampled breath or blanks was standardized and closely monitored. Finally, multiple blanks were collected during each session, allowing experimental discrimination of contaminants from exhaled VOCs. Taken together they provided a high degree of confidence in our ability to discern on-breath VOCs from environmental contaminants and use only the former for classification. The detailed clinical characterization of cases adds to the strength of the study, allowing us to evaluate the impact of disease severity on exhaled VOCs and classification performance.

A study limitation is the reduced characterization of subjects allocated to the control group, with no laboratory blood data available. The subjects may have been affected by a liver disease at a preclinical stage and misclassified as false positives, influencing the classification performance. However, establishing the accuracy of a breath test for cirrhosis requires a large population and goes beyond the exploratory nature of this study. Another limitation is the unstandardized dietary intake and exposure to exogenous compounds before testing. On one hand, this represents clinical reality; on the other hand, it could have impacted the discriminatory performance of these compounds. Limonene is a monoterpene isolated from a variety of plant sources.[39] 2-pentanone has been found in cheddar cheese[40] and ripe bananas.[41] Selenium, in its different forms, is a micronutrient essential for cellular function and is abundant in plant-based foods.[42] Although these compounds accumulate in the body when liver function is impaired,[10] subjects at earlier stage cirrhosis, and with random exposure lower than hepatic clearance, may be misclassified as false negative and affect test sensitivity. On the other hand, control subjects exposed to these compounds shortly before testing may result as false positive and impair test specificity. Consistent with this hypothesis, the three false positives allocated by our model in the test set were above the 75th percentile for limonene or 2-pentanone,

indicating that they probably ingested these compounds shortly before breath collection. These observations suggest that standardizing compound exposure would be vital to increasing the performance of a breath test so that it can be extended to earlier stage liver diseases such as nonalcoholic steatohepatitis (NASH).[5]

Classification with combined VOCs outperformed that measured with single compounds, indicating that multiple compounds better represent the complexity of hepatic metabolic pathways. Terpenes, and terpenoids such as limonene and eucalyptol, are mainly metabolized by the CYP system,[15,43] which is well established to be downregulated in chronic hepatic conditions including NASH.[44–46] 2-pentanone and other ketones may be converted to 2-pentanol,[47] by αα-alcohol dehydrogenase (αα-ADH),[48] and to a lesser extent to 3-Hydroxy-2-pentanone and 2,3-pentanediol.[47] Consistent with those observations, liver samples obtained from patients with NASH had reduced ADH and aldehyde dehydrogenase (ALDH) activity.[49] Elevated breath levels of alkenes may derive from lipid peroxidation of unsaturated fatty acids as a consequence of persistent inflammation and oxidative stress affecting the cirrhotic liver.[37] Indole is generated by the catabolism of tryptophan mediated by gut bacteria.[37] Impaired hepatic clearance may explain elevated levels of indole in cirrhosis. Alkylbenzenes with a long alkyl group may be oxidized to the corresponding carboxylic acids and undergo β-oxidation to form benzoic acid, which is then converted to hippuric acid. This metabolic pathway was found downregulated in subjects with cirrhosis.[50] Dimethyl selenide is an excretion product of selenium metabolism.[42] Impaired organic and inorganic selenium biotransformation explains downregulation of dimethyl selenide in the breath of cirrhotic patients. Consistent with this hypothesis, plasma selenium concentration was reduced in patients with cirrhosis to an extent related to disease severity.[51] Cirrhosis comorbidities may also influence the amount of exhaled VOCs. Increased levels of limonene and 2-pentanone in subjects with cirrhosis and portal hypertension compared to those without portal hypertension, suggest that altered blood flow to the liver contributes to reduce the hepatic ability to clear these compounds. Similarly, obese patients with increased levels of indole, may have alterations of gut bacteria leading to increased production of this compound. That, coupled with reduced hepatic clearance leads to greater elevation of levels in the breath. Although, information on comorbidities was collected, the study design was not optimal to address those questions.

Collectively, the evidence indicates that breath analysis looking at specific VOCs has potential to assess functional alterations of specific metabolic pathways associated with chronic liver diseases. Although there is no clinical need for an additional diagnostic test for cirrhosis, current diagnostic modalities have limitations as screening tools. Consistently, more than 50% of the subjects receive a diagnosis after suffering decompensating events.[1,2,52] A breath-based test could be used as a screening tool either in primary care, or for at home self-testing, such that lifestyle changes and therapeutic interventions could be implemented before the first episode of decompensation.

In summary, this study identified a set of exhaled VOCs with alterations that seem to be driven primarily by functional impairment of the liver. The results underpin earlier observations that downregulation of different hepatic metabolic pathways occurring in cirrhosis and early stages of liver disease may be the underlying mechanism. Interestingly, most of the VOCs were of exogenous origin. Further investigation will establish if adjustment of the exposure to these

VOCs allows detection of more subtle metabolic alterations that occur in earlier stages of liver diseases such as NASH.

Conflict of interest

Giuseppe Ferrandino, Giovanna De Palo, Antonio Murgia, Owen Birch, Rob Smith, Ahmed Tawfike, Olga Gandelman, Max Allsworth, and Billy Boyle, are/were employees of Owlstone Medical Ltd.

Author contributions

Conceived and designed the study (MPvdS, MA, OG, MA, MH, RCF, VKS, BB), developed analytical methodology to meet requirements for quantitation of breath samples (RS, AM), coordinated the clinical trial (AKT), recruited patients and collected breath samples (ID-B, GK, AML, AG), analyzed data (GF, GDeP, OB, AT, AS), drafted the manuscript (GF); All authors contributed to critical revision of the manuscript for important intellectual content and approved the final manuscript.

Ethical statement

This cross-sectional case-control study was part of Owlstone Medical and Cancer Research UK (CRUK) PAN-study (NCT03756597 U.S. National Library of Medicine), which was approved by the ethics committee of the East of England – Cambridge East Research Ethics Committee (REC reference: 18/EE/0041. IRAS ID: 237560) and all participants provided written informed consent.

Data sharing statement

The supplementary tables, figures, and methods used to support the findings of this study are included within the supplementary information files.

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