Research Article

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Non-alcoholic steatohepatitis: A non-invasive diagnosis by analysis of exhaled breath

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Background & Aims: Histological evaluation of a liver biopsy is the current gold standard to diagnose non-alcoholic steatohepatitis (NASH), but the procedure to obtain biopsies is associated with morbidity and high costs. Hence, only subjects at high risk are biopsied, leading to underestimation of NASH prevalence, and undertreatment. Since analysis of volatile organic compounds in breath has been shown to accurately identify subjects with other chronic inflammatory diseases, we investigated its potential as a non-invasive tool to diagnose NASH.

Methods: Wedge-shaped liver biopsies from 65 subjects (BMI 24.8–64.3 kg/m^2) were obtained during surgery and histologically evaluated. The profile of volatile organic compounds in pre-operative breath samples was analyzed by gas chromatography–mass spectrometry and related to liver histology scores and plasma parameters of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Results: Three exhaled compounds were sufficient to distinguish subjects with (n = 39) and without NASH (n = 26), with an area under the ROC curve of 0.77. The negative and positive predictive values were 82% and 81%. In contrast, elevated ALT levels or increased AST/ALT ratios both showed negative predictive values of 43%, and positive predictive values of 88% and 70%, respectively. The breath test reduced the hypothetical percentage of undiagnosed NASH patients from 67–79% to 10%, and of misdiagnosed subjects from 49–51% to 18%.

Conclusions: Analysis of volatile organic compounds in exhaled air is a promising method to indicate NASH presence and absence. In comparison to plasma transaminase levels, the breath test significantly reduced the percentage of missed NASH patients and the number of unnecessarily biopsied subjects.
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Keywords: NAFLD; NASH; Obesity; Liver biopsy; Exhaled air; Volatile organic compounds.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; VOC, volatile organic compounds; HbA_{1c}, glycated hemoglobin; LR, likelihood ratio; PV, predictive value; GC–MS, gas chromatography–mass spectrometry.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide, affecting one in three adults, and one in ten adolescents in the USA [1,2]. NAFLD is present in the majority of patients with metabolic risk factors such as obesity and type 2 diabetes mellitus (T2DM) [1]. While steatosis, the early stage of NAFLD, is considered to be benign and reversible, progression towards more advanced stages often occurs. These advanced stages, referred to as non-alcoholic steatohepatitis (NASH), are characterized by inflammation [3,4]. Importantly, NASH is in turn associated with the development of hepatic fibrosis, cirrhosis, hepatocellular carcinoma, and an increased risk of liver failure and liver-related mortality [3,4]. It is therefore clinically relevant to differentiate between patients with sole hepatic steatosis and those suffering from NASH, at an early stage.

Currently, a liver biopsy remains necessary to accurately diagnose NASH and to assess its severity [5,6]. However, the procedure to obtain a liver biopsy is invasive and associated with considerable discomfort, costs, and morbidity; significant complications are encountered in 0.5% of cases [7,8]. In order to optimize the risk-benefit ratio, it is advocated to obtain a needle biopsy from all obese patients with clinical risk factors, and a per-operative biopsy from all morbidly obese patients undergoing abdominal surgery [9]. Apart from obesity, acknowledged risk factors are elevated plasma levels of alanine aminotransferase (ALT), an elevated ratio of aspartate aminotransferase (AST) to ALT (AST/ALT ratio), insulin resistance, hypertension, sleep apnea, and increased plasma levels of

triglycerides [10]. However, performing liver biopsy procedures based upon these risk factors leads to a selection bias in clinical practice, especially since mainly the presence of obesity and plasma levels of aminotransferases are taken into account. On the one hand, these plasma levels are often maintained within the normal range despite advanced disease [11], resulting in an underestimation of NASH prevalence, as well as undertreatment. On the other hand, if the indication to obtain a liver biopsy is based upon elevated aminotransferase levels, a large proportion of biopsies are obtained from subjects who do not suffer from NASH, since elevated AST and/or ALT levels are not specific for the presence of this liver disease.

In view of (1) the clinical relevance of NASH, (2) the difficulties of selecting the appropriate population to biopsy, and (3) the biopsy-related burden, a less invasive method to identify patients with NASH is urgently required. Such a method could be the analysis of volatile organic compounds (VOC) in exhaled breath. VOC are considered as markers of oxidative stress and can indicate the presence of reactive oxygen species that derive, for example, from peroxidation of polyunsaturated fatty acids [12]. Components in exhaled air have been previously shown to reflect the presence of inflammatory diseases affecting the airways [13,14] and liver [15–17]. Hence, analysis of VOC in exhaled air may be useful for predicting NASH presence. In this pilot study, we found that subjects with NASH can be accurately distinguished from those without NASH based upon analysis of VOC in exhaled breath.

Patients and methods

Study design

Sixty-five subjects were included consecutively between October 2007 and May 2011, before they underwent laparoscopic abdominal surgery; either cholecystectomy or primary bariatric surgery. Subjects ranged from overweight to severely obese with a BMI range of 24.8–64.3 kg/m^2. The laparoscopic abdominal surgery was performed either at the Maastricht University Medical Centre or the Atrium Medical Centre Parkstad by the same surgeon (JWG). Exclusion criteria were acute, recent, and chronic inflammatory diseases (e.g., M. Crohn, colitis), other known liver diseases (such as viral hepatitis), consumption of >10 g alcohol daily, and use of medication associated with NAFLD (e.g., steroids, amiodarone, valproate, methotrexate). The previously mentioned risk factors for NASH such as BMI, hypertension, and sleep apnea were evaluated. This study was approved by the Medical Ethical Committees of both the Maastricht University Medical Centre and the Atrium Medical Centre Parkstad, and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written informed consent was obtained from all subjects.

Sample collection and analysis

Breath samples

Breath samples from all 65 patients were collected at the end of the afternoon prior to the day of the surgery. The complete analytical procedure and instrumental analysis have been published previously [13,18]. Briefly, all subjects exhaled into a resistance-free 5 L plastic bag (Tedlar bag, SKC Ltd, Dorset, UK). The VOC were trapped within 24 h after sampling, by deflating the bag into a sorption tube filled with carbograph 1TD/Carbopack X (Markes International Inc, Cincinnati, OH). For analysis, the VOC were released by thermal desorption and injected into a gas chromatograph (Trace GC, Thermo Fischer Scientific, Austin, TX) connected to a time-of-flight mass spectrometry (Tempus Plus, Thermo Fischer Scientific) [18].

Blood samples

Preoperative fasting venous blood samples could be obtained on the morning of the surgery from 61 subjects and were processed as previously described [19]. Plasma levels of C-reactive protein (CRP), ALT, AST, glucose, insulin, total cholesterol, HDL, LDL, triglycerides, free fatty acids and HbA_{1c} were measured according to the protocol of the Department of Clinical Chemistry of the Maastricht University Medical Centre. The upper limit of normal ALT levels was 35 IU/L for women and 45 IU/L for men [20], while an AST/ALT ratio >1 was considered to be elevated [21].

Liver biopsies

Wedge-shaped liver biopsies of at least 15 by 10 mm were obtained intra-operatively from all patients, by the same surgeon (JWG), and processed as previously described [22]. All biopsies contained at least five portal tracts to allow for correct evaluation of the hepatic architecture. No overt pathologic condition other than NAFLD was observed. Steatosis, hepatocellular ballooning, lobular and portal inflammation, Mallory's hyaline, and fibrosis were scored according to both the Brunt scoring system [5] and the NAS activity score according to Kleiner *et al.* [6], by an experienced liver pathologist (AD) blinded to the clinical context and laboratory parameters. Liver biopsies that were evaluated as healthy or steatotic did not show any sign of portal or lobular inflammation, hepatocyte ballooning, or fibrosis (n = 26). In contrast, livers showing signs of steatosis and inflammation were defined as NASH (n = 39) and were further evaluated according to the Brunt classification and NAS activity score according to Kleiner *et al.* [5,6].

Data processing and analysis

Processing of data

Detailed descriptions of the data handling procedures have been previously reported [18]. Briefly, gas chromatography and mass spectrometry (GC–MS) chromatograms of all breath samples were recorded. Retention times were normalized by calculating retention indices, relative to toluene and using easily recognizable component peaks, to correct for chromatographic drifting. The beginning and end of each run (retention index either <0.15 or >2.8) were removed because of noisy mass spectra at the beginning of the chromatograms and column bleeding at the end of each run. The remaining data, containing almost 4800 different chromatographic peaks as determined by retention time and mass spectrum combined with a relative intensity, were transformed into Excel files. The measured mass spectra were compared to one another at the same retention time. The resemblance of the original spectra determines whether or not peaks at the same retention time represent the same component. Intensities under the detection limit were set at 0%.

Statistical analysis of the GC–MS data

The data matrix was analyzed by a stepwise discriminant analysis by a leave-one-out cross-over approach, using Statistical Package for Social Sciences 19.0.0 (IBM SPSS Software Inc., Chicago, IL). All but one of the chromatograms were included to construct the discriminant function. The one that was left out was subsequently used to predict the group it belonged to. This was repeated until every chromatogram had been left out once; all samples have been classified. Based upon 33 components, the discriminant functions that are optimal in terms of differentiation between both groups are not necessarily the best predictors for unknown samples, because of obvious overfitting. Therefore, the number of variables was gradually diminished until a reasonable small number of components with sufficient predictive power remained. This reduction in components was reached by repeating the analysis from the original large dataset by leaving the least informative components out, one by one.

Statistical analysis of clinical data

Statistical analysis was performed using SPSS and Prism 5.0 (GraphPad Software Inc., San Diego, CA) for Windows. Differences between groups were analyzed by the Mann Whitney *U* test or the Kruskal Wallis-test followed by Dunn's post-testing. A p <0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean.

Results

Population characteristics

The body mass index (BMI) ranged from 24.8 to 64.3 kg/m^2 (mean 43.7 kg/m^2), population characteristics are summarized in Table 1. NASH was diagnosed in 39 subjects (60%). The average plasma ALT and AST levels were higher in subjects with NASH

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