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PAPER

Breathomics—exhaled volatile organic compound analysis to detect hepatic encephalopathy: a pilot study

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Abstract

The current diagnostic challenge with diagnosing hepatic encephalopathy (HE) is identifying those with minimal HE as opposed to the more clinically apparent covert/overt HE. Rifaximin, is an effective therapy but earlier identification and treatment of HE could prevent liver disease progression and hospitalization. Our pilot study aimed to analyse breath samples of patients with different HE grades, and controls, using a portable electronic (e) nose.

42 patients were enrolled; 22 with HE and 20 controls. Bedside breath samples were captured and analysed using an uvFAIMS machine (portable e-nose). West Haven criteria applied and MELD scores calculated.

We classify HE patients from controls with a sensitivity and specificity of 0.88 (0.73–0.95) and 0.68 (0.51–0.81) respectively, AUROC 0.84 (0.75–0.93). Minimal HE was distinguishable from covert/overt HE with sensitivity of 0.79 and specificity of 0.5, AUROC 0.71 (0.57–0.84).

This pilot study has highlighted the potential of breathomics to identify VOCs signatures in HE patients for diagnostic purposes. Importantly this was performed utilizing a non-invasive, portable bedside device and holds potential for future early HE diagnosis.

Abbreviations

AUROC	Area under receiver operator curve
BAD	Bile acid diarrhoea
GCMS	Gas chromatography and mass spectrometry
HCC	Hepatocellular carcinoma
HE	Hepatic encephalopathy
IBD	Inflammatory bowel disease
MELD	Model for end stage liver disease
uvFAIMS	Ultra-violet field asymmetric ion mobility spectroscopy
VOCs	Volatile organic compounds

1. Introduction

Hepatic encephalopathy (HE) is a progressive but reversible neuropsychiatric condition and is the hallmark

of decompensated liver disease [1]. The clinical spectrum of HE is broad with varying degrees of symptoms and effects on intellectual, cognitive and motor function. The distinction of the various classes of HE are currently made clinically via the use of the West Haven criteria (see table 1). Clinical presentation ranges from no impairment of cognitive function (Grade 0), to minimal HE (MHE) which has imperceptible clinical symptoms, requiring cognitive testing to elucidate, to minor cognitive impairment (Grade I), to the more apparent confusion and coma (Grade II–IV). West Haven criteria are commonly utilized but are subject to inter-observer variability [2]. A recent attempt to clarify the diagnosis by the International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN) guidelines led to the re-classifying of Minimal and Grade I HE into 'covert HE' (CHE), with Grades II to IV as 'overt HE' (OHE), but scoring still based on the West Haven criteria [2].

Table 1. West Haven classification.

Stage	Definition
0	Lack of detectable changes in personality or behaviour. Minimal changes in memory, concentration, intellectual function, and coordination. Asterixis are absent.
1	Trivial lack of awareness. Shortened attention span. Impaired addition or subtraction. Hypersomnia, insomnia, or inversion of sleep pattern. Euphoria, depression, or irritability. Mild confusion. Slowing of ability to perform mental tasks. Asterixis can be detected.
2	Lethargy or apathy. Minimal disorientation. Inappropriate behaviour. Slurred speech. Obvious asterixis. Drowsiness, lethargy, gross deficits in ability to perform mental tasks, obvious personality changes, inappropriate behaviour, and intermittent disorientation, usually regarding time.
3	Somnolent but can be aroused, unable to perform mental tasks, gross disorientation about time and place, marked confusion, amnesia, occasional fits of rage, present but incomprehensible speech.
4	Coma with or without response to painful stimuli.

The development of HE is associated with a poorer prognosis, even accounting for a patient's Model for End Stage Liver Disease (MELD) score [3]. The prevalence of minimal HE is reported between 30%–84% in patients with liver cirrhosis, with profound effects on daily functioning and nearly 50% of minimal HE patients may be unfit to maintain employment [1]. Therefore earlier detection of minimal HE and the prevention of overt HE are clearly of vital importance.

Current regimes for treatment and secondary prevention of various grades of HE include non-absorbable disaccharides (e.g. lactulose) and non-absorbable antibiotics e.g. Rifaxamin [1]. Non-absorbable disaccharides are fermented in the colon resulting in the formation of lactic and acetic acid which converts ammonia into ammonium which is not systemically absorbed and excreted in the stool. These non-absorbable disaccharides are currently recommended as 1st line therapy for overt HE [4]. There is increasing evidence to support their use in the treatment of minimal HE, with significant reduction in abnormal psychological testing, blood ammonia levels and progression to overt HE, including a meta-analysis of five studies [1].

Rifaxamin, meanwhile, acts by inhibiting bacterial RNA/protein synthesis, thus modulating their activity and reducing intestinal ammonia and toxin formation [1]. It has been demonstrated to be a superior treatment option than lactulose alone to overt HE [5], and to work best when combined with lactulose to treat overt HE [6]. It has since been demonstrated to improve neuropsychometric functioning, reduce ammonia levels and improve Health Related Quality of Life (HRQoL) scores and improve driving function [7, 8].

While these treatments have been demonstrated to improve symptoms and daily functioning of patients with minimal HE and overt HE, most will only receive treatments after they have presented with overt HE. Earlier detection of these clinical states may allow

treatment to be started earlier and improve the long terms clinical outcomes for these patients. Thus the clinical challenge is to detect those with minimal HE using non-invasive and reliable tools.

The detection of volatile organic compounds (VOCs) has been a rapidly expanding area of research in recent years. The study of the potential use of VOC patterns in bodily secretions (breath, urine and faeces) as non-invasive biomarkers to help identifying various disease states in multiple body systems has shown considerable promise [9–19]. The mechanism by which VOCs are generated is the subject of on-going research. Their generation within the body can be the result of metabolic derangement, toxin or teratogen exposure and finally microbiological process [20, 21]. Within the GI tract their production represents the results of fermentation of dietary non-starch polysaccharides and thus the complex interaction of diet, gut epithelial cells, human gut microflora and invading pathogens [22–24]. These fermentation products can exist in the gaseous phase and are present in exhaled air, sweat, urine and faeces [25]. Their presence in bodily secretions from sites other than the GI tract (sweat, exhaled air and urine) is possible due to the altered gut permeability afforded in certain disease states. This allows the transfer of the VOCs into circulating blood, and from there they can either; pass into the lungs via the right ventricle (volatiles which are highly diffusible will then preferentially cross the alveolar membrane and exit in exhaled breath) or the urine (via filtration after circulatory delivery to the kidneys) [21, 25].

There is now increasing evidence that gut microbiota play a major role in the pathogenesis of liver disease ranging from non-alcoholic fatty liver disease to cirrhosis and hepatocellular carcinoma (HCC) [26]. We believe that VOCs represent a patient specific bio-signature, which is affected by a variety of factors including

host microbiome, disease state and environmental factors such as diet.

There are a number of approaches to measure VOCs in breath. Most early studies employed GCMS (gas chromatography mass spectrometry), through a separate capture and pre-concentration approach (usually a tedlar bag or metal pipe followed by subsequent capture on tenax tube and desorption into the GCMS system) [27]. The large number of steps together with limited chemical information captured and the expense of the equipment makes this approach of limited clinical use. More recently, SIFT-MS (selective ion flow tube-mass spectrometer) has been the method of choice for breath analysis [28]. It is highly sensitive and selective, but has a unit cost even greater than GCMS and is significantly more bulky. Both of these approaches require specialized facilities, services and trained staff to operate. There have also been a number of studies employing electronic (e) noses. These instruments can be made portable, relatively low-cost and use air as the carrier [9]. However, they are limited to sensor drift, humidity dependence and low sensitivity. More recently, FAIMS (field asymmetric ion mobility spectrometer) has shown promise in the medical domain, using the movement of molecules in high electric fields. Such instruments can work at room temperature and pressure, use air as the carrier gas and have reduced drift due to measuring physical properties of molecules (instead of chemical in an electronic nose) [14]. Usually, these instruments require a Ni-63 radioactive source, which requires special licenses in many countries. However, the uvFAIMS system (deployed in this work) removes this need, making it more applicable to such studies. We believe that this is the first medical work reported using this instrument.

The aim of this study is to determine whether HE could be distinguished from healthy controls by analysis of exhaled VOCs (breathomics). We also sought to determine whether it could have potential to distinguish covert from overt HE.

2. Materials and methods

2.1. Subjects

A total of 42 patients were recruited for this pilot study; 22 patients with HE (13 covert and 9 with overt HE as well as 20 healthy controls without clinical or biochemical evidence of liver disease. Healthy controls were chosen to confirm that the breath profiles of those with liver disease are distinct from the healthy. All the liver patients had disease secondary to alcohol injury. Severity of disease and grading was recorded according to West Haven and the updated ISHEN criteria (table 1) as well as MELD scores. Patients' demography and diets were recorded. Patients with type 2 diabetes or other gastrointestinal conditions (inflammatory bowel disease, coeliac disease and cancer) were excluded from the study. The demographics of the subjects are shown in table 2.

Table 2. HE patient demographics.

Demographic	Liver patients (n = 22)	Controls (n = 20)
Sex (Male: Female)	15: 7	6: 14
Mean Age (Range; SD)	55 (30–80; 15)	31 (23–54; 10)
HE grade (Covert & Overt)	13:9	NA
Mean MELD score (Range; SD)	13 (6.4– 19; 3.7)	NA
HE prophylaxis (Y/N)	20:2	NA
current smokers (Y/N)	3: 19	1: 19
Mean BMI (Range; SD)	26 (16–40; 6.3)	24 (18–3; 3.2)

2.2. Specimen collection

Morning breath samples were taken from both patients and controls after a period of at least a 2 hour fast and absence of cigarette smoking. The specimens were collected using a 3 litre Tedlar® bag (Thames Restek, UK) which had a ¼ inch OD nozzle tube and threaded valve for sample capture. A custom made breath capture device was constructed to allow sampling. The breath capture device allows patients to breathe naturally through a mouth piece into the attached Tedlar® bag. The device comprises of a filter, to ensure controlled environmental standards for sampling, to which is connected to a 2 way valve. The two way valve allows the patient to inhale clean air (which first passes through the filter) and then exhale into the tedlar bag. These valves also had custom made PTFE adaptors attached to allow for connection to disposable mouthpieces, whilst the other adaptors connect to an additional three way tap between the exhaled breath path and the Tedlar bag. This tap is then attached to the breath bag via disposable tubing and allows for the isolation of end-tidal breath in the bags, which has higher VOC content. All samples were obtained from patients directly and placed in a freezer (−20 °C) as soon as possible. The samples were then shipped to the University of Warwick with a total of one hour to reach room temperature before analysis (including the transport time). Once defrosted the sample bags were attached to the FAIMS via the custom unit as described in the next section. All samples were tested on the same day as capture.

2.3. Analysis

Analysis was undertaken using an ultra-violet Field Asymmetric Ion Mobility Spectroscopy (uvFAIMS, Lonestar, Owlstone UK). This system achieves separation of chemical components on the basis of differences in molecular mobilities in a high electric field. FAIMS allows gas molecules to be separated and analysed at atmospheric pressure and room temperature. Once the sample is ionized, the resulting ions are passed between two metal plates and then an asynchronous high voltage waveform is applied to these plates, subjecting the ionized molecules to high electric fields. The difference in movement of these ions within this high electric field can be measured, thus

resulting in a separation of the complex mixture. The field dispersion (ion mobility) is then tracked to form a characteristic plume.

The Lonestar was modified with a custom set up designed specifically for breath sampling. This custom made unit comprises an inlet port for the Tedlar® bags, compressed air running through a mass-flow controller (MFC) and an external pump on the instrument exhaust. The use of a MFC allowed for greater control of the air : sample ratio to optimize the analysis conditions. The flow rate of the sample was set by controlling the difference between the pump (set to 1.8 l min^{-1}) and the compressed air line (1.5 l min^{-1}) this results in a vacuum and the sample inlet then draws 300 ml min^{-1} of the breath sample into the machine for analysis. Each sample was run to collect 2 matrices of data, using approximately 2.3 l of sample.

2.4. Statistical methods

The extracted data was then analysed using a previously developed analysis pipeline based on a 2D wavelet transform and threshold to remove background noise. This was followed by feature selection to identify key variables (using a Wilcoxon rank-sum test applied separately to each feature), with the resultant feature set used to separately train four classifiers (sparse logistic regression, Random Forest, Support Vector Machine and Gaussian Process). Sensitivity, specificity, and Area Under Receiver Operator Curve (AUROC) were calculated in leave-one-out cross validation. Sparse logistic regression gave the best overall performance, so we report those results unless otherwise stated. We note that the study of multiple classifiers can be important as our experience with a range of FAIMS data sets shows that different classifiers can be best-performing in each case.

2.5. Ethics

Ethical approval was obtained from the Warwickshire Research and Development Department and Warwickshire Ethics committee (09/H1211/38). Written informed consent was obtained from all patients who participated in the study.

3. Results

There was a range of severity of liver disease as reflected by the MELD score. There were 13 patients with covert HE and 9 patients with overt HE. The demographics of the subjects are covered in table 2 and the medications of the liver patients and controls in table 3.

Classification of those with HE and controls was achieved with sensitivity and specificity of 0.88 (95% Confidence Interval (CI): 0.73–0.95) and 0.68 (95% CI: 0.51–0.81) respectively, and AUROC of 0.84 (95% CI: 0.75–0.93)—figure 1(a). Further analysis to separate covert HE from overt HE revealed a sensitivity of 0.79 (95% CI: 0.49–0.95) and specificity of 0.50 (95% CI: 0.37–0.63), AUROC 0.71 (95% CI: 0.57–0.84)—figure 1(b).

Table 3. Medications of HE patients and controls.

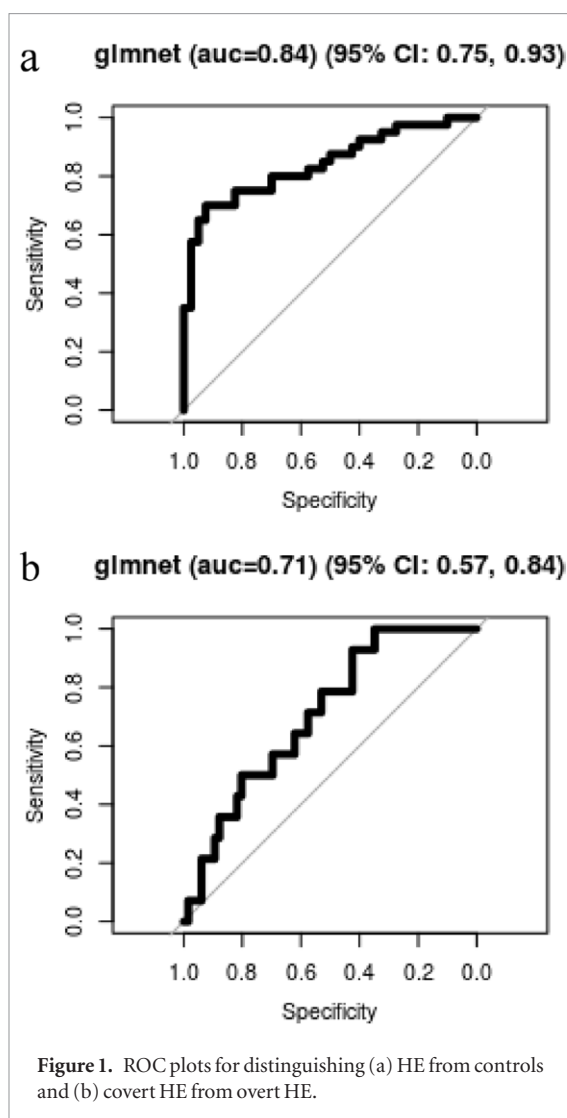
Medications	Liver patients (n = 22)	Controls (n = 20)
Gastrointestinal:		
Thiamine/Vitamin B	14	
Co-strong/Folate		
Proton Pump Inhibitors	9	
Lactulose	5	
Pabrinex	2	
Chlordiazepoxide	2	
Spirolactone/Frusemide	4	
Carvedilolol/Beta Blocker	1	
Antiemetics	1	
Ursodeoxycholic acid	1	
Cardiovascular:		
Antihypertensives	2	2
Statins	2	
Aspirin	1	
Neurological:		
Selective Serotonin	5	
Reuptake Inhibitors		
Antipsychotics	2	
Anti-convulsants	1	
Opioids	2	
Respiratory:		
Inhalers	2	2
Endocrine:		
Thyroxine	0	1

There was no statistically significant differences between differing HE grades (as per older West Haven criteria) in this pilot study, AUROC 0.61 (95% CI: 0.43–0.79). However, this may be due in part to the even smaller sample size.

4. Discussion

This pilot study provides initial evidence that breath VOC analysis has potential application as a diagnostic aid in distinguishing HE of all grades from healthy controls subjects. It may also have potential as an aid to distinguish covert HE from overt HE. This finding was through the detection of a unique gas phase bio-odorant fingerprint found in the breath of patients with HE, detected here by uv FAIMS analysis. It also expands on previous work into the non-invasive detection of many luminal GI diseases by VOC detection and more recently the non-invasive detection of NAFLD and NASH/NASH-C [9, 11–15].

Exhaled VOCs analysis performed reasonably well as a clinical tool when distinguishing HE patients from controls, suggesting a discernible VOC profile for the condition. The sensitivity and specificity for distinguishing HE from the healthy controls were 0.88 and 0.68, AUROC 0.84 (95% CI: 0.75–0.93). Within HE patients, covert HE could be distinguished from overt HE with a sensitivity of 0.79 and specificity of 0.50, AUROC 0.71 (95% CI: 0.57–0.84).



uvFAIMS is highly sensitive, portable instrument that detects both VOCs and gasses and is able to detect chemicals down to the parts per trillion (ppt) level (depending on their proton affinity). The instrument is sensitive to ammonia, but due to the range of molecular mobilities used in the analysis ammonia may only be one of a number of chemicals that play an important role. However, the role of ammonia testing in the diagnosis of HE has long been in question. It has value in the management of acute liver failure, but is not useful for the evaluation or screening of HE in patients with CLD as it does not confirm or exclude HE, and levels do not correlate with the degree of encephalopathy [29]. This technology does not allow for specific quantification of the chemicals that separate the groups nor is it necessary. It highlights rather the potential for real time separation of VOC profiles utilizing non-invasive technology (which is portable; all analysis was carried out at the bedside) to aid in the earlier detection of covert HE, and could help prevent reliance on time consuming neuropsychometric testing to identify this clinical condition. Consequently Lactulose or Rifaximin treatment could potentially be initiated at an earlier stage to hopefully provide better long term outcomes for CLD patients complicated by HE.

The non-invasive detection of disease specific volatile organic compounds (VOCs) patterns, detectable in urine, breath, sweat and faeces has become a rapidly developing field in recent years, termed 'fermentomics'. Detection of these gas phase biomarkers (VOCs/gasses) has increasingly been recognized as non-invasive means to positively diagnose patients with a wide range of diseases including pulmonary, infectious, metabolic and gastrointestinal diseases. Exhaled VOC analysis by more conventional GC-MS (gas chromatography and mass spectrometry) technology has been demonstrated to aid in the distinction of not just cancer from non-cancer patients but also multiple cancer subtypes including lung, breast, prostate and colorectal cancer [9, 10]. More recently, urinary VOCs have also been demonstrated to have potential diagnostic value in liver disease, particularly non-alcoholic fatty liver disease, in the distinction of fatty liver disease from healthy controls and also Cirrhotic Non-alcoholic steatohepatitis (NASH-C), from non-cirrhotic NASH [15].

Exhaled biomarkers have also been used in liver disease for the detection of hepatocellular carcinoma and fatty liver disease in humans [16–18]. Recent animal studies have shown that Electronic-nose technology is able to detect acute liver failure in rats with high classification accuracy [19]. VOCs are believed to be a physiological by-products of colonic fermentation and represent the result of the complex interaction between the gut epithelial cells, faecal flora, mucosal integrity and invading pathogens [12]. This provides supportive evidence for the role for gut microbial dysbiosis in the pathophysiology of liver disease, which has been suggested in other studies [26, 30, 31].

It should be noted that FAIMS technology, unlike technology based around Mass Spectrometry, does not allow for specific quantification of the chemicals that separate the groups, instead it represents the overall 'smell print' of the VOC cocktail. The value of FAIMS is the potential for real time separation of VOC profiles utilizing non-invasive technology (which is portable, breath analysis can be carried out at the bedside) to aid in the detection of HE and the distinction of the various grades of HE. This is of great clinical relevance. FAIMS is significantly cheaper technology than SIFT-MS, is a much smaller unit and does not require any specialized infra-structure making it more applicable to a medical setting [32].

Although the sample size in this proof of principle study is small, the HE patients show good reclassification accuracy within the statistical analysis. This suggests a discernible VOC profile for the HE, and particularly covert HE from overt HE. As this was a pilot study, we have not controlled for age or sex but previous work has not shown differences in VOC profiling based on sex, age, or medication differences [11, 13, 33].

Given the small sample size in this pilot study, larger validation studies are needed to confirm the reproducibility of our findings in those with all grades of HE and matched controls (liver disease without HE). Thus

breathomics in HE does hold potential for rapid, portable and non-invasive diagnosis for a condition, which, as yet is difficult to diagnose.

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