Breath Biopsy Assessment of Liver Disease Using an Exogenous Volatile Organic Compound—Toward Improved Detection of Liver Impairment

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INTRODUCTION: Liver cirrhosis and its complication — hepatocellular carcinoma (HCC) — have been associated with increased exhaled limonene. It is currently unclear whether this increase is more strongly associated with the presence of HCC or with the severity of liver dysfunction.

METHODS: We compared the exhaled breath of 40 controls, 32 cirrhotic patients, and 12 cirrhotic patients with HCC using the Breath Biopsy platform. Breath samples were analyzed by thermal desorption–gas chromatography–mass spectrometry. Limonene levels were compared between the groups and correlated to bilirubin, albumin, prothrombin time international normalized ratio, and alanine aminotransferase.

RESULTS: Breath limonene concentration was significantly elevated in subjects with cirrhosis-induced HCC (M: 82.1 ng/L, interquartile range [IQR]: 16.33–199.32 ng/L) and cirrhosis (M: 32.6 ng/L, IQR: 6.55–123.07 ng/L) compared with controls (M: 6.2 ng/L, IQR: 2.62–9.57 ng/L) (P value = 0.0005 and 0.0001, respectively) with no significant difference between 2 diseased groups (P value = 0.37). Levels of exhaled limonene correlated with serum bilirubin ($R^2 = 0.25$, $P = 0.0016$, $r = 0.51$), albumin ($R^2 = 0.58$, $P = 5.3e-8$, $r = -0.76$), and international normalized ratio ($R^2 = 0.29$, $P = 0.0003$, $r = 0.51$), but not with alanine aminotransferase ($R^2 = 0.01$, $P = 0.36$, $r = 0.19$).

DISCUSSION: Exhaled limonene levels are primarily affected by the presence of cirrhosis through reduced liver functional capacity, as indicated by limonene correlation with blood metrics of impaired hepatic clearance and protein synthesis capacity, without further alterations observed in subjects with HCC. This suggests that exhaled limonene is a potential non-invasive marker of liver metabolic capacity (see Visual abstract, Supplementary Digital Content 1, http://links.lww.com/CTG/A388).

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A387 and http://links.lww.com/CTG/A388

unfortunately, in the western countries, as few as 30% of the cases are detected at early stages despite the establishment of semiannual surveillance protocols for the at-risk populations (7). These data indicate a pressing need for more efficient screening tests for the early detection of chronic hepatic injury and HCC.

Analysis of volatile organic compounds (VOCs) in exhaled breath represents an emerging diagnostic approach with the potential to develop safe, non-invasive tests for the early detection of cancer (8,9). Exhaled VOCs are low molecular weight compounds that can be endogenous, resulting from physiological or pathological metabolic processes (10), or exogenous (EVOCs), resulting from dietary or environmental exposure. Dietary EVOCs are absorbed and reach breath through blood circulation, diffusing into the lungs via gaseous exchanges between blood and air (8) and can be used to assess metabolic functions in vivo.

Limonene is an EVOC taken-up through the consumption of plant-derived foods, especially citrus in the form of fruits, flavored foods, and beverages (11). It is rapidly absorbed and metabolized in the liver by the enzymes CYP2C9 and CYP2C19 presumably to transcarveol and perillyl alcohol, respectively (12). High concentrations of limonene have been found in the breath of cirrhotic patients because of reduced hepatic clearance (13–18). This reduction is attributed to the downregulation of CYP2C9 and CYP2C19, which, in turn, increases the half-life of limonene in the bloodstream, leading to higher abundance in the breath (13–18). O’Hara et al. (17) found a moderate increase of breath limonene in patients affected by HCC with underlying cirrhosis. Furthermore, proteomic profiling of resected early-stage liver primary tumors and comparison with paired non-tumor liver tissues showed a general downregulation of the CYP 450 system, including CYP2C9 and CYP2C19, in the cancer tissues (19).

Given these findings, the effects of patients having HCC in addition to cirrhosis on limonene concentrations in breath need further investigation. Understanding this in more detail is crucial to shed light on the utility of a limonene breath test. We therefore investigated, in cirrhotic patients, the impact of disease severity, measured as Child-Pugh (CP) class, on exhaled limonene levels and ascertained whether the presence of HCC, in cirrhotic patients with matching CP class, independently modulates exhaled limonene.

METHODS
Study design and subjects
This study is a cross-sectional case-control study, part of Owlstone Medical’s PAN-study (NCT03756597). This study 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.

Subjects younger than 30 years of age were recruited from the clinical research facility at Addenbrooke’s Hospital (Cambridge) or through the Cambridge BioResource. Cirrhotic patients had an established diagnosis according to EASL and AASLD guidelines (20,21). Disease severity was classified using the CP, the United Kingdom Model for End-Stage Liver Disease, and the Model for End-Stage Liver Disease scoring systems (22,23). Per protocol, only patients with CP A or B were eligible for this study, regardless of disease etiology. Subjects with HCC were identified through semiannual surveillance imaging of cirrhotic risk population and confirmed by either cytology/histology or cross-sectional radiology, as defined in the European Association for the Study of the Liver HCC guidelines (21) and classified according to the Barcelona-Clinic Liver Cancer staging system (24). No patients with HCC were receiving any anticancer treatment at the time of sampling.

Control subjects had no known liver disease and were excluded if they were under investigation or had a history of malignancy in the past 2 years.

Breath sampling
Breath collection was performed in a single room at the Addenbrooke’s Hospital (Cambridge, UK) for all the subjects involved in the study. Breath samples were collected by adsorption onto 1/4” × 3 1/2” inert-coated stainless-steel tubes with Tenax TA/carbograph 5TD adsorbent material (Markes International, Llantrisant, UK) through a ReCIVA Breath Sampler (Owlstone Medical, Cambridge, UK) (25). Before sampling, the tubes were conditioned in a TC-20 (Markes International) by a N2 flow at 20 psi and 320 °C for 4 hours. Before sampling, the system was allowed to calibrate and adjust to the breathing pattern of the subject. Approximately 1.5 L of breath was sampled per tube at 225 mL/min. Ambient contamination was minimized by using the CASPER Portable Air Supply (Owlstone Medical) (25). The tubes were stored at a temperature of 4–8 °C for no more than 4 weeks before limonene was analyzed.

No dietary restrictions were applied to any participant. To generate a crude estimate of limonene intake, an unvalidated food questionnaire was administered to all cirrhotic subjects to classify them into daily and non-daily consumers of limonene-containing foods.

Limonene measurements
The tubes containing breath samples were purged in a TD-100 (Markes International). Samples were first desorbed at 210 °C and focused onto a cold trap U-T12ME-2S, Material/Emission, C4-C32 (Markes international) at 20 °C. Cryofocused analytes were then desorbed at 300 °C for 3 minutes and purged by helium into the GC column (TraceGOLD TG-624SiM5; Thermo Fisher Scientific, Waltham, MA), with a sample flow split ratio of 10:1 and temperature gradient steps of 40 °C for 1 minute, 270 °C with a rate of 10 °C/min, and 300 °C with a rate of 30 °C/min for 5 minutes. Mass detection was performed using a Q Exactive GC Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) scanning from m/z 30 to m/z 450 with a resolving power of 60,000. Electron ionization voltage was set at 70 V. The ion source and heater temperatures were, respectively, fixed at 230 and 250 °C.

Limonene identification and peak integration were performed with the Chromelone Chromatography Data System (Thermo Fisher Scientific) by injecting a limonene standard (62118; Sigma Aldrich, Gillingham, UK). Total ion chromatograms were subjected to a NIST library search and the product ion signals with m/z values of 67, 79, 93, 107, 121, and 136, and an elution time of 11 minutes was assigned to limonene. The product ion at m/z 67 was used as a quantifier by comparison with a limonene standard curve (5–1,500 ng on-tube, Supplementary Digital Content 1, http://links.lww.com/CTG/A387). Limonene on breath corresponding to peaks below 5 ng have been extrapolated. No baseline correction was applied to the chromatograms. Limonene values were normalized to the exact volume of collected breath as recorded by the ReCIVA Breath Sampler system. Equipment
and site blanks were also analyzed to estimate potential contaminations.

**Data analysis**
The data were analyzed by using JMP version 15 (SAS Institute, Cary, NC, 1989–2019). Limonene concentration is expressed as ng/L of breath and reported as median and interquartile range (IQR). The other parameters are reported as median, minimum, and maximum. Distribution of breath limonene concentration for each group was assessed using a Shapiro-Wilk test and found to be non-normally distributed, so non-parametric tests were used for intergroup comparisons. A Mann-Whitney U test was used to test for significant changes between the groups. Limonene and other parameters were Log10 transformed to obtain normal distribution. Least square regression was used to correlate breath limonene levels with blood metrics, Model for End-Stage Liver Disease, and United Kingdom Model for End-Stage Liver Disease scoring systems. A P value lower than 0.05 was regarded as statistically significant. A limonene receiver operating characteristic plot was built to estimate diagnostic accuracy. The cutoff point was selected as the concentration of limonene corresponding to the Youden index (highest [sensitivity — (1 — specificity)]).

**RESULTS**

**Subject characteristics**
Breath samples were collected from 40 cirrhosis- and HCC-free controls (m: age 62 [34–81] years, m/f [21/19]), 32 patients with cirrhosis (m: 56.5 [35–78] years, m/f [20/12]), and 12 patients with cirrhosis complicated by HCC (m: 69.5 [55–79] years, m/f [7/5]). Study subject details are provided in Table 1. The following observations can be made. The unbalanced sex ratio within each group reflects the higher prevalence of HCC in male patients (26). Across all subjects, the exhaled limonene levels were not affected by sex (P value = 0.308), as previously described (17,27). Significant differences in age were observed between the study groups (P values: 0.019, 0.006, and 0.0002, respectively, for cirrhosis vs healthy, HCC + cirrhosis vs healthy, and cirrhosis vs HCC + cirrhosis). No significant differences were observed for blood metrics between the study groups (Table 1).

**Exhaled limonene levels**
Median exhaled limonene levels in patients with cirrhosis (m: 32.6 ng/L, IQR: 6.55–123.07 ng/L) and patients with cirrhosis complicated by HCC (m: 82.1 ng/L, IQR: 16.33–199.32 ng/L) were significantly higher than those of the controls (m: 6.22 ng/L, IQR: 2.62–9.57 ng/L) at P values of 0.0001 and 0.0005, respectively. The presence of HCC in study subjects with cirrhosis did not influence the exhaled limonene levels (P value = 0.37) (Figure 1 and Supplementary Digital Content 2, http://links.lww.com/CTG/A387). Therefore, all subjects with cirrhosis and cirrhosis complicated by HCC were gathered in one group for all the subsequent analysis, increasing the statistical power of those analyses.

An exploratory receiver operating characteristic analysis, detailed in Figure 2, resulted in an area under the curve of 0.78 for

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<th>Table 1. Patient characteristics</th>
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<td><strong>Healthy</strong></td>
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<td>No. of patients</td>
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<td>Age median [range] years</td>
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<tr>
<td>Male/Female</td>
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<td>Child-Pugh class A/B/C/na</td>
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<td>MELD median [range]</td>
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<td>UKELD median [range]</td>
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<td>BCLC 0/A/B/C/na</td>
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<tr>
<td>Total bilirubin median [range] (µmol/L)</td>
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<td>Serum albumin median [range] (g/L)</td>
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<td>INR median [range] (%)</td>
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<td>ALT median [range] (IU/L)</td>
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<td>ALP median [range] (IU/L)</td>
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<td>Creatinine (µmol/L)</td>
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<td>Sodium (mM)</td>
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<td>Limonene level [median, IQR] (ng/L)</td>
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aCirrhosis vs healthy.
bHCC + Cirrhosis vs healthy.
cCirrhosis vs HCC + Cirrhosis.

ALT, alanine aminotransferase; ALP, alkaline phosphatase; BCLC, Barcelona-Clinic Liver Cancer; HCC, hepatocellular carcinoma; INR, international normalized ratio; IQR, interquartile range; MELD, Model for End-Stage Liver Disease; UKELD, United Kingdom Model for End-Stage Liver Disease.
the prediction of cirrhosis. At a threshold of 10.2 ng/L, an optimal overall accuracy was achieved (Youden index) with a sensitivity of 0.73 and 1-specificity of 0.23.

Association with disease severity
Stratification of subjects by CP score (23) shows that exhaled limonene levels in class B (median: 159.9 ng/L, IQR: 46–580 ng/L) are significantly higher compared with those in class A (median: 12.6 ng/L, IQR: 46–580 ng/L) (P value = 0.0117) (Figure 3). Consistent with this result, a least square regression shows that breath limonene correlates with bilirubin ($R^2 = 0.25$, P value = 0.0016, $r = 0.51$) (Figure 4a), albumin ($R^2 = 0.58$, P value = 5.3e-8, $r = −0.76$) (Figure 4b), and international normalized ratio (INR) ($R^2 = 0.29$, P value = 0.0003, $r = 0.51$) (Figure 4c). However, limonene does not correlate with alanine aminotransferase (ALT) ($R^2 = 0.01$, P value = 0.36, $r = 0.19$), a biomarker of liver damage (Figure 4d).

Impact of exposure and medications
The amount of ingested limonene depends on the dietary intake. Class stratification of the disease population in daily and non-daily citrus product consumers shows that patients ingesting citrus products on a daily basis have higher breath limonene (m: 131.9 ng/L, IQR: 10.7–367.9 ng/L) compared with non-daily consumers (m: 15.4 ng/L, IQR: 4.9–43.5 ng/L) (P value = 0.0093) (see Supplementary Digital Content 3, http://links.lww.com/CTG/A387).

Drugs interacting with CYP2C9 and CYP2C19 may modulate their activity (28,29) and affect limonene clearance. Therefore, we recorded medications taken by the patients within 1 week before breath sampling. Patients taking CYP2C9/19 substrates showed significantly increased breath limonene concentration compared with those not taking these medications (M: 74.0 and 10.2 ng/L, respectively) (P value = 0.0053). On the contrary, patients taking CYP2C9/19 inhibitors showed no significant difference compared with patients not taking those drugs (P value = 0.25) (M: 50.7 and 11.2 ng/L, respectively). Antacids, antibiotics, and lactulose also showed no effects on breath limonene levels (P value = 0.41) (see Supplementary Digital Content 4, http://links.lww.com/CTG/A387).

Detailed examination of the impact of comorbidities such as diabetes, portal hypertension, and increased body mass index on the exhaled limonene levels revealed no differences (data not shown).

**DISCUSSION**
In this pilot study, we have established that exhaled limonene levels are elevated in subjects with liver cirrhosis, regardless of the presence of HCC. Our data point toward the impact that cirrhosis has on liver function, being the primary driver for the elevation of breath limonene levels. These observations are in line with previous observations suggesting that limonene is primarily metabolized by the CYP450 enzymatic system (12). This implies that...
an exhaled limonene test could be a non-invasive way to evaluate liver functional capacity. Our results confirm and expand on previous findings by O’Hara et al. (17) who demonstrated that limonene levels are elevated in subjects with HCC and cirrhosis and a study by Fernandez Del Rio et al. (14) which showed that restoring hepatic function by liver transplant brings breath limonene levels to baseline in the following weeks. Similarly, Sinha et al. (30) found high levels of limonene in the breath of patients with non-alcoholic steatohepatitis (NASH) cirrhosis, compared with those of patients with non-alcoholic fatty liver disease, further supporting our conclusion that cirrhosis is the primary driver for the elevation of breath limonene levels.

In this study, we have significantly expanded on this concept by showing that limonene correlates with established blood metrics of liver function, but not with ALT, a biomarker of liver damage. In agreement with our results, Sinha et al. (30) also mentioned a negative correlation between breath limonene and serum albumin. A difference can be observed with a study by Friedman et al. (15) that showed a correlation between lung air limonene and prothrombin time, but not with serum bilirubin and albumin. This discrepancy with our results may be explained by different approaches used to compare limonene with blood metrics. Here, we performed a linear regression analysis instead of stratifying the patients based on their breath limonene levels.

A strong aspect of our study is the diligent analysis of exhaled limonene levels. First, we ensured that the inhaled breath was purified air, isolated from any possible environmental limonene contamination. Second, the volume of sampled breath was standardized and closely monitored. Finally, extensive analytical validation was performed to assure that the sampled levels of limonene could be reliably quantified. Taken together, this gives us a high degree of confidence in our ability to provide absolute quantitation of limonene on breath. The detailed clinical characterization of cases furthermore adds to the strength of this study allowing us to evaluate the impact of disease severity and medication on exhaled limonene.

A limitation of the study is the unstandardized dietary intake of limonene. On one hand, this represents clinical reality; on the other hand, it could explain the relatively high number of false negatives based on a threshold of 10.2 ng/L exhaled limonene (27%). We tried to correct for this difference by administering a questionnaire attempting to quantify limonene intake. Importantly, this questionnaire was not validated and can therefore only provide a qualitative measure of limonene intake. A difference was observed between study subjects with low and high limonene intakes. Interestingly, 17 patients who consumed citrus products less than daily showed breath limonene >10.2 ng/L. The likely cause of these elevated levels is that, with reduced liver function, unmetabolized limonene is stored in fat tissue (31) and hence persists in the body for a long time (14). These observations undoubtedly indicate that standardizing limonene exposure would be vital to increasing performance of a limonene breath test and extend this approach to earlier stages of liver diseases such as NASH.

Our study supports the notion that the differences between control and diseased subjects observed in this study reflect differences in the activity of the CYP450 enzyme system. It is well

![Figure 4. Correlation of breath limonene levels with blood metrics related to liver function. Least square regression analysis of Log10 transformed limonene against (a) bilirubin, (b) albumin, (c) international normalized ratio (INR), and (d) alkaline phosphatase. ALT, alanine aminotransferase.](image-url)
established that chronic hepatic conditions result in down-regulation of the CYP450 system (32,33), reduction of liver functional units (34), reduced portal blood flow, and impaired liver extraction capacity (34–36). The exact molecular mechanism for this is still unclear (32), but interleukin 6 (IL-6), a proinflammatory cytokine, may play an important role in this process. Increased levels of this cytokine were found in patients with liver conditions predisposing to HCC (37–41), including hepatic cirrhosis (34). Furthermore, IL-6 treatment of human primary hepatocytes downregulates the expression of the CYP450 system, including CYP2C9 and CYP2C19 (42), the limonene metabolizing enzymes (12). Consistent with these in vitro data, clearance of CYP450-metabolized drugs correlates inversely with plasma IL-6 levels in patients with various types of cancer (43). The reduction of functional liver units is a consequence of replacement of parenchymal tissue with fibrotic tissue that also alters the vascular architecture (34). These modifications lead to progressive sinusoidal capillarization, endothelial cells denervation, and deposition of basement membrane components within the perisinusoidal space (34). Taken together, these alterations compromise the effective extraction capacity of the liver (44), including its ability to extract limonene.

Given the lipophilicity of limonene, an adipose tissue compartment model may best describe breath limonene pharmacokinetics (2,14,31). In a condition of reduced liver function, normal environmental exposure can overcome hepatic clearance, resulting in limonene accumulation in the fat. Ultimately, this results in prolonged elevated levels of limonene in the blood and therefore the breath. In healthy individuals, the hepatic metabolic capacity to biotransform limonene is sufficient to prevent the accumulation of limonene in the adipose tissue, resulting in rapidly decreasing breath levels after exposure.

An important mechanistic observation in this study is the fact that exhaled limonene levels correlate positively with bilirubin and INR, negatively with albumin, and do not correlate with ALT. We can speculate that this underpins the relation between exhaled limonene and the functional capacity of the liver. Bilirubin is removed by the liver through conjugation, and hence, increased levels of this substance may indicate an impaired ability to do so. Normal INR and albumin values depend on the protein synthesis capacity of the liver that is impaired in cirrhosis. Finally, ALT is released during the lysis of liver cells without it directly reflecting a functional liver parameter. Taken together, this evidence points in the direction that exhaled limonene is a non-invasive way of evaluating liver metabolic capacity by providing a proxy measurement of CYP2C9 and CYP2C19 metabolism.

Given the observed increase in liver disease, particularly NASH, there is an ever more pressing need for non-invasive tools that can easily detect liver impairment. A breath test is an attractive proposition from the perspective of being a non-invasive test that would be easily provided at the point of care. This study suggests that a breath test can complement currently available blood tests and provide clinicians with additional tools to guide their clinical decision-making. Further validation of a breath test for limonene in an intention to diagnose population in combination with blood and imaging tests could provide a valuable means of monitoring the increased number of patients.

In summary, this study has found clearly elevated levels of limonene in the breath of subjects with liver cirrhosis. This seems to be driven primarily by functional impairment of the liver, underpinning earlier observations that downregulation of the CYP450 system occurring in cirrhosis may be the underlying mechanism. Further evaluation of a limonene breath test will help elucidate its potential to aid clinical decision-making in subjects with hepatic diseases.

CONFLICTS OF INTEREST
Guarantor of the article: Marc P. van der Schee, MD, PhD.
Financial support: None to report.

Study Highlights

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<td>Diagnosis of liver dysfunction remains challenging because of the lack of reliable biomarkers.</td>
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<td>Breath limonene concentrations are generally found to be higher in patients with cirrhosis compared with healthy controls.</td>
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<th>WHAT IS NEW HERE</th>
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<tr>
<td>Limonene has been independently confirmed as a breath biomarker associated with cirrhosis. However, onset of HCC over underlying cirrhosis has no effect on the limonene concentrations in the breath.</td>
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<tr>
<td>Elevated breath limonene is associated with the severity of liver dysfunction and correlates with biomarkers reflecting disease severity, hepatic clearance, and protein synthesis capacity, but not with a biomarker that reflects liver damage.</td>
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<th>TRANSLATIONAL IMPACT</th>
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<td>Exhaled limonene represents a potential biomarker for the non-invasive detection of chronic liver diseases and associated impairments of hepatic function.</td>
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ACKNOWLEDGMENT
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REFERENCES


