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Breath biomarkers and non-alcoholic fatty liver disease: Preliminary observations

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Abstract

Breath biomarkers have the potential to offer information that is similar to conventional clinical tests or they are entirely unique. Preliminary data support the use of breath biomarkers in the study of liver disease, in particular non-alcoholic fatty liver disease (NAFLD). It was evaluated whether breath ethanol, ethane, sulfur compounds and acetone would be associated with hepatic histopathology amongst morbidly obese patients presenting for bariatric surgery. Breath samples were collected during a preoperative visit and compared with liver biopsies obtained during the surgery. A Student's two-tailed *t*-test was used to compare differences between the two groups. Linear regression was used to analyse associations between the concentrations of breath molecules and independent predictor variables. It was found that breath ethanol, ethane and acetone can be useful biomarkers in patients with NAFLD. In particular, breath ethanol can be associated with hepatic steatosis, and breath acetone can be associated with non-alcoholic steatohepatitis.

Keywords: *Breath, biomarker, non-alcoholic fatty liver, liver disease*

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Introduction

Breath biomarkers have the potential to offer information that is similar to conventional clinical tests or unique. Until recently, research in breath biomarkers has been hindered by technical factors related to the reliable measurement of breath biomarkers found only in very low concentrations (picomoles per litre). However, advances in breath collection, concentration, storage and analysis increasingly have made breath analysis practical, reliable, reproducible and, quite possibly, clinically relevant. The collection of exhaled breath enjoys major advantages since it is non-

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invasive and safe for both study subjects and medical personnel. Further, results can be available immediately, and serial measurements are easy.

Preliminary data support the use of breath biomarkers in the study of liver disease, in particular non-alcoholic fatty liver disease (NAFLD). NAFLD is a common and potentially serious form of chronic liver disease that occurs in patients who do not abuse alcohol (Clark et al. 2002). NAFLD, which is prevalent in morbidly obese patients (Wanless & Lentz 1990, Youseff & McCullough 2002), can progress to significant hepatic inflammation (non-alcoholic steatohepatitis, NASH), and is a major cause of 'cryptogenic cirrhosis' (Poonawala et al. 2000). The pathogenesis of NAFLD is uncertain, but since its histopathology is markedly similar to alcoholic fatty liver disease, it may share common pathogenic mechanisms (Tilg et al. 2000). Indeed, by using a murine model of NASH, Cope et al. (2000) demonstrated that even in the absence of ethanol ingestion, ethanol could be detected in exhaled breath and that breath ethanol could be reduced by treatment with a poorly absorbed antibiotic. Further research by Nair et al. (2001) found greater breath ethanol concentrations to be associated with obesity in humans. Thus, endogenous ethanol, perhaps produced by gut flora, may contribute to the pathogenesis of NAFLD, even in the absence of exogenous alcohol intake.

In addition to ethanol, there are various other substances detectable in breath that could be useful in understanding the pathophysiologies of liver diseases. For example, since oxidative stress is involved in the pathogenesis of NAFLD (Diehl et al. 2005) and breath ethane is an investigational marker of oxidative stress (Risby & Sehnert 1999), it is possible that this biomarker could be useful diagnostically in patients with NAFLD. Preliminary data have also suggested that sulfur compounds and acetone may be of interest in liver disease. For example, Sehnert et al. (2002) found that breath carbonyl sulfide and carbon disulfide can distinguish between hepatocellular versus biliary patterns of injury, and can stage the severity of disease. However, since this study included patients with many kinds of liver disease, the authors were unable to establish whether a particular disease might have a unique breath pattern. Moreover, liver histopathology was not included. Finally, acetone is abundant in human breath and is formed by decarboxylation of acetoacetate as a result of lipolysis (Miekisch et al. 2004). Breath acetone is increased in uncontrolled diabetes (Lebovitz 1995) and diet-induced ketosis, where it has also been shown to approximate plasma beta-hydroxybutyrate closely (Musa-Veloso et al. 2002). Previously, the present authors found that acetone increased in ob/ob mice between 14 and 24 weeks of age, but not in lean controls (Cope 2002, p. 108). Since insulin resistance, including impaired insulin-mediated suppression of lipolysis, is common in patients with NAFLD (Sanyal et al. 2001), plasma-free fatty acids and beta-hydroxybutyrate may be abnormal even in the absence of overt ketosis (Sanyal et al. 2004). Accordingly, it was speculated that NAFLD patients may have abnormal concentrations of breath acetone.

The goal of the present study was to evaluate whether breath biomarkers correlate with liver histopathology in a group of obese patients at risk for NAFLD. The following were hypothesized:

- Breath ethanol will be increased in patients with more severe hepatic steatosis and hepatic inflammation.
- Breath ethane will be increased in patients with more severe hepatic inflammation.

- Breath sulfur compounds will be increased in patients with more severe hepatic fibrosis.
- Breath acetone will be increased in patients with more severe hepatic steatosis, inflammation and steatohepatitis.

Finally, the present study compares breath biomarkers with the standard serum liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Materials and methods

The Johns Hopkins University Institutional Review Board approved the study. Study subjects gave informed written consent.

Study subjects

The study population was comprised of 48 patients presenting for Roux-en-Y gastric bypass (RYGB) between January 2001 and November 2003. Patients presenting at the authors' bariatric centre underwent an extensive clinical preoperative assessment, including an evaluation by an internist, psychiatrist, specialized nutritionist and the operating surgeon. The work-up included documentation of the patient's medical history, physical examination, medications and routine laboratory work. Since ethanol was an analyte molecule in the proposed study, the use of any alcohol was an exclusion criterium in the selection of study subjects. Some of these study subjects had been previously diagnosed to be diabetics, but at the time of study their diabetes was under control. The same clinical investigator reviewed all patient charts and recorded patient data.

Breath collection and analysis

Breath samples were collected during a preoperative visit. Subjects were asked to relax and breathe through their mouth. A single mixed expired breath sample was collected in a Tedlar (3L, SKC, PA) gas sampling bag attached to a three-way non-rebreathing valve. A capnometer was placed between the valve and the gas sampling bag and the end tidal carbon dioxide was recorded during the collection of the sample. The sample of breath was returned to the laboratory for analysis. Aliquots of the collected breath were sampled onto duplicate adsorbent tubes packed with three sequential beds of Carbopack X, Carboxen-1018 and Carboxen-1019 (Supelco Corp., Bellefonte, PA, USA) at a constant flow of 80 ml min^{-1}. During this collection the concentration of carbon dioxide was measured and this steady-state concentration was used to correct for dilution. Adsorbent tubes were analysed by two-stage thermal desorption-gas chromatography (Perkin Elmer Corp., Norwalk, CT, USA) and the concentrations of ethane, acetone, isoprene and ethanol were determined. Additional samples of collected breath were analysed for carbon monoxide and for total volatile sulfur compounds. Carbon monoxide was determined electrochemically (LR250, Logan Research Ltd., Rochester, UK) and total volatile sulfur compounds in breath were quantified electrochemically (Halimeter RH-17 Series, Interscan Corp., Chatsworth, CA, USA) with a sensor that responds to any sulfur-containing volatile species. The composition of collected breath has been found to remain constant in the Tedlar gas

sampling bags for at least 1 week and for more than 6 months when adsorbed onto the adsorbent tubes. However, all samples were analysed within 24 h of collection.

Liver biopsies

All patients underwent a routine intraoperative wedge liver biopsy at the start of RYGB, as is the authors' standard practice because they are at high risk for liver disease. Biopsy specimens were fixed and stained with hematoxylin and eosin and Masson's trichrome in a standard manner. Biopsies were read by a single expert hepatopathologist (M. T.) blinded to clinical information. The biopsies were graded and staged according to the Brunt method (Brunt et al. 1999). For the purposes of hepatopathologic analyses, the pathologist evaluated the presence of the following:

1. Steatosis, graded from 0 (none) to 3 (severe).
2. Inflammation, graded from 0 (none) to 3 (severe).
3. Fibrosis, staged from 0 (none) to 4 (cirrhosis).

In addition, an overall assessment was made and cases were further classified as with or without non-alcoholic steatohepatitis (NASH).

Statistical analyses

Since mild steatosis is common in patients with morbid obesity and has uncertain pathological significance (Matteoni et al. 1999), patients with grade 0 or 1 were compared with patients with grades 2 or 3. However, since any inflammation or fibrosis is considered pathological, the presence was compared with the absence of these findings. Clinical and laboratory associations with each of the histological components of NAFLD were assessed using linear regression analysis. Age, BMI, AST and ALT were evaluated as continuous variables. A Shapiro–Francia w' test was used to test for normality of distribution. Breath ethanol and acetone concentrations were skewed. Therefore, log transformation was used to make distributions more normal. In addition, patients with ethanol concentrations below the median of the total were analysed separately from those above the median (Figure 1). For data where the distributions were normal, a Student's two-tailed t-test was used to compare differences between two groups. Linear regression was used to analyse associations between breath molecule concentration and independent predictor variables. For all statistical tests, $p \leq 0.05$ or below was considered significant. All statistical analyses were performed using the STATA 8.2 computerized statistical package (Stata Corporation, College Station, TX, USA).

Results

A summary of the demographics is shown in Table I. The mean age of the 48 subjects was 44 years (range 27–64 years); 46 were white and 41 were female. The mean body mass index (BMI) was 52 kg m^{-2}. Forty-four subjects had at least some degree of steatosis, with 25 subjects having grade 2 or 3 (moderate to severe) steatosis. In addition, 33 subjects had some degree of inflammation and 27 subjects had quantifiable fibrosis. No patients had cirrhosis. Twenty-four patients had NASH.

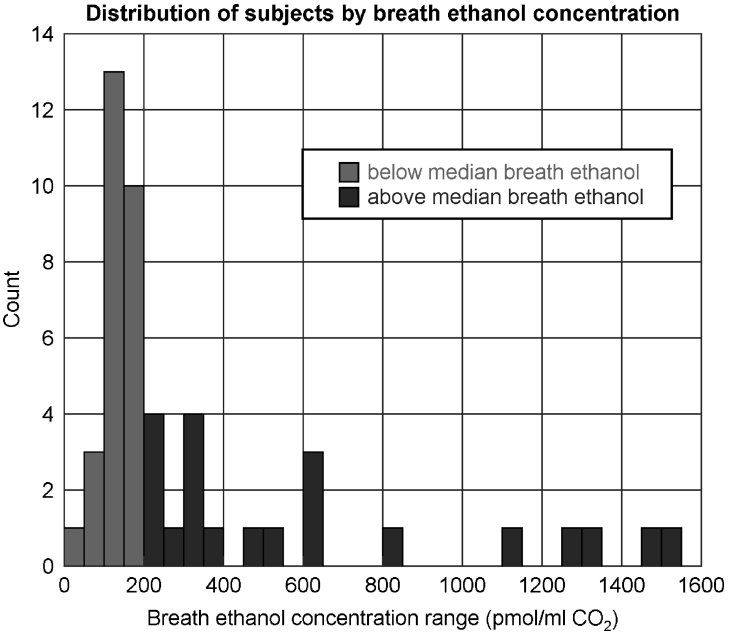

Figure 1. Breath ethanol was non-normally distributed, with a majority of subjects ($n=27$) having low breath ethanol, and the others ($n=21$) having significantly more.

Table I. Characteristics of 48 subjects presenting for Roux-en-Y gastric bypass.

Age (years)	44 (range 27–64)
Female	41 (85%)
Caucasian	44 (91%)
BMI (kg m^{-2})	52 (range 37–90)
ALT (IU l^{-1})*	30 (SD = 20)
AST (IU l^{-1})†	21 (SD = 11)
Steatosis	*Grade:*
	0: 4 (8%)
	1: 19 (40%)
	2: 11 (23%)
	3: 14 (29%)
Inflammation	*Grade:*
	0: 15 (31%)
	1: 24 (50%)
	2: 6 (13%)
	3: 3 (6%)
Fibrosis	*Stage:*
	0: 21 (44%)
	1: 22 (46%)
	2: 4 (8%)
	3: 1 (2%)
	4: 0 (0%)
NASH[#]	24 (50%)

Values are means, except as noted. ALT and AST values were available on 45 subjects; the BMI was available on 47.

*Alanine aminotransferase; †aspartate aminotransferase; [#]non-alcoholic steatohepatitis.

There were no significant associations between breath ethanol, ethane, or sulfur compounds and age, gender or BMI.

Breath ethanol

Breath ethanol was skewed to the right, with a majority of subjects ($n=27$) having low breath ethanol, and the others ($n=21$) having significantly more (Figure 1). When all 48 subjects were evaluated together, no significant association was found between breath ethanol and any histological outcome, including steatosis, inflammation, fibrosis and NASH or high AST or ALT.

However, breath ethanol can be affected by many factors, including the time since food intake, the composition of recent food intake, ambient room ethanol and ingestion of ethanol (Nair et al. 2001, Cope et al. 2004). Therefore, it is quite possible that breath ethanol was elevated in the 21 subjects with higher breath ethanol due to one of these reasons except the ingestion of alcohol. Therefore, we re-analysed the data and excluded subjects who had a breath ethanol concentration above the median (200 pmol ml^{-1} CO_2), and included only those below this cut-off ($n=27$). The distribution within this group was normally distributed with a mean breath ethanol concentration of 135 (SD = 43) pmol ml^{-1} CO_2. Within this group, it was found that subjects with moderate to severe steatosis (grades 2 or 3, $n=16$) had significantly higher breath ethanol compared with subjects with less steatosis (grades 0 or 1, $n=11$): 149 (SD = 39) pmol ml^{-1} CO_2 versus 114 (SD = 42) pmol ml^{-1} CO_2, $p=0.03$, (Figure 2). Furthermore, within the group of 27, subjects with more hepatic inflammation (grades 2/3, $n=6$ versus grades 0/1, $n=21$) had a trend towards more breath ethanol (161 (SD = 32) pmol ml^{-1} CO_2 versus 127 (SD = 44), $p=0.09$). However, subjects with steatohepatitis ($n=15$) did not have more breath ethanol (146 (SD = 38) pmol ml^{-1} CO_2 versus 121 (SD = 47) pmol ml^{-1} CO_2, $p=0.15$) and there was no association between the presence of fibrosis and breath ethanol.

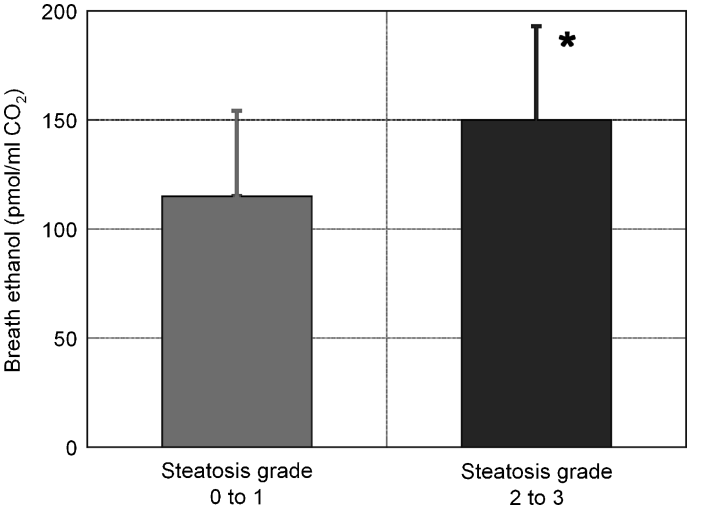

Figure 2. Within the group of low ethanol producers ($n=27$), subjects with moderate to severe steatosis (grades 2 or 3, $n=16$) had significantly higher breath ethanol compared with subjects with less steatosis (grades 0 or 1, $n=11$): 149 (SD = 39) pmol ml^{-1} CO_2 versus 114 (SD = 42) pmol ml^{-1} CO_2, $p=0.03$.

Breath ethane

Breath ethane was normally distributed in the 48 subjects. The mean breath ethane was 30 (SD = 16) pmol ml^{-1} CO_2. Subjects with hepatic inflammation had a breath ethane of 32 pmol ml^{-1} CO_2 versus 25 pmol ml^{-1} CO_2 ($p = 0.17$). Similarly, subjects with NASH did not have higher breath ethane than those without NASH (34 versus 27, $p = 0.14$). There was no association between breath ethane and hepatic steatosis, fibrosis, or high AST or ALT.

Breath sulfur compounds

There was no association between breath sulfur compounds and any histological outcome or high AST or ALT.

Breath acetone

Table II summarizes the associations between breath acetone concentration and the histological findings. There was a tendency for acetone concentration to be significantly increased in patients with steatosis grade 2 or 3 compared with steatosis grade 0 or 1. In addition, breath acetone concentration was significantly increased in the breath of subjects with histologically confirmed steatohepatitis versus those without steatohepatitis. Moreover, the acetone concentration was significantly increased in patients with fibrosis stage 1 and 2 compared with patients without fibrosis. Furthermore, six patients with grade 3 inflammation had a significantly increased concentration of acetone compared with patients with no inflammation. Finally, there was a significant association of breath acetone concentration with serum AST ($\beta = 1.02$ pmol ml^{-1} CO_2, 95% CI 1.01–1.03, $p < 0.001$), and with serum ALT ($\beta = 1.01$ pmol ml^{-1} CO_2, 95% CI 1.0–1.02, $p < 0.004$).

Table II. Association of acetone concentration with histological findings.

Histology	Histological grade or stage	Frequency	Mean acetone concentration (pmol ml^{-1} CO_2)*	95% Confidence intervals†	p value for difference‡
Steatosis	0, 1	22	675	552–826	
	2, 3	25	919	707–1195	0.065
Inflammation	0	15	659	498–873	
	1	24	809	649–1007	
	2	6	1310	720–2684	0.013
	3	3	708	127–3627	
NASH	no	24	641	530–775	
	yes	24	996	774–1282	0.006
Fibrosis	0	21	620	523–735	
	1	22	943	698–1275	0.014
	2	4	1149	782–1687	0.041
	3	1	994		

*Geometric mean; †anti-log of 95% CI; ‡stages (1–3) compared with 0, unless otherwise indicated.

Discussion

Consistent with our first hypothesis, it was found that among morbidly obese patients presenting for RYGB, subjects with more severe hepatic steatosis had significantly elevated breath ethanol. Notably, these findings existed only among a sub-group of low breath ethanol subjects. This finding corroborates earlier work by Cope et al. (2000) that endogenous ethanol, as measured in exhaled breath, may be associated with hepatic pathology. Since the present study evaluated subjects at only one point in time, causality cannot be inferred, and the pathogenic mechanisms remain uncertain. Nevertheless, this research is consistent with an emerging theme in implicating gut flora in the pathogenesis of NAFLD (Schwimmer & Lavine 2003, Solga & Diehl 2003). Such research has led some investigators to postulate that deliberate alterations in gut flora may have therapeutic implications (Nardone & Rocco 2004).

In addition, breath ethane had some association with more severe hepatic inflammation and the presence of NASH, though statistical significance was not reached. A greater sample size may be necessary to evaluate more fully our hypothesis that breath ethane would associated with inflammation. Reliable, non-invasive measurements of hepatic inflammation do not yet exist, and standard serum markers for hepatic necro-inflammation and injury, including AST and ALT, correlate poorly with biopsy results in many liver diseases (Green & Flamm 2002). Accordingly, there is a critical need for a more robust biomarker of hepatic inflammation. It remains uncertain whether breath ethane will be useful in this capacity.

No association in this study was found between sulfur compounds and any histological outcome or serum transaminases. However, we did not measure individual sulfur compounds, e.g. carbonyl sulfide, methanethiol, dimethyl sulfide and carbon disulfide, separately. Since the liver metabolizes some sulfur compounds, we may have missed true differences in hepatic function by measuring only total volatile sulfur compounds.

Finally, breath acetone was found to be associated with NASH, hepatic fibrosis, ALT and AST. This finding is consistent with breath data from murine models of NASH (Cope 2002), and may be due to increased fatty acid oxidation through increased expression of P450 enzymes, e.g. P4502E1 (Chalasani et al. 2003, Lieber 2004). The increase in acetone concentration may also result from a decrease in d-3-hydroxybutyrate dehydrogenase activity, and/or from reduced levels of NADH (H+) in hepatocyte mitochondria. Either case would result in a back-up of acetoacetate and hence increased acetone concentrations. It is possible that a combination of acetone and beta-hydroxybutyrate levels could serve as early markers of insulin sensitivity and help distinguish simple steatosis from NASH. Note, however, that acetone physiology may be affected by many factors, including blood glucose and insulin levels, diabetes control, medication use, time since last meal, recent exercise, etc. (Laffel 1999). Since the present study lacked this potentially relevant information, the findings must be viewed as very preliminary and will need to be further evaluated.

A major strength of the present study is the review of all hepatic histopathology by a single expert hepatopathologist blinded to clinical data. Further, the study was able to compare breath biomarkers with a range of NAFLD hepatic histopathology, not just cirrhosis.

The study has several limitations, including a small sample size, a lack of detailed information on time since the last food intake and meal composition, and a lack of detailed information on co-morbid conditions, including pulmonary function. No

control study population was studied since it is unethical to obtain liver biopsies for research purposes. However, only four of the 48 subjects had normal liver pathology based upon histopathology. Further, it is possible that we missed cases of viral hepatitis or other causes of liver disease among the study subjects. In addition, blood analyses were performed at several different laboratories, making comparison of ALT and AST across the group difficult.

Finally, since the subjects were morbidly obese patients presenting for RYGB, these results may not be applicable to other patient populations. Note, however, that the subjects were part of a larger consecutive series of 189 patients presenting for RYGB that have been previously described (Solga et al. 2005). The baseline characteristics and degree of hepatic pathology in these 48 subjects are similar to this larger series, and are also consistent with other published series (Ong et al. 2005). Accordingly, we believe the group of patients in this study is representative of morbidly obese patients presenting for bariatric surgery.

Conclusion

Breath ethanol, ethane and acetone may be useful biomarkers for investigating patients with NAFLD. In particular, breath ethanol may be a risk factor for the development of hepatic steatosis, and breath acetone may be associated with NASH and fibrosis. Larger, prospective longitudinal studies are needed.

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