BREATH® BIOPSY

Dynamic Limonene Breath Testing Maximizes Classification Performance for Subjects with Cirrhosis

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Aims

- Assess diagnostic performance of a dynamic limonene breath test for cirrhosis detection.
- Investigate limonene exhalation kinetics after oral administration.
- Explore the correlation of limonene bioavailability with cirrhosis severity.

1. Background and Objectives

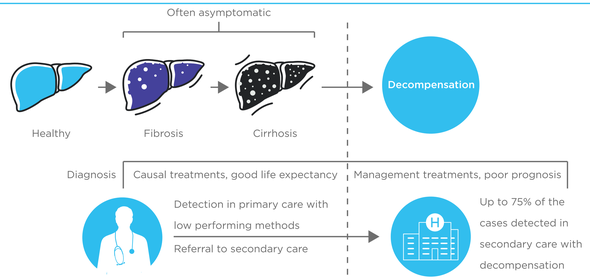

Figure 1: Progression of chronic liver disease to cirrhosis is often asymptomatic. Patients that are diagnosed at earlier stages benefit from causal treatments and can achieve a life expectancy similar to that of the healthy population. On the contrary, patients diagnosed with episodes of decompensation (liver failure) have a poor prognosis. Methods to detect cirrhosis in primary care suffer from low performance and most tests performed are relegated to secondary or tertiary care. This short diagnostic pathway results in reports of up to 75% of patients receiving a diagnosis of cirrhosis only upon decompensation[1].

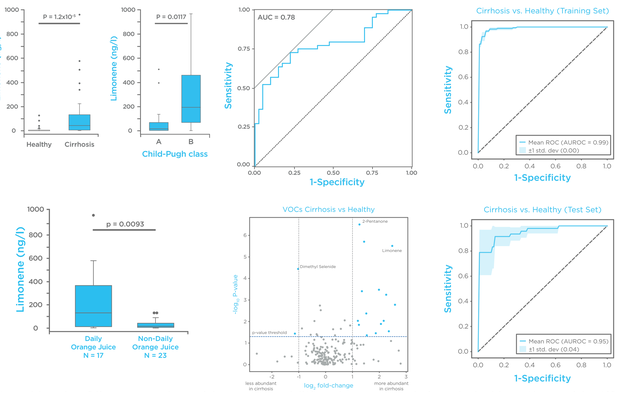

Figure 2: Altered levels of limonene alongside certain additional compounds in exhaled breath can identify subjects with cirrhosis compared to controls, demonstrating high diagnostic performance. However, limonene and the other compounds originate from the diet· therefore the amount of these compounds in the breath depends on the subject's diet[2,3]. Therefore, diet is a confounding factor that needs to be controlled to maximize the diagnostic performance of compounds in exhaled breath for cirrhosis.

References

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3. Ferrandino G, De Palo G, Murgia A, Birch O, Tawfike A, Smith R, et al. Breath Biopsy((R)) to Identify Exhaled Volatile Organic Compounds Biomarkers for Liver Cirrhosis Detection. J Clin Transl Hepatol 2023;11(3):638-648. doi: 10.14218/JCTH.2022.00309. PMID: 36969895
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2. Methods

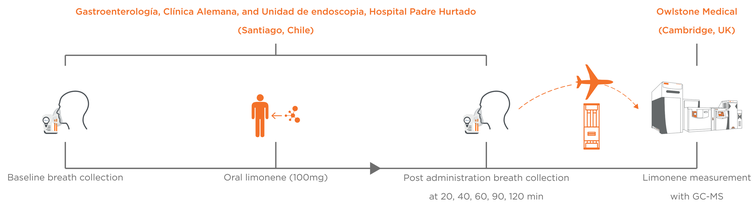

Figure 3: This cross-sectional case-control study was approved by "Comité Ético Científico de la Facultad de Medicina Clínica Alemana Universidad del Desarollo" and all participants provided written informed consent. All of the procedures were conducted in compliance with applicable guidelines for the ethical conduct of the study with their origins in the Declaration of Helsinki. Presence or absence of cirrhosis was established using ultrasound according to EASL and AASLD guidelines. Subjects older than 18 years old and weighing more than 60 kg were randomly enrolled between January 2022 and December 2022 and instructed before the experiment to fast for ≥10 h, to not consume alcohol the previous day, and to not brush their teeth or use mouthwash in the previous 2 h. Breath Biopsy® samples were collected before and after ingestion of 100 mg limonene. Samples were shipped to Owlstone Medical (UK) for analysis using gas-chromatography mass spectrometry (GC-MS). Absolute limonene quantification was obtained against a standard curve.

3. Results

Table 1	Control	Cirrhosis	p-values
Number of patients	29	29	-
Age median [IQR] years	43 [38-57]	59 [54-67]	< 0.001
Sex n (%)	M - 11 (38%) F - 18 (62%)	M - 9 (31%) F - 20 (69%)	-
Height median [IQR] cm	163 [158-170]	160 [156-170]	0.32
Weight median [IQR] Kg	75 [64-84]	78 [68-85]	0.50
BMI median [IQR]	26.8 [23.9-31.2]	27.7 [26.0-32.8]	0.23
Waist circumference median [IQR] (cm)	90 [81.5-104]	106 [97-113]	0.036
Child-Pugh class	-	23 (78%), 5 (16%), 2 (6%)	-
MELD median [IQR]	-	10 [7.2-12.8]	-
FIB4 median [IQR]	1.4 [0.8-3.1]	2.3.1 [1.9-4]	p < 0.001
APRI median [IQR]	0.2 [0.2-0.3]	0.6 [0.4-0.9]	p < 0.001
Platelets median [IQR] *10³/L	240 [216-294]	147 [107-209]	p < 0.001
Total bilirubin median [IQR] (µmol/L)	8.2 [6.5-12.9]	14.3 [8.2-18.4]	p < 0.001
Serum Albumin median [IQR] (g/L)	45 [44-45]	40 [37-44.5]	p < 0.001
INR median [IQR]	1 [1-1.06]	1.2 [1.0-1.4]	p < 0.001
ALT median [IQR] (IU/L)	18 [15-26]	28.5 [18.7-38]	p < 0.001
AST median [IQR] (IU/L)	21 [18.5-24]	35 [27.2-45.7]	p < 0.001
GGT median [IQR] (IU/L)	24 [16-32]	76 [59.2-108.7]	p < 0.001
ALP median [IQR] (IU/L)	85 [71.5-101.5]	117 [94.5-153]	p < 0.001
Creatinine median [IQR] (mg/dL)	0.78 [0.68-1.93]	0.74 [0.64-0.87]	0.31
Sodium median [IQR] (mM)	141 [138.7-142.5]	142 [139.5-142.5]	0.65

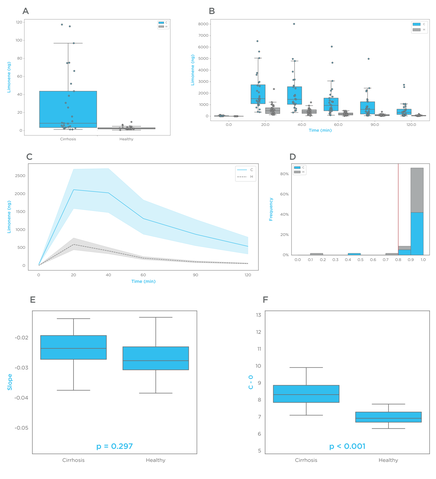

Table 2. Limonene exhalation kinetic parameters			
Parameter	Control	Cirrhosis	p-value
Cmax (ng) median [IQR]	595 [361 - 903]	2077 [1051 - 4260]	< 0.001
Log10 C_0 median [IQR]	6.9 [6.69 - 7.29]	8.4 [7.9-8.9]	< 0.001
Tmax n (%) 20 min 40 min 60 min 90 min 120 min	18 (62.1%) 11 (37.9%) 0 0 0	13 (44.8%) 12 (41.4%) 2 (6.9%) 1 (3.4%) 1 (3.4%)	-
AUC (0-90 min) ng*min/400ml median [IQR]	27107 [17605 - 34946]	121437 [57921 - 202733]	<0.001
Slope	-0.027 [-0.031; -0.023;]	-0.025 [-0.027 -0.019]	0.072

Figure 4: After overnight fasting, subjects with cirrhosis showed significantly elevated levels of breath limonene as expected[4] (A). Limonene administration induced a spike on breath with significantly higher levels in subjects with cirrhosis at all of the tested timepoints (B). A higher limonene bioavailability was observed in the cirrhosis group (C). A semi logarithmic presentation of exhaled limonene profile showed a first order decay with > 90% of the subjects showing an R2 > 0.8 (D). The slope of the control and cirrhosis group showed no significant difference (E) while the cirrhosis group showed higher c0 (intercept with y axes) (F).

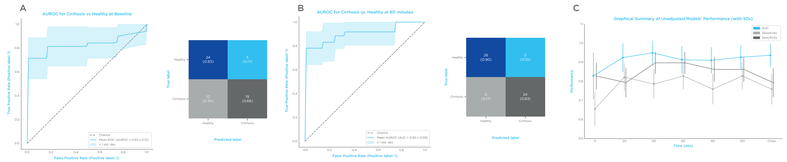

Figure 5: Levels of limonene on breath at baseline after an overnight fast showed high diagnostic/ classification performance for subjects with cirrhosis as reported previously[4] (A). All of the timepoints post administration showed improved classification performance compared to baseline, with the best performing time point being at 60 minutes (B). Importantly, limonene administration boosted sensitivity, the main parameter affected at baseline (C).

Table 3. Summary of limonene diagnostic performances at different timepoints				
Timepoint (min)	AUROC	Sensitivity/ Specificity (%)	+/- Predictive Values (%)	+/- Likelihood Values (%)
0	0.83 ± 0.12	0.66 ± 0.09/ 0.83 ± 0.07	79.17/70.59	3.8/0.42
20	0.92 ± 0.07	0.82 ± 0.095/ 0.79 ± 0.08	79.88/81.62	3.97/0.23
40	0.94 ± 0.06	0.79 ± 0.08/ 0.9 ± 0.06	88.37/80.71	7.6/0.24
60	0.91 ± 0.07	0.83 ± 0.07/ 0.9 ± 0.06	88.89/83.87	8.0/0.19
90	0.91 ± 0.07	0.76 ± 0.08/ 0.86 ± 0.06	84.62/78.12	5.5/0.28
120	0.93 ± 0.06	0.83 ± 0.07/ 0.86 ± 0.06	85.71/83.33	6.0/0.2

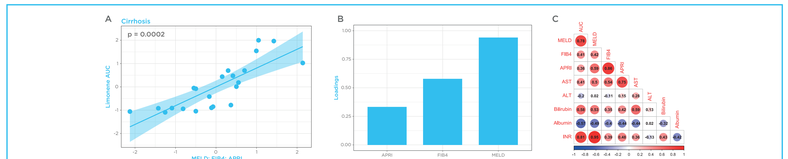

Figure 6: Limonene bioavailability measured as area under the curve (AUC) in subjects with cirrhosis correlates with disease severity estimated using MELD, and risk of advanced fibrosis estimated using FIB4 and APRI. Canonical correspondence analysis (CCA) score plot using the first canonical variates of limonene bioavailability, estimated as AUC, and scoring parameters. Each projected data point represents the combined information of limonene AUC and scoring systems of one cirrhotic patient (A) . Canonical loadings of the scoring systems representing their correlation between the original variable and its canonical variate and express the contribution of each variable to the overall correlation (B). Correlation matrix visualizing the Spearman correlation between indicated variables (C). Intensity of the red and blue colors indicate strength of respectively positive and negative correlations, while circle size represents the significance test between the variables. A total of six subjects with incomplete metadata were excluded from analysis.

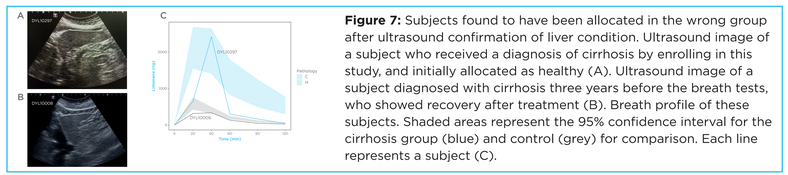

Figure 7: Subjects found to have been allocated in the wrong group after ultrasound confirmation of liver condition. Ultrasound image of a subject who received a diagnosis of cirrhosis by enrolling in this study, and initially allocated as healthy (A). Ultrasound image of a subject diagnosed with cirrhosis three years before the breath tests, who showed recovery after treatment (B). Breath profile of these subjects. Shaded areas represent the 95% confidence interval for the cirrhosis group (blue) and control (grey) for comparison. Each line represents a subject (C).

4. Conclusions and Next Steps

- Dynamic breath analysis enhances classification performance for cirrhosis opens up the opportunity to implement a reliable test in primary care.
- Alterations of limonene breath kinetics in cirrhosis resemble the changes induced by drugs with high hepatic extraction.
- Correlation of limonene with disease severity suggests applications for non-invasive monitoring of therapeutic interventions.

- Identify additional compounds with low hepatic extraction to assess their accuracy in reporting on metabolic alterations.
- Explore applications for disease progression and regression.
- Investigate similar approaches in earlier stages of liver disease such as non-alcoholic steatohepatitis (NASH).