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Research Paper

Volatile Biomarkers in Breath Associated With Liver Cirrhosis — Comparisons of Pre- and Post-liver Transplant Breath Samples

R. Fernández del Río [a], M.E. O'Hara [a,*], A. Holt [b], P. Pemberton [c], T. Shah [b], T. Whitehouse [c], C.A. Mayhew [a]

[a] School of Physics and Astronomy, University of Birmingham, Birmingham B15 2TT, UK
[b] Department of Hepatology, University Hospital Birmingham NHS Trust, Birmingham B15 2TH, UK
[c] Critical Care and Anaesthesia, University Hospital Birmingham NHS Trust, Birmingham B15 2TH, UK

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ABSTRACT

Background: The burden of liver disease in the UK has risen dramatically and there is a need for improved diagnostics.
Aims: To determine which breath volatiles are associated with the cirrhotic liver and hence diagnostically useful.
Methods: A two-stage biomarker discovery procedure was used. Alveolar breath samples of 31 patients with cirrhosis and 30 healthy controls were mass spectrometrically analysed and compared (stage 1). 12 of these patients had their breath analysed after liver transplant (stage 2). Five patients were followed longitudinally as in-patients in the post-transplant period.
Results: Seven volatiles were elevated in the breath of patients versus controls. Of these, five showed statistically significant decrease post-transplant: limonene, methanol, 2-pentanone, 2-butanone and carbon disulfide. On an individual basis limonene has the best diagnostic capability (the area under a receiver operating characteristic curve (AUROC) is 0.91), but this is improved by combining methanol, 2-pentanone and limonene (AUROC curve 0.95). Following transplant, limonene shows wash-out characteristics.
Conclusions: Limonene, methanol and 2-pentanone are breath markers for a cirrhotic liver. This study raises the potential to investigate these volatiles as markers for early-stage liver disease. By monitoring the wash-out of limonene following transplant, graft liver function can be non-invasively assessed.

1. Introduction

The publication of the 2014 Lancet Commission on liver disease has highlighted how the burden of liver disease in the UK has risen sharply over the past few decades and that it poses a major public health issue (Williams et al., 2014). It is the only major cause of mortality and morbidity which is on the increase in England, while at the same time decreasing in most other European countries, with cirrhosis accounting for 83% of deaths (Davies, 2012). It is the third biggest cause of premature mortality, with three quarters of liver deaths due to alcohol (Williams et al., 2014). Liver disease has a widespread effect not only to the patient, encompassing physical and psychological morbidity and mortality, but also incurring significant societal costs. One of the main difficulties is that often patients do not present symptoms or signs until the disease is advanced. Even then diagnosis is difficult and the symptoms and signs are often general and can be mistaken for other pathologies. Non-invasive diagnostic techniques currently used, namely serum biomarkers and transient elastography (TE) are not ideal. Serum biomarkers are not liver specific and TE results require an expert clinician for interpretation (Castera et al., 2015).

Among the ten key recommendations in a recent Lancet report is to strengthen the detection of early-stage liver disease, which is essential to reduce disease progression (Williams et al., 2014). Analysis of volatiles in the breath has the potential to deliver this, but only if chemical compounds can be found that are unambiguously associated with a diseased liver.

To date, the use of breath volatiles for medical diagnosis has met with limited success. Confounding factors, such as volatiles present in the environment, contamination in the sampling procedures and poor sampling methods, have meant that there is a great deal of uncertainty in volatile discovery (Kwak and Preti, 2011). Problems of bias and false

Abbreviations: AID, autoimmune liver disease; ALD, alcoholic liver disease; AUROC, area under receiver operator curve; CD, cryptogenic disease; GC, gas chromatography; HBV, hepatitis B virus; HCC, hepatocellular cancer; HCV, hepatitis C virus; ITU, intensive treatment unit; LQ, lower quartile; MS, mass spectrometry; OPU, out-patient clinic; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; ppbv, parts per billion by volume; ppmv, parts per million by volume; PTR-MS, proton transfer reaction mass spectrometry; ROC, Receiver operating characteristics; TAC, transplant assessment clinic; TE, transient elastography; UKELD, United Kingdom model for end-stage liver disease; UQ, upper quartile; VOC, volatile organic compounds; VMR, volume mixing ratio; BMI, body mass index.

* Corresponding author.
E-mail addresses: r.fernandezdelrio@bham.ac.uk (R. Fernández del Río), M.E.OHara@bham.ac.uk (M.E. O'Hara), Andrew.holt@uhb.nhs.uk (A. Holt), pembo@doctors.ork (P. Pemberton), Tahir.Shah@bham.ac.uk (T. Shah), TonyWhitehouse@uhb.nhs.uk (T. Whitehouse), c.mayhew@bham.ac.uk (C.A. Mayhew).

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discovery in biomarker discovery research have been widely reviewed (Broadhurst and Kell, 2006; Ransohoff, 2005).

Previous studies investigating breath volatiles in patients suffering with liver disease have proposed a large number of possible biomarkers (Millonig et al., 2010; Morisco et al., 2013; Sehnert et al., 2002; Solga et al., 2006; Tangerman et al., 1983; Van den Velde et al., 2008; Dadamio et al., 2012; Friedman et al., 1994; Hanouneh et al., 2014; Khalid et al., 2013, 2014; Shimamoto et al., 2000; Vollnberg et al., 2009; Verdam et al., 2013), but generally different studies report different volatiles. Various GC–MS studies found raised levels of many volatiles in the breath of patients with liver disease, including dimethyl sulfide, acetone, 2-butanone, 2-pentanone, β-pinene, α-pinene, and limonene (Van den Velde et al., 2008; Dadamio et al., 2012; Friedman et al., 1994; Khalid et al., 2013). Studies using soft chemical ionization mass spectrometric techniques have reported volatiles such as acetaldehyde, ethanol, isoprene, benzene, methanol, 2-butanone, 2- or 3-pentanone, heptadienol, and a monoterpene (limonene) (Morisco et al., 2013). Although the results of these studies are extremely encouraging, few volatile organic compounds (VOCs) are common to more than two or three studies and it is not useful to have hundreds of putative markers. Furthermore some volatiles, which have been proposed as biomarkers for liver disease, such as isoprene, acetone and ethanol, are not specific enough because they are possible biomarkers for other diseases or arise from numerous normal metabolic processes. If breath analysis is to progress to clinical utility, then markers must be definitively associated with the disease in question.

All previous studies can be regarded as hypothesis-generating, in that they do not follow up in a second group to confirm the putative biomarkers. We report here a two-stage breath biomarker discovery process: breath samples from a group of patients suffering from liver disease are first compared to breath samples from healthy controls; post-transplant breath samples are then compared with a sub-cohort of these patients who went on to have a liver transplant. A set of putative volatile markers is first determined by comparing patients with controls, and then pre- and post-transplant breath samples are examined to look for intra-individual differences in these volatiles. In this way, this study is hypothesis-led and uses patients as their own controls, thereby reducing the risk of false discovery. Furthermore, the use of patients' companions as controls and of room air samples minimizes the influence of any exogenous volatiles present in the home and hospital as confounding factors.

2. Methods

2.1. Patients, Controls and Hospital Room Air

Patients were recruited at the University Hospital Birmingham from either the transplant assessment clinic or in wards after being admitted with hepatic encephalopathy. 31 patients suffering from liver disease participated in the pre-transplant measurements (F/M 8/23, mean age 55 years, min–max 27–71 years). There were a number of etiologies and 11 patients had more than one condition: alcoholic liver disease (N = 13), hepatocellular cancer (N = 10), cryptogenic (N = 4), hepatitis C (N = 5), primary sclerosing cholangitis (N = 4), primary biliary cirrhosis (N = 2), autoimmune liver disease (N = 1), hepatitis B (N = 3), non-alcoholic steatohepatitis (N = 1), and non-alcoholic fatty liver disease (N = 1). Of these 31 patients, 12 went on to have a liver transplant (F/M 4/8, mean age 48 years, min–max 27–58 years). One additional patient (F, age 53 years) was recruited into the post-transplant study. Table 1 summarises the details of the 13 patients who had a liver transplant (12 from the pre-group), with patients being identified by sex (F or M) and a number. In addition to pre-transplant diagnostics, definitive diagnoses by histopathological examination of the explanted liver are provided. All but one (F2) were diagnosed with cirrhosis by standard liver function tests and biopsy. F2 was admitted suffering with hepatic encephalopathy, and histopathology of the explanted liver gave a diagnosis of severe hepatitis with multiacinar necrosis. Supplementary Table 1 shows demographic information for all patients including medications they were taking at the time of the pre-transplant sample.

For 28 pre-transplant measurements, breath samples from the patients' companions were taken. For the other three, two came alone to clinic and the other's companion declined to take part. Two additional controls were therefore recruited, one was a ward nurse and the other was a visitor to the hospital in ITU. These controls, while not related to the patient, had been in the same room for several hours prior to sampling so that confounding factors associated with volatiles present in the room environment were taken into consideration. In total 30 controls (F:M 23:7, mean age 44 years, min–max 20–75 years) took part in the study. The larger number of females in the control group arose due to the tendency of men coming to the clinic accompanied with their wives. While this means the control group is not ideally matched, there is no consistent evidence of dependences of volatile breath composition on sex (Kwak and Preti, 2011; Ellis and Mayhew, 2014). In confirmation of this we also found no correlation between sex and VOCs in either our control or patient groups. We consider that inhaled VOCs have a greater potential to confound biomarker discovery. As the majority of the companions were living with the patients, they provided an ideal control for exposure to exogenous volatiles in the home environment. VOCs inhaled at home, or in transit, may well still be present in breath for hours or days after inhalation and the biological half-life of inhaled VOCs is not well known (Beauchamp, 2011).

All study subjects were asked to complete a detailed questionnaire which included details on their home environment, diet, smoking status, health and medications. Participants were asked if they had consumed fruit and fruit juices and fruit flavoured drinks as a normal part of their diet, and, if so, to provide details on quantity and how long before the breath sampling these had been consumed.

Hospital room air was collected every time breath samples were taken so that any exogenous volatiles, such as isopropanol coming from hand gels resulting in product ions at m/z 43 and m/z 61, could be taken into consideration.

2.2. Breath Sampling Protocol

There is no agreed standard for the collection of breath for volatile analysis and uncontrolled breath sampling has been shown to be unreliable (Schubert et al., 2001; O'Hara et al., 2008). Therefore, capnography controlled sampling was used to collect only the alveolar phase of the breath. Subjects were in a relaxed state throughout the measurements and were either in a seated or lying position. They were asked to breathe normally into a gas tight respiratory system (Intersurgical Limited) containing an in-line CO_2 mainstream sensor connected to a fast-time response capnometer (Capnogard 1265 Novametrix Medical Systems Inc.). A 100 ml glass syringe (Sigma-Aldrich) was coupled to the tubing using a 3-way luer-lock stopcock (Braun Medical Limited). When the alveolar plateau on the capnograph was observed, a breath sample was manually drawn from the subject's breath stream into the syringe. Three to four breaths samples were collected for each 100 ml syringe, and four replicates of these were taken for each subject. Glass syringes were used, because our tests showed that they have no contaminating volatiles. Fig. 1 schematically shows the sampling system used.

After collection, the syringes were sealed using the luer lock fitting. They were transported from hospital to laboratory (a 10 minute outdoor walk) in an opaque storage box. Once at the laboratory, the syringes were placed inside an incubator set at 40 °C.

All samples were mass spectrometrically analysed within 2 h of collection. For the measurements, syringes were taken out of the incubator and immediately placed into a purpose designed heating bag (Infroheat, Wolverhampton) maintained at a constant temperature of 40 °C in order to limit condensation, which could otherwise lead to volatile