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Non-Invasive Distinction of Non-Alcoholic Fatty Liver Disease using Urinary Volatile Organic Compound Analysis: Early Results

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ABSTRACT

Background & Aims: Non-Alcoholic Fatty Liver Disease (NAFLD) is the commonest cause of chronic liver disease in the western world. Current diagnostic methods including Fibroscan have limitations, thus there is a need for more robust non-invasive screening methods. The gut microbiome is altered in several gastrointestinal and hepatic disorders resulting in altered, unique gut fermentation patterns, detectable by analysis of volatile organic compounds (VOCs) in urine, breath and faeces. We performed a proof of principle pilot study to determine if progressive fatty liver disease produced an altered urinary VOC pattern; specifically NAFLD and Non-Alcoholic Steatohepatitis (NASH).

Methods: 34 patients were recruited: 8 NASH cirrhotics (NASH-C); 7 non-cirrhotic NASH; 4 NAFLD and 15 controls. Urine was collected and stored frozen. For assay, the samples were defrosted and aliquoted into vials, which were heated to 40±0.1°C and the headspace analyzed by FAIMS (Field Asymmetric Ion Mobility Spectroscopy). A previously used data processing pipeline employing a Random Forrest classification algorithm and using a 10 fold cross validation method was applied.

Results: Urinary VOC results demonstrated sensitivity of 0.58 (0.33 - 0.88), but specificity of 0.93 (0.68 – 1.00) and an Area Under Curve (AUC) 0.73 (0.55 – 0.90) to distinguish between liver disease and controls. However, NASH/NASH-C was separated from the NAFLD/controls with a sensitivity of 0.73 (0.45 - 0.92), specificity of 0.79 (0.54 - 0.94) and AUC of 0.79 (0.64 - 0.95), respectively.

Conclusions: This pilot study suggests that urinary VOCs detection may offer the potential for early non-invasive characterisation of liver disease using 'smell prints' to distinguish between NASH and NAFLD.

Key words: FAIMS – NAFLD – NASH – cirrhosis – screening – urine – volatile organic compounds.

Abbreviations: BAD: bile acid diarrhea; BMI: body mass index; E-nose: Electronic nose; FAIMS: Field Asymmetric Ion Mobility Spectroscopy; GCMS: Gas Chromatography and Mass Spectrometry; HCC: Hepatocellular carcinoma; IBD: Inflammatory Bowel Disease; IMS: Ion Mobility Spectroscopy; NAFLD: Non-Alcoholic Fatty Liver Disease; NASH: Non-Alcoholic Steatohepatitis; NASH-C: Cirrhotic Non-Alcoholic Steatohepatitis; SIBO: small intestinal bacterial overgrowth; TE: Transient elastography ; UC: ulcerative colitis; VOCs: volatile organic compounds

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is currently the most common cause of chronic liver disease in the developed world. This epidemic is set to even overtake the health burden of hepatitis C worldwide [1]. This increase is due to a variety of factors such as the marked rise in obesity, more sedentary lifestyle, unhealthy diets, insulin resistance and type 2 diabetes [1, 2]. NAFLD represents a spectrum of liver diseases from simple fatty infiltration (steatosis) to fat infiltration with inflammation (NASH), and finally NASH with cirrhosis (NASH-C) [3, 4]. The true prevalence of NAFLD has proved hard to estimate; figures of 20-30% have been quoted [5] and one large European study found NAFLD in 94% of obese patients (body mass index, BMI >30), 67% of overweight patients (25 >BMI < 30), and 25% of normal weight patients [6]. They also found that the overall prevalence of NAFLD in type 2 diabetes patients ranged from 40 to 70% [6].

Previously, NAFLD pathogenesis was thought to follow a two hit hypothesis, the first of which involving hepatic lipid

accumulation and insulin resistance, with the second step comprising oxidative stress followed by lipid peroxidation contributing to the progression from simple steatosis to NASH [7]. Recent treatment developments have been based on the role of antioxidants and have shown some promise [8, 9]. However, there is now increasing evidence that gut microbiota play a major role in the pathogenesis of liver disease ranging from NAFLD to cirrhosis and hepatocellular carcinoma (HCC) [10]. Gut microbiota have been shown to affect the regulation of energy homeostasis and ectopic fat deposition, hence their implication in metabolic diseases. The alteration in metabolic and fat profiling can lead to abnormal fat deposition in the liver promoting steatohepatitis [11]. There is also growing understanding that small intestinal bacterial overgrowth (SIBO) plays an important role in progression of NALFD to NASH and eventually cirrhosis [12]. It has been demonstrated that NASH resulting from SIBO following jejunoileal bypass can be reversed with use of antibiotics (metronidazole) [13]. It has also been shown that combination of pro/prebiotics given to NAFLD patients can improve cytokine levels (TNF-α) and oxidative stress markers as well as the liver function tests [14-17].

Current investigations for NAFLD diagnosis are based on elevated liver enzyme levels, identification of steatosis usually via liver imaging: ultrasound, computerised tomography (CT) or transient elastography (TE, Fibroscan), with confirmation of diagnosis of NASH, with or without cirrhosis, via liver biopsy. Transient elastography has developed over recent years as a non-invasive measure of liver fibrosis by assessing liver stiffness. It has the advantage of being non-invasive, painless and easy to perform; however, it is user dependent. Inter-observer agreement was found to be significantly reduced in patients with lower degrees of fibrosis, steatosis and those with raised BMI [18]. These factors affect TE's ability to provide a reliable diagnosis in some cases. Given the improvements in treatment and the potential to reverse non-cirrhotic liver damage, new non-invasive investigative modalities to aid in the detection of pre-cirrhotic NAFLD patients would be of great benefit.

In recent years, the detection of disease specific patterns of volatile organic compounds (VOCs) in urine, breath, sweat and faeces has been a rapidly developing novel tool to allow non-invasive detection of many disease states. Such chemical analysis can be undertaken with a broad spectrum of different analytical instruments. For example, analysis of patients' breath using GCMS (Gas Chromatography and Mass Spectrometry) has revealed VOC patterns which can distinguish not just cancer from non-cancer patients, but also cancer subtypes including lung, breast, prostate and colorectal cancer [19].

Analysis of urinary VOCs has also distinguished colorectal cancer from healthy controls using FAIMS (Field Asymmetric Ion Mobility Spectrometry) technology [20]. VOC patterns in urine have been analyzed by E-nose (Electronic nose) and also FAIMS (a type of E-nose), to distinguish between not only Inflammatory Bowel Disease (IBD) and healthy controls but between active and quiescent disease for both IBD subtypes [21]. Patients with significant pelvic radiotherapy induced gastrointestinal side effects have also been identified in this manner [22]. More recent developments include the detection of bile acid diarrhoea (BAD) and distinction of coeliac disease from irritable bowel syndrome by urinary VOC analysis [23, 24]. Thus, gas phase biomarkers have been of significant interest as a method of positively diagnosing patients with a wide range of diseases relevant to the field of gastroenterology [25].

VOCs have been found to be perturbed in many physiological and pathological states including different diets and numerous diseases. The exact mechanism of VOC generation is the subject of ongoing research but their production in the gastrointestinal tract is believed to be the result of the fermentation of dietary non-starch polysaccharides. This would indicate that they represent the complex interaction of colonocytes, human gut microflora and invading pathogens [26]. The resultant products of fermentation, 'the fermentome' can exist in the gaseous phase and are present in exhaled air, sweat, urine and faeces [23, 25, 27-29]. Their presence in bodily secretions from sites other than the gastrointestinal tract (sweat, exhaled air and urine) is presumed possible due to the altered gut permeability afforded in certain disease states [27]. We believe that VOCs represent a bio-signature specific to a patient that is affected by a variety of factors such as genetics, disease state and environmental factors such as diet.

The aim of this pilot study was to determine whether urinary VOCs could distinguish between various stages of fatty liver disease (NAFLD, NASH and NASH-C) and controls.

METHODS

Subjects

A total of 34 patients were recruited prospectively for this study. Eight patients had histologically confirmed cirrhosis, NASH-C, 7 had non-cirrhotic NASH (NASH confirmed by use of Fibroscan), 4 had NAFLD (confirmed on liver ultrasound) and 15 were healthy controls without clinical or biochemical evidence of liver disease. In order to reduce confounding factors as much as possible given our small sample size we only included stable cirrhotic patients from the liver outpatient clinics; none of whom had recently or were currently receiving a course of antibiotics. Patients with type 2 diabetes or other gastrointestinal conditions (IBD, coeliac disease) were excluded from the study. The mean age of the cohort of patients and volunteers tested, which consisted of 15 males and 19 females, was 62 years (SD 8.2 years). The characteristics of the subjects are shown in Table I.

Study design

This was a prospective cohort study, with recruitment of sequential patients attending the Gastroenterology and Liver outpatient clinics at University Hospital Coventry & Warwickshire, UK. Urine was collected from patients and controls in standard universal sterilin specimen containers (Newport, UK) and frozen to -80°C, after collection, for subsequent batch analysis.

Analysis

This was undertaken using FAIMS (Owlstone Lonestar (UK)). Post collection, urine specimens were frozen at -80°C (within 2 hours) and stored for later batch analysis. Prior to

Table I. Characteristics of liver disease patients and healthy controls (SD – standard deviation)

Disease group	Sample size (n)	Male/ Female (ratio)	Mean age in years (SD)	Mean cigarettes smoked /day(SD)	Weekly alcohol intake in units (SD)	Mean BMI Kg/m^2 (SD)
Volunteers	15	36/64	61.1 (10.8)	0.5 (0)	8.2 (20.9)	27.2 (3.6)
NAFLD	4	1/3	62.8 (8.8)	0 (0)	2.5 (3.3)	25.3 (2.2)
NASH	7	71/29	58 (6.9)	0 (0)	4.2 (7.3)	31 (2.6)
NASH-Cirrhosis	8	1/1	70 (4.4)	0 (0)	0.6 (1.1)	32.5 (4.5)

analysis they were left to thaw in a laboratory fridge overnight and aliquoted into appropriate sample bottles (as described below). The samples were then analyzed as previously described [30]; an abbreviated summary is provided below.

FAIMS (Field Asymmetric Ion Mobility Spectroscopy) achieves separation of complex chemical mixtures based on differences in molecular mobilities within a high electric field. It allows gas molecules to be separated and analyzed at atmospheric pressure and room temperature. In FAIMS the sample is first ionised and the resulting ions are passed between two metal plates to which an asynchronous high voltage waveform is applied. This subjects the ionised 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.

A sample of 5 ml of urine was aliquoted into a standard 22 mL borosilicate glass vial (Fisher Scientific, UK). The vials are inserted into the Owlstone ATLAS sampler unit, which then heats the sample to 40°C. The Lonestar was set up in a pressurised configuration with a flow rate of 2 L/min. The dispersion field was stepped through 51 equal settings between 0 and 90% (the dispersion field is related to the magnitude of the electric field) and for each dispersion field the compensation voltage stepped was between +6V and -6V in 512 steps (a method of precisely measuring the molecular movement in the high electric field).

Statistical methods

Analysis of FAIMS data followed the same pipeline for consistency. Due to the very large datasets produced by the FAIMS (52, 224 in total) the data was concatenated into a 1D array. A wavelet transform (Daubechies D4) was then used to extract important features (wavelet transforms are a type of compression used regularly in audio and vision application - they are very good at separating subtle changes in one frequency that is being swamped by a different, much larger signal). Once completed, coefficients below a given threshold were removed on the basis that these are dominated by noise. Next, a Wilcoxon rank-sum test was used on the training set to identify the features that were most informative in discriminating between different samples. An in-depth machine learning analysis was then carried out using a 10-fold cross-validation. The Random Forest algorithm (implemented in the R package 'randomForest', version 4.6-10, R version 3.1.1) (http://cran.ro-project.org/web/packages/randomForest/index.html) was applied inside the cross-validation loop. From previous studies we have shown that Random Forest provides the best performance for these types of datasets and this was applied here [20-24].

Ethics

Scientific and ethical approval was obtained from the Warwickshire Research & Development Department and Warwickshire Ethics Committee 09/H1211/38. Written informed consent was obtained from all patients who participated in the study.

RESULTS

The characteristics of the NAFLD, NASH, NASH-C and healthy control patients are described in Table I. No statistically significant difference between the groups was noted. However, there was female gender predominance in our control group.

Sensitivity and specificity for the various groups were then calculated. Due to the small sample size we extracted each disease group in turn and then compared them to the other remaining samples including controls. The Random Forest algorithm was then applied to analyze the data, with a 10-fold cross validation to ensure robustness and avoiding type 2 errors.

The analysis showed that urinary VOCs were able to distinguish samples of patients with liver disease (NASH, NASH-C or NAFLD) from the healthy control samples with 0.58 (0.33 – 0.88) sensitivity but specificity of 0.93 (0.698 – 1.00) and Area Under the Receiver Operating Curve, AUROC 0.73 (0.55 – 0.90) (Fig. 1).

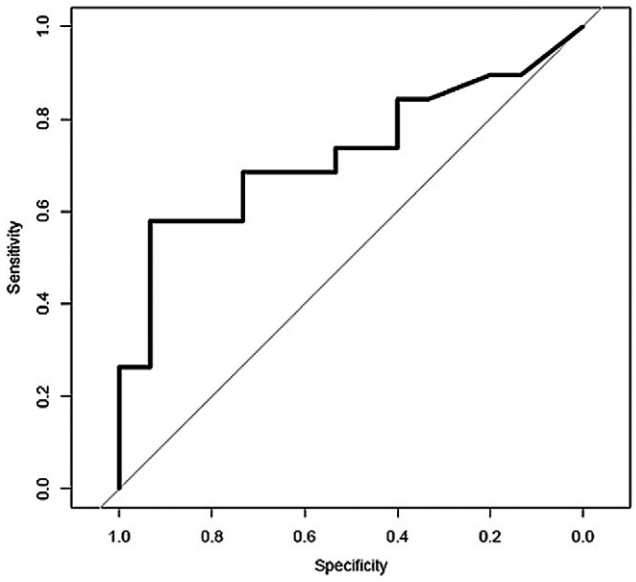

Fig. 1. AUC of liver disease vs controls: 0.73 (95% CI: 0.55-0.90).

Similarly it was also able to separate NASH (both NASH with and without cirrhosis) from NAFLD with sensitivity of 0.73 (0.45 - 0.92), specificity of 0.79 (0.54 - 0.94) and AUROC of 0.79 (0.64 - 0.95) (Fig. 2).

Urinary VOCs' smell prints were also able to distinguish NAFLD from controls as well as separating NASH-C from NASH alone (without cirrhosis). Table II highlights the sensitivities and specificities for various fatty liver conditions - NASH, NASH-C and NAFLD and for healthy controls.

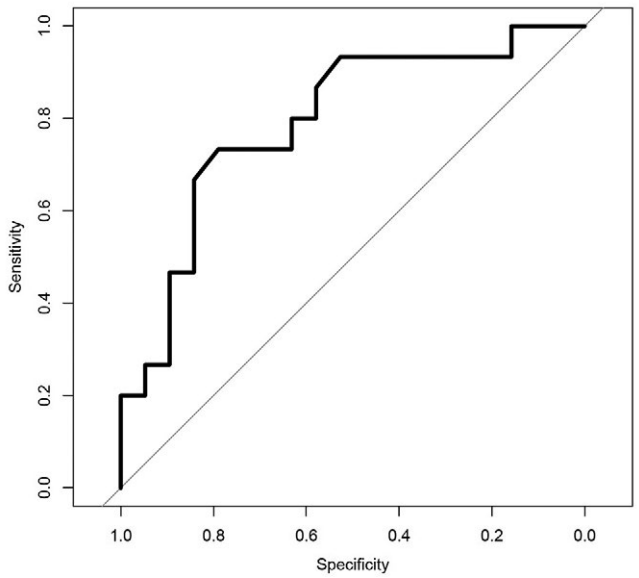

Fig. 2. AUC of NASH (including cirrhosis) vs NAFLD: 0.79 (0.64 - 0.95).

DISCUSSION

This pilot study provides initial evidence that urinary VOCs have potential application as an alternative non-invasive diagnostic test to distinguish between the various stages of NAFLD compared with healthy control subjects. This was achieved through the detection of unique gas phase bio-odorant fingerprints found in urine. Moreover it expands on previous research on VOC patterns in urine of patients with luminal gastrointestinal disease [25].

VOCs are believed to be gaseous by-products of colonic fermentation, the result of a complex interaction between the colonocyte cells, human faecal flora, mucosal integrity and invading pathogens [28]. These usurp into bodily fluids and as a result, VOCs are detected in urine, faeces and breath. Consequently they have huge potential as biomarkers to aid in the assessment of gastrointestinal and even metabolic diseases. Changes found in the pattern of VOCs are reflective of changes and variations within the gastrointestinal environment. This provides supportive evidence for the role of gut microbial dysbiosis in the pathophysiology of NAFLD and NASH, which has been suggested in other studies [10- 12].

All patients within the cirrhosis group had histologically confirmed cirrhosis on liver biopsy. The non-cirrhotic NASH group had NASH confirmed by Fibroscan but without liver biopsy. We selected patients (for this proof of principle study) in whom there was no clinical suspicion of cirrhosis and Fibroscan stiffness scores were below the range normally found in cirrhotic patients.

When comparing the liver disease groups with healthy controls, the relatively low sensitivity but contrasting high specificity suggests that the chemicals that are varied by the presence of liver disease are dissimilar in the various fatty liver conditions. This would suggest one of three possibilities: 1) the results seen are due to modulation of the whole chemical profile of the disease state rather than an individual chemical; 2) chemicals produced in some subjects have a similar mobility to the chemical of interest; 3) the sensitivity of our system is insufficient to detect the target molecules in some of the patient cohort. This will strongly depend on the proton affinity of the molecules in question.

Although the sample size in this proof of principle study is small, data from the various subgroups of liver disease (NAFLD, NASH, NASH-C) are tightly clustered and have a high reclassification accuracy. This suggests that there may be a discernible VOC profile for the various stages of NAFLD. Within this pilot study we have not controlled for age or sex but in our previous work neither of these variables affected VOC profiling [20-24]. All diabetic patients were excluded and only stable cirrhotic patients were included, none of whom were receiving or had recently received any antibiotics.

CONCLUSIONS

The unique chemical 'fingerprint' pattern produced by the different disease states demonstrated in this pilot study suggests there is potential for urinary VOCs as a non-invasive adjunctive diagnostic tool for patients with NAFLD, NASH and NASH-C. It also provides some evidence that the detection of these urinary VOCs could represent a way forward in the search to

Table II. Sensitivity, specificity and AUROCs of urinary VOCs in distinguishing between liver disease and controls. 95% confidence intervals are given in brackets

Disease group	FAIMS Sensitivity	FAIMS Specificity	FAIMS AUROC
NAFLD	0.75 (0.19 - 0.91)	0.93 (0.68 – 1.00)	0.82 (0.52 – 1.00)
NASH	0.67 (0.22 - 0.96)	0.93 (0.68 – 1.00)	0.81 (0.57 – 1.00)
NASH-C	0.44 (0.14 - 0.79)	0.93 (0.68 -1.00)	0.63 (0.36 - 0.90)
NASH-C/NASH vs NAFLD & controls	0.73 (0.45 - 0.92)	0.79 (0.54 - 0.94)	0.79 (0.64 - 0.95)
Liver disease (NAFLD, NASH, NASH-C) vs Controls	0.58 (0.33 - 0.88)	0.93 (0.68 -1.00)	0.73 (0.55 - 0.90)

find an alternative approach to the diagnosis and monitoring of patients with confirmed fatty liver disease, especially in cases where patients are unwilling to undergo liver biopsy or those in whom liver biopsy is deemed unsuitable. Further larger scale studies would need to be undertaken to confirm that the patterns shown here are also present in larger cohorts and to identify the chemicals involved.

Conflicts of interest: No conflict to declare.

Authors' contribution: R.A., J.C.: conception of idea and final review for academic content; R.A., M.McM.: review of clinical data and writing of manuscript; S.W., N.O'C.: patient recruitment and review of clinical data; J.S., R.S.: statisical support and review of data; K.D.B., C.U.N.: review of manuscript for intellectual content.

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