ORIGINAL ARTICLE

NMR spectroscopy metabolomic profilling of exhaled breath condensate in patients with stable and unstable cystic fibrosis

Paolo Montuschi1, Debora Paris2, Dominique Melck2, Vincenzina Lucidi3, Giovanni Ciabattoni4, Valeria Raia5, Cecilia Calabrese6, Andrew Bush7, Peter J Barnes8, Andrea Motta2

ABSTRACT

Background Metabolomics could provide new insights into the pathophysiology of cystic fibrosis (CF) by identifying profiles of endogenous metabolites.

Objectives To investigate whether metabolomics of exhaled breath condensate could discriminate between patients with unstable CF, stable CF and healthy subjects, and whether selected metabolites were responsible for between-group differences.

Methods Twenty-nine patients with stable CF, 24 with unstable CF and 31 healthy subjects (age 9–24 years) participated in a cross-sectional study. Metabolomics was performed with high-resolution nuclear magnetic resonance spectroscopy. Partial least squares-discriminant analysis was used as classifier. The results were validated in a second independent study.

Results Intraclass correlation coefficients for between-day and technical repeatability were 0.93 and 0.96, respectively. Bland–Altman analysis showed good within-day repeatability. Correct classification rate of CF (n=53) vs healthy subjects (n=31) was 96% (R2=0.84; Q2=0.79). Model validation with a testing sample set obtained from subjects not included in the primary analysis (23 CF and 25 healthy subjects) showed a sensitivity of 91% and a specificity of 96%. The classification rate of stable CF (n=29) vs unstable CF patients (n=24) was 95% (R2=0.82; Q2=0.78). Model external validation in 14 patients with stable CF and 16 with unstable CF showed a sensitivity of 86% and a specificity of 94%. Ethanol, acetate, 2-propanol and acetone were most discriminant between patients with CF and healthy subjects, whereas acetate, ethanol, 2-propanol and methanol were the most important metabolites for discriminating between patients with stable and unstable CF.

Conclusions Nuclear magnetic resonance spectroscopy of exhaled breath condensate is reproducible, discriminates patients with CF from healthy subjects and patients with unstable CF from those with stable CF, and identifies the metabolites responsible for between-group differences.

Key messages

What is the key question?

▶ Does nuclear magnetic resonance (NMR) spectroscopy of exhaled breath condensate (EBC) discriminate between patients with unstable cystic fibrosis (CF), stable CF and healthy controls, and are selected metabolites responsible for between-group differences?

What is the bottom line?

▶ NMR spectroscopy of EBC is a reproducible technique which discriminates patients with unstable CF, stable CF and healthy subjects and also unravels the metabolic changes that characterise CF.

Why read on?

▶ To learn how NMR-based metabolomics of EBC provides a unique opportunity for investigating the pathophysiology of CF and identifies a panel of unbiased potential biomarkers of CF. These markers may be useful endpoints in future studies.

INTRODUCTION

Airway inflammation plays a central role in the pathophysiology of cystic fibrosis (CF).1 It is not clear whether the exuberant airway inflammation in CF is due entirely to chronic bacterial infection, whether the CF airway epithelium is inherently proinflammatory in the absence of infection2 and whether there is an excessive response to bacterial infection3 or a defect in the resolution of inflammation.4 Breath volatile compounds including hydrogen cyanide have been measured by mass spectrometry techniques in patients with CF and compared with healthy subjects.5 6 Exhaled breath condensate (EBC) is a non-invasive technique for studying the composition of airway lining fluid.7 Relatively few data on EBC metabolite profiles are available, and information on EBC metabolite composition is often obtained by analysing single inflammatory molecules. Metabolomics involves the detection of metabolites in a biological fluid usually using high-resolution nuclear magnetic resonance (NMR) spectroscopy.8 This approach enables a specific quantitative description of the low molecular mass endogenous metabolites in biological sample, provides a metabolic ‘fingerprint’ which can be used for classification purposes and focuses on the most important regions of the NMR spectrum for
further analysis. The identification of specific molecules and their relative variations might provide further insights into the pathophysiology of CF.

The presence of discriminating metabolites in a NMR spectrum can be tested with multivariate statistical algorithms which allow a thorough comparison of datasets.

Metabolomics of EBC with NMR spectroscopy discriminates between children with mild-to-moderate persistent asthma and healthy children and between patients with chronic obstructive pulmonary disease and healthy subjects. Using NMR spectroscopy, chlorinated and brominated tyrosine residues were detected in the sputum of patients with CF but not in healthy subjects. Metabolomic profiles of bronchoalveolar lavage fluid from children with CF are correlated to the degree of airway inflammation.

The primary objectives of the present study were to verify whether (1) EBC metabolomic analysis with NMR spectroscopy discriminates between patients with unstable CF, stable CF and healthy controls and (2) selective metabolites are responsible for between-group differences.

METHODS

Subjects

Twenty-nine patients with stable CF, 24 patients with unstable CF and 31 age-matched healthy controls were studied. Healthy subjects had no history of respiratory or other diseases. They had no upper airway infections nor had received any medication in the previous 4 weeks. The diagnosis of CF was based on clinical, radiological and genotypic characteristics and an abnormal sweat test (sweat chloride ≥60 mmol/l). Patients with stable CF had no change in cough or shortness of breath, no requirement for oral or intravenous antibiotics and no significant spirometry change in the previous 4 weeks. Patients with unstable CF were assessed before treatment for pulmonary exacerbation, defined by the presence of at least two of the following signs: oral temperature >38°C, more frequent coughing, increased sputum volume, appetite or weight loss, absence from school or work due to illness (at least 3 of preceding 7 days) and symptoms of upper respiratory tract infection, associated with at least one of the following: decrease in forced vital capacity (FVC) of at least 10%; increase in respiratory rate of at least 10 breaths/min; peripheral blood neutrophil count of ≥15 000/mm³. All subjects were non-smokers (see also online supplement).

Study design

The study was cross-sectional. Subjects attended the CF Unit outpatient clinic of Ospedale Pediatrico Bambino Gesù, Rome, Italy on one occasion for clinical examination, EBC collection, spirometry, chest x-ray and blood sampling. Between-day repeatability was assessed in five healthy and 11 CF subjects, among those who participated in the principal study by collecting three EBC samples within 7 days of the first. From the same subjects, separate EBC samples were collected twice within the same day for assessing within-day repeatability. External validation of the classification model was performed by testing EBC samples obtained from subjects not included in the primary analysis and collected in a different centre under similar experimental conditions (CF Unit, University of Naples Federico II, Italy) (see table E1 in online supplement). Informed consent was obtained from parents and adult subjects, and age-appropriate assent from children. The study was approved by the local ethics committees.

EBC sampling

Before EBC collection, subjects refrained from eating for at least 3 h. EBC was collected using a condenser (Ecoscreen, Jaeger, Hoechberg, Germany), which has a saliva trap to reduce the chance of salivary contamination, in a windowless clinic facility without disinfectant dispensers; CF patients had not used wipes prior to the study. Saliva was also obtained. EBC was immediately transferred into 10 ml glass vials, closed with 20 mm butyl rubber lined with polytetrafluoroethylene septa and crimped with perforated aluminium seals. Before sealing, volatile substances were removed from samples by a gentle nitrogen gas flow for 5 min (see online supplement). Samples were then frozen in liquid nitrogen to immediately ‘quench’ metabolism and preserve the metabolite concentrations.

NMR sample preparation

EBC and saliva samples were rapidly defrosted. 70 μl of reference standard D₂O solution (containing 0.1 mM sodium 3-trimethylsilyl [2,2,3,3-²H₄] propionate (TSP) and sodium azide at 3 mM) were added to 650 μl of sample.

NMR spectroscopy measurements

Spectra were recorded on a 600 MHz Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a CryoProbe. One-dimensional (1D) 1H-NMR and two-dimensional (2D) clean total correlation spectroscopy (TOCSY) spectra were recorded using a standard pulse sequence. The estimated detection limit was 0.14 μM. Full details are given in the online supplement.

Spectral and statistical analysis

There are no data in the literature for a sample size calculation in a NMR-based metabolomic study. The spectral 0.12–8.60 ppm region of the high-resolution 1H-NMR spectra was automatically data reduced to integrated regions (buckets) of 0.02 ppm each using the AMIX 3.6 package (Bruker BioSpin GmbH, Rheinstetten, Germany). To account for possible metabolite concentration variations due to dilution, each bucket was normalised to the corresponding total spectral area or spectra were referenced to a known TSP concentration (0.1 mM) added to the samples. No difference was observed between these approaches.

To examine EBC metabolite clustering in an unsupervised manner, we applied the spectral filtering orthogonal signal correction routine with partial least squares-discriminant analysis (PLS-DA).

Within-day repeatability of NMR spectroscopy was assessed according to the method of Bland and Altman. Between-day and technical repeatability were assessed with ANOVA for repeated measures and expressed as intraclass correlation coefficient (ICC).

Data were expressed as mean±SEM after assessing for normality with the D’Agostino-Pearson omnibus normality test. One-way ANOVA and unpaired t tests were used for comparing groups. Significance was defined as a p value of <0.05.

RESULTS

Patient characteristics

The characteristics of the study subjects are shown in table 1. Forced expiratory volume in 1 s (FEV₁) and FVC were reduced in patients with CF compared with healthy subjects (table 1). Patients with unstable CF had lower FEV₁ and higher systemic...
Cystic fibrosis

Table 1  Subject characteristics*

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>Stable CF patients</th>
<th>Unstable CF patients</th>
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<tbody>
<tr>
<td>N</td>
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<td>24</td>
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<tr>
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<td>11/13</td>
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<td>FEV₁, % predicted</td>
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<td>p</td>
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<tr>
<td>FVC, % predicted</td>
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<td>p</td>
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*Data are expressed as mean±SEM. One-way ANOVA and unpaired t tests were used for comparing groups. Significance was defined as p<0.05.
†Compared with healthy subjects.
‡Compared with patients with stable CF.
§Genotype was not determined in one patient with stable CF and in two patients with unstable CF.

Inflammation as reflected by serum C reactive protein and white blood cell counts than those with stable CF (table 1).

NMR spectroscopy of EBC discriminates between patients with CF and healthy controls

The EBC NMR profiling of patients with stable and unstable CF was compared with that in healthy controls. Figure 1 shows representative EBC 1D spectra of a healthy subject (figure 1A), a patient with stable CF (figure 1B) and a patient with unstable CF (figure 1C). Spectra resonances were assigned to single metabolites by resorting to 2D 1H-1H TOCSY and 1H-13C heteronuclear single quantum coherence experiments (not shown) and compared with the Human Metabolome Database (http://www.hmdb.ca/) and published chemical shift data.

PLS-DA showed a strong regression model (96%) between healthy subjects and patients with CF (figure 2A) in which data variation is well explained by the model (R²=0.82, Q²=0.78; figure 5A).

To validate this model, a sample set obtained from subjects not included in the primary analysis (14 patients with stable CF and 16 with unstable CF; table E1 in online supplement) was tested blindly. The model correctly identified 12 out of 14 patients with stable CF (86% accuracy) and 15 out of 16 patients with unstable CF (94% accuracy) with a sensitivity of 91%, a specificity of 96%, a positive likelihood ratio (LR+) of 23 and a negative likelihood ratio (LR–) of 0.08.

NMR profiling of EBC discriminates between patients with stable and unstable CF

PLS-DA yielded a model of patients with stable (n=29) versus unstable (n=24) CF which resulted in three predictive and three orthogonal components (R²=0.82, Q²=0.78; figure 5A).

Four metabolites differentiate classes

The variables responsible for between-class differences observed in the score plot can be identified from the loading plot (see
Figure E1 in online supplement) which reports the spectral ‘buckets’ containing metabolite resonances. The variables of importance plot (VIP) describes the most important metabolites in making the model accurate. The first 14 buckets (corresponding to 11 metabolites) separate patients with CF from healthy control subjects.

Figure 2 Patients with cystic fibrosis (CF) versus control subjects. Partial least squares-discriminant analysis (PLS-DA) with the spectral filtering orthogonal signal correction (OSC) of exhaled breath condensate metabolites in patients with CF compared with healthy control subjects. (A) Score plot showing the degree of separation of the model between healthy subjects (closed circles) and patients with CF (open circles). t[1] and t[2] represent the scores (the ‘first two partial least squares components’) of the model, which are sufficient to build a satisfactory classification model. The model showed a strong regression (96%). (B) Variables of importance plot (VIP) which represents the 14 most important ‘buckets’ (11 metabolites) generating this model \((R^2 = 0.84\) and \(Q^2 = 0.79\)). Metabolite assignments are indicated together with the corresponding chemical shift. Ethanol and 2-propanol are reported more than once as their different chemical groups resonate at different frequencies. The x-axis reports the buckets, identified with chemical shift (in ppm) and metabolite name, and is labelled ‘VAR-ID’ (variable identity); the y-axis, labelled ‘VIP[2]’, shows the strength of the labelled metabolites in the classification between patients with CF versus healthy subjects. Error bars represent 95% CIs.

Figure 3 Stable versus unstable cystic fibrosis (CF). (A) Partial least squares-discriminant analysis (PLS-DA) with the spectral filtering orthogonal signal correction (OSC) of exhaled breath condensate metabolites in subjects with stable CF (blue dots) compared with subjects with unstable CF (red dots). t[1], t[2] and t[3] along the axes represent the scores (the ‘first three partial least squares components’) of the model, which are sufficient to build a satisfactory classification model. (B) Variables of importance plot (VIP) which represents the nine most important metabolites generating this model \((R^2 = 0.82\) and \(Q^2 = 0.78\)). Metabolite assignments are indicated together with the corresponding chemical shift. Ethanol and 2-propanol are reported more than once as their different chemical groups resonate at different frequencies. The x-axis reports the buckets, identified with chemical shift (in ppm) and metabolite name, and is labelled ‘VAR-ID’ (variable identity); the y-axis, labelled ‘VIP[2]’, shows the strength of the labelled metabolites in the classification between patients with stable versus unstable CF. Error bars represent 95% CIs.

Not all metabolites shown in figures 2B and 3B are required for between-group classification. The first four metabolites—namely acetate, ethanol, 2-propanol and acetone signals at 1.93, 1.19, 1.17 and 2.25 ppm, respectively—discriminate between patients with CF and healthy subjects \((R^2 = 0.81, Q^2 = 0.75\); figure 2B). Likewise, ethanol, acetate, 2-propanol and methanol signals at 1.19, 1.93, 1.17 and 3.57 ppm, respectively,
Cystic fibrosis

discriminate between patients with stable and unstable CF ($R^2=0.80$, $Q^2=0.65$; figure 3B). These data suggest that a limited number of metabolites can be used to pinpoint some of the metabolic changes in CF.

Figure 4 represents the difference between the coefficients of variation plots of the four selected metabolites between healthy subjects and patients with CF (ie, healthy subjects minus patients with CF) (figure 4A), and the difference between patients with stable and unstable CF (figure 4B). Acetate is higher in healthy subjects than in patients with CF whereas ethanol, 2-propanol and, to a much lesser extent, acetone are higher in patients with CF than in healthy subjects (figure 4A). Within CF patients, ethanol and 2-propanol are higher in those with stable CF whereas acetate and methanol are higher in patients with unstable CF (figure 4B). These data indicate that selective profiles of EBC metabolites might be useful for identifying biomarkers of CF. Estimated metabolite concentrations are reported in table E2 in the online supplement.

**NMR spectra of EBC are reproducible**

All peaks were used for assessing within-day repeatability. Each spectrum was subdivided into six regions which were integrated and normalised to the total spectrum area (see online supplement). The SD was within ±1.96 SD in 94 out of 96 samples (see figure E3 in online supplement), indicating a good within-day repeatability.

Between-day repeatability was expressed as ICC of the 4.4–0.4 ppm spectral region which contains all the relevant signals shown in figures 2B and 3B. The ICC was 0.93.

Technical repeatability was assessed by repeating NMR spectroscopy on three different samples (one from a healthy subject, one from a patient with stable CF and one from a patient with unstable CF) 10 times consecutively. The ICC for the 4.4–0.4 ppm spectral region was 0.96.

**No effect of salivary contamination or cleaning solution on EBC profiles**

Saliva and EBC spectra were different, indicating no significant salivary contamination of EBC (figure 5). No peak was observed in the spectrum of the sodium hypochlorite solution (5.55 mM) used for disinfection of the EBC collection set-up (not shown), indicating that the cleaning procedure does not affect the results.

**Correlations**

There was no correlation between metabolomic data and spirometry in any study group. In the CF groups there was no correlation between EBC metabolites and type of bacterial infection or genotypes. In patients with stable CF there was no correlation between metabolomic profiles and tobramycin or human recombinant DNase treatment. Assessing other correlations was difficult because of the small numbers of patients in some subgroups (table 1).

**DISCUSSION**

The novel features of our study are: (1) the demonstration that NMR-based metabolomics can be applied to EBC to discriminate between patients with CF and age-matched healthy control subjects and, more interestingly, between patients with stable and unstable CF; (2) the use of high resolution 2D-NMR spectroscopy which enables the highly specific identification of the EBC metabolites that account for between-group differences; (3) the external validation of these results in independent datasets; and (4) the largest application of NMR spectroscopy to CF to date.

The technique has good within-day, between-day (ICC=0.93) and technical repeatability (ICC=0.96). A detection limit of 0.14 µM, notably low for NMR-based metabolomics, indicates that it can be exploited for a more complete profiling of EBC metabolites.

Unsurprisingly given the complexity of CF profiles of metabolites rather than a single metabolite are needed to enable between-group discrimination. In previous studies, several metabolites have been identified in the exhaled breath...
metabolites to either the host or microbial metabolome and the site of origin of the metabolites. Indeed, if the metabolites are normal bacterial flora or the pathogens characteristic of CF may have been the source of some of the signals. Determining whether EBC metabolites are of host or microbial origin is difficult owing to the heterogeneous population of CF pathogens, the lack of specificity of many metabolites to either the host or microbial metabolome and the complexity of the host–microbial interactions in CF. Further studies are required to establish the specific origin of EBC metabolites, including the effect of anaerobic, fungal or mycobacterial infections. However, the aim of this study was to determine whether disease states could be differentiated, not the site of origin of the metabolites. Indeed, if the metabolites are discriminatory, their precise site of origin is less important to the clinician.

NMR-based metabolomics of EBC may be a useful contribution to the definition and management of a CF infective exacerbation, although longitudinal studies are required to confirm this. Ascertaining whether, and after how long, intravenous antibiotic treatment in patients with unstable CF reverts EBC metabolic profiles to those observed in patients with stable CF was beyond the scope of the present study, but would clearly need to be performed to evaluate the clinical utility of the measurements. We plan to perform a study to address this issue.

In conclusion, NMR spectroscopy of EBC can be used to discriminate patients with CF from healthy subjects and medications (healthy subjects received no treatment). It is not ethical to stop treatment in patients with CF. Pharmacological treatment is less likely to be responsible for the differences in EBC metabolites between patients with unstable and stable CF observed in our study as the numbers treated with inhaled corticosteroids (ICS) and short-acting β agonists (SABA) were similar in the two groups (table 1), indicating that ICS/SABA treatment has little effect on the results. There were no differences in a subgroup of stable CF patients between those who were and were not being treated with either inhaled tobramycin or recombinant human DNase (see Results, Subgroup analysis in online supplement). However, the cross-sectional design of this study precludes definitive conclusions on the effects of these drugs in patients with CF. Large prospective controlled studies to establish the effect of pharmacological treatment on EBC metabolites in patients with CF are required.

Acetic acid, acetone, ethanol, 2-propanol and methanol have previously been detected in exhaled breath in healthy subjects. Using 1H-NMR, acetone was detected in the bronchoalveolar lavage fluid of children with CF with varying levels of inflammation. 2-Propanol, an enzyme-mediated product of reduction of acetone, was demonstrated in a breath sample from one patient with CF infected with *Pseudomonas aeruginosa*. We speculate that the elevated EBC ethanol concentrations in patients with CF may be related to the reduced capacity of *Pseudomonas aeruginosa* to oxidise ethanol to acetate, whereas the elevated EBC 2-propanol concentration might be due to bacterial metabolism and/or increased lipolysis and lipid peroxidation. Elevated EBC acetate concentrations in healthy subjects could reflect metabolism of oral resident bacteria such as *Streptococcus mutans*, which degrades pyruvate into end products of metabolism including acetate and lactate.

There are other factors to be considered in the interpretation of the results. NMR-based metabolomics is not affected by the cleaning procedure used in this study. We cannot ascertain the source(s) of EBC metabolites. Salivary and EBC NMR spectra are completely different, suggesting that salivary contamination is unlikely. Furthermore, EBC metabolite signals are obtainable in tracheostomised patients in whom there can be no salivary contamination. Normal bacterial flora or the pathogens characteristic of CF may have been the source of some of the signals. Determining whether EBC metabolites are of host or microbial origin is difficult owing to the heterogeneous population of CF pathogens, the lack of specificity of many metabolites to either the host or microbial metabolome and the complexity of the host–microbial interactions in CF. Further studies are required to establish the specific origin of EBC metabolites, including the effect of anaerobic, fungal or mycobacterial infections. However, the aim of this study was to determine whether disease states could be differentiated, not the site of origin of the metabolites. Indeed, if the metabolites are discriminatory, their precise site of origin is less important to the clinician.

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In conclusion, NMR spectroscopy of EBC can be used to discriminate patients with CF from healthy subjects and
between patients with unstable and stable CF. This technique allows identification of unbiased potential biomarkers of CF, some of which may be useful surrogate endpoints in clinical trials, and also unravels the metabolic changes that characterise this disease.

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Competing interests None.

Ethics approval Ethics approval was provided by Ospedale Pediatrico Bambino Gesù, Rome and University of Naples Federico II, Italy.

Contributors Conception and design: PM. Analysis and interpretation: AM, DP, DM, FM, GC, VL, VR, CC. Drafting the manuscript for important intellectual content: PM, AB, PJdB, AM, VL. Final approval of the version to be published: FM, AB, PJdB, AM, VL.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

NMR Spectroscopy Metabolomic Profiling of Exhaled Breath Condensate in Patients with
Stable and Unstable Cystic Fibrosis

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Valeria Raia, Cecilia Calabrese, Andrew Bush, Peter J. Barnes, and Andrea Motta
INTRODUCTION

Volatile compounds produced by different isolates of *Pseudomonas aeruginosa* cultures *in vitro* from patients with cystic fibrosis (CF) have been measured with selected ion flow tube mass spectrometry [E1].

METHODS

Subjects

Healthy control subjects were recruited from staff or children of staff of Ospedale Pediatrico Bambino Gesù, Rome, Italy (principal study) or Department of Pediatrics, University Federico II, Naples, Italy (validation study). Healthy control subjects were at least 6 years of age, had negative skin prick test results, no history of asthma, atopic disease, respiratory diseases or other diseases and were able to perform reproducible spirometry. Healthy subjects were non-smokers and they were not exposed to passive smoking, were judged to be in good, stable physical and mental health based on medical history, physical examination, and routine laboratory data, and appeared to be able to successfully complete this study.

CF patients were recruited among those who were attending the CF Unit, Ospedale Pediatrico Bambino Gesù, Rome, Italy (principal study) or CF Unit, University Federico II, Naples, Italy (validation study). Subjects were recruited from 15 February 2008 to 15 December 2008.

Pulmonary exacerbation of CF was defined by the presence of at least two of the following signs or symptoms based on Ramsey criteria: fever (oral temperature >38°C), more frequent coughing (increase of 50%), increased sputum volume (increase of 50%), loss of appetite, weight loss of at least 1 kg, absence from school or work (at least 3 or preceding 7 days) due to illness, symptoms of upper respiratory tract infection. These symptoms had to have been associated with at least one of the following 3 additional criteria: decrease in FVC of at least 10%; an increase in respiratory rate of at least 10 breath per minute; a peripheral blood neutrophil count of 15000 per cubic millimetre or more [E2].
CF patients with fungal or mycobacterial infections were excluded from the study to reduce the risk of cross-contamination. Data regarding anaerobes are not presented as these bacteria are very difficult to isolate.

**Study design**

The 16 subjects in whom between-day and within-day repeatability was assessed were chosen among those subjects included in the primary analysis group. EBC samples for externally validating the model were collected from a group of 25 healthy subjects and 30 patients with CF (14 stable and 16 unstable) who were not included in the primary analysis and who were recruited in a different centre (CF Unit, University of Naples Federico II, Italy) (Table E1). For validating the classification model “CF patients vs healthy control subjects”, EBC samples were obtained from 23 patients with CF (11 stable and 12 unstable) who were chosen among those 30 CF patients recruited in the CF Unit, University of Naples Federico II, Italy. EBC samples were collected under similar experimental conditions using the same type of condenser (Ecoscreen, Jaeger, Hoechberg, Germany) [E3] (see EBC sampling).

**EBC sampling**

Subjects were asked to wash their mouth thoroughly before collecting EBC, to breathe tidally through a mouthpiece into a two-way non-rebreathing valve for 15 minutes wearing a nose-clip, and to stop breathing into the mouthpiece and swallow every time they felt salivation. An average of 1.5 ± 0.2 ml (mean ± SD) of EBC was collected in 15 minutes of tidal breathing. EBC sampling was performed as previously described.[E3] Previous experiments showed no difference with spectra acquired after a variable time of nitrogen exposure (1, 3, 5, 10, 15 and 20 min).[E3] However, as 1-min interval appeared to be too short to avoid systematic errors, a 3-min interval was chosen. Samples were not dried out to avoid their precipitation, with a possible loss of nonvolatile compounds, and/or formation of aggregates upon dissolving the dried condensate for NMR.
measurements. Sealed samples were then frozen in liquid nitrogen, so as to immediately “quench” metabolism and preserve the metabolite concentrations. [E3] The samples were stored at -80°C until metabolomic analysis.

**NMR sample preparation**

To provide a field frequency lock, 70 μL of a D₂O solution [containing 1 mM sodium 3-trimethylsilyl [2,2,3,3-²H₄] propionate (TSP) as a chemical shift reference for ¹H spectra, and sodium azide at 3 mM], were added to 630 μL of EBC reaching 700 μL of total volume.

**NMR spectroscopy measurements**

One-dimensional (1D) ¹H-NMR spectra were collected at 300 K with the excitation sculpting pulse sequence to suppress the water resonance. [E4] Two-dimensional (2D) clean total correlation spectroscopy (TOCSY) [E5] spectra were recorded using a standard pulse sequence, and incorporating the excitation sculpting sequence for water suppression. Both 1D and 2D spectra were referred to 0.1 mM TSP, assumed to resonate at δ = 0.00 ppm. Spectra were referred to the lactate doublet (βCH₃) resonating at 1.33 ppm for ¹H, and 20.76 ppm for ¹³C.

Detection limit for metabolomic analysis of EBC by NMR spectroscopy was calculated by integrating EBC spectra that were normalized to 0.1 mM TSP. The analysis of 10 spectra estimated an average concentration of 0.14 ± 0.04 μM for the phenylalanine peaks, among the lowest detected signals.

**Pulmonary function testing**

Forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) were measured by spirometry (Quark PFT2; Cosmed; Rome, Italy) and the best of three manoeuvres, expressed as percentage of predicted values, was chosen.
Measurement of serum C-reactive protein (CRP)

CRP was measured with the Tina-quant CRP particle enhanced immunoturbidimetric method, an automated high-sensitive CRP method, performed using a COBAS INTEGRA 400 analyzer (Roche Diagnostics, Basel, Switzerland). The analytic measurement range is 0–160 mg/L with automatic dilution for results up to 1600 mg/L.

Spectral and statistical analysis

For classification purposes, we carried out a multivariate statistical data analysis using projection methods. The integrated data reduced format of the spectra was imported into Soft Independent Modeling of Class Analogy (SIMCA)-P+ 12 package (Umetrics, Umeå, Sweden). Principal Component Analysis (PCA) was first applied to detect EBC metabolite clustering in an unsupervised manner. To reinforce classification, the spectral filtering orthogonal signal correction (OSC) routine [E6] with the partial least squares-discriminant analysis (PLS-DA) was applied.

We obtained two regression models: the first is a regression between all the spectral data (healthy and CF subjects) and the disease state by choosing the healthy state as the one-component Y matrix; the second regression model was constructed for CF samples only by using the “stable CF” as the one-component Y matrix. In addition, for each filter a permutation test (n = 200) was carried out to test possible overfit of the model.

For Projection methods like PLS-DA there are no standardized methods for evaluating the power of the analysis. The power of principal component regression (PCR) analysis has been approached with parametric or Monte Carlo methods. However, the results are not completely reliable as those for classical statistical methods. Therefore, we consider our study as a pilot study for which no a priori power analysis was possible. The data obtained could be used for an a posteriori power analysis, but the current methods appear not sufficiently robust to warrant a satisfactory analysis for PLS-DA. On the other hand, the permutation and the validation tests done
within the PLS-DA have confirmed the existence and validity of the model and avoided the overfitting problem.

RESULTS

Table 1. Subject characteristics

Inhaled budesonide at a dose of 400 µg once daily and inhaled beclometasone at a dose of 200 µg b.i.d. were given via a spacer device. Inhaled salbutamol at a dose of 200 µg b.i.d. was given using a metered dose inhaler. Tobramycin (Tobi®; Dompé; Milano, Italy) was administered b.i.d. as tobramycin inhalation solution (300 mg per 5 ml) aerosolized with a jet nebulizer (PARI LC PLUS; Pari; Starnberg, Germany).

Recombinant human DNase (dornase alfa) (Pulmozyme®; Roche; Milano, Italy) was administered once a day as 2.5 mg of recombinant human DNase in 2.5 mL buffered solution using a nebulizer.

Missing data: Patients with stable CF: Forced Expiratory Volume in one second (FEV₁), n = 2; Forced Vital Capacity (FVC), n = 2; Body Mass Index (BMI), n = 3; White Blood Cells (WBC), n = 2.

NMR spectroscopy of EBC discriminates between patients with cystic fibrosis and healthy controls

Applying PCA, a sample classification of 55% (samples correctly classified into different regions) was obtained.

The signal observed at 3.65 ppm (a one-proton area “septet”) corresponds to 2-propanol and cannot be confused with the potential hydrogen cyanide signal (shift at 3.6 ppm), which is a broad “singlet”, or 1-propanol (shift at 3.58 ppm). The signal at 3.65 ppm is coupled to the six-proton area doublet at 1.17 ppm, and unequivocally stems from the O-CH of 2-propanol, while the signal at 3.58 ppm stems from the O-CH₂ group and is part of the 1-propanol spin system in the databank (http://www.chem.wisc.edu/areas/reich/handouts/nmr-h/hdata.htm).
NMR spectra of EBC are reproducible

Due to the high number of variables, we subdivided each spectrum in 6 regions (region 1: 8.6–6.6 ppm; region 2: 6.6–5.2 ppm; region 3: 4.4–3.4 ppm; region 4: 3.4–2.4 ppm; region 5: 2.4–1.4 ppm; region 6: 1.4–0.4 ppm), while region 4.40–5.20 ppm, containing the residual water resonance, was excluded. All regions were integrated and normalized to the total spectrum area to avoid possible variation of metabolite concentrations due to differences in volume during EBC collection. We obtained 6 parameters (the integrated fractional regions) for each spectrum obtained from 16 subjects so that 96 values were used to assess the within-day repeatability of EBC metabolomics. For assessing within day repeatability, Bland-Altman test was applied to single regions.

For assessing between-day repeatability that was expressed as intraclass correlation coefficient (ICC), three EBC samples, collected from the same 16 subjects (11 CF patients and 5 healthy subjects) on day 1, 3 and 7, were analyzed with NMR spectroscopy. The 4.4–0.4 ppm spectral area which contains all the relevant signals shown in Figure 2B and 3B was integrated and normalized to the total spectrum area.

Subgroup analysis

Due to number limitation, subgroup analysis was only possible for inhaled tobramycin and DNase treatment in stable CF patients (Table 1, main manuscript). No difference in metabolic profiles in EBC was observed between CF patients who were being treated with tobramycin or DNase and CF patients who were not receiving the drug.

DISCUSSION

NMR spectroscopy of EBC has recently been questioned based on the possible EBC contamination due to the pentapotassium-bis-(peroxomonosulfate) bis(sulfate) (30%) and citric acid (5%) solution (Descogen, Antiseptica, Pulheim, Germany) used for cleaning the EBC collection setup.[E10] However, the absence of any NMR spectroscopy peaks in the disinfectant solution (sodium
hypochlorite at 3.55 mM) (Milton Pharmaceutical UK Limited, Gloucester, United Kingdom) that we used demonstrates that, under our experimental conditions, the cleaning procedure does not affect EBC metabolomics.

Differences in the EBC metabolic profiles between CF patients and healthy controls are much greater than those between stable and unstable CF patients and likely preclude discrimination within the CF group when the three groups are compared simultaneously.

Methanol was selectively elevated in EBC in patients with unstable CF, but the origin and significance of methanol is not yet sufficiently understood.

There are no quantitative between group differences in EBC metabolites in our study (Table E1). These data are presented in the online supplementary material and not in the main manuscript as quantitative assessment of EBC metabolites was not the primary objective of this study. Several factors might be responsible for the lack of between group differences in metabolite concentrations in our study including between group differences in drug treatment (e.g., vitamin E, glucocorticoids, antibiotics) and within group biological heterogeneity of CF patients. Further studies, focused on the quantitative assessment of EBC metabolites, are warranted and will require careful selection of patients to minimise within group biological variability and stratification of factors potentially affecting metabolite concentrations (e.g., drug treatment). On the other hand, the possibility to classify study groups by applying PLS-DA to NMR-based metabolomic EBC data in the presence of such biological heterogeneity and confounding factors emphasizes the discriminative power of this technique.

References


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<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>Stable CF patients</th>
<th>Unstable CF patients</th>
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<tbody>
<tr>
<td>n</td>
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<td>14</td>
<td>16</td>
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<tr>
<td>Age, yr</td>
<td>15 ± 0.8</td>
<td>14 ± 0.9</td>
<td>15 ± 0.7</td>
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<td>Sex, F/M</td>
<td>12/13</td>
<td>7/7</td>
<td>8/8</td>
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<tr>
<td>FEV1, % predicted</td>
<td>111.8 ± 2.7</td>
<td>107.1 ± 3.2(#)</td>
<td>54.2 ± 1.9(§)</td>
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<tr>
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<td>p &lt; 0.001</td>
<td>p &lt; 0.014</td>
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<td>FVC, % predicted</td>
<td>115.3 ± 3.5</td>
<td>109.0 ± 2.0(#)</td>
<td>70.4 ± 1.7(§)</td>
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<td>p &lt; 0.017</td>
<td>p &lt; 0.013</td>
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<td>BMI (kg/m²)</td>
<td>20.9 ± 0.6</td>
<td>20.0 ± 0.4</td>
<td>19.0 ± 0.8</td>
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<td>CRP (mg/L)</td>
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<td>0.4 ± 0.1</td>
<td>2.8 ± 0.3(§)</td>
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<td>p &lt; 0.0001</td>
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<td>WBC ((\times 10^9)/L)</td>
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<td>7.1 ± 0.6</td>
<td>11.7 ± 0.5(§)</td>
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<td>p &lt; 0.004</td>
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BMI, body mass index; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CRP, C reactive protein; FEV₁, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroids; WBC, peripheral white blood cell counts.

*Data are expressed as n or mean ± SEM. One-way ANOVA and unpaired t test were used for comparing groups. Significance was defined as a value of p < 0.05.

#Compared with healthy subjects; §compared with stable CF patients.

^Genotype was not determined in one patient with stable CF and in two patients with unstable CF.

Age range: healthy subjects: 10-22 years; stable CF patients: 12-17 years; unstable CF years: 11-22 years. Patients with CF with concomitant allergy and/or asthma were excluded from the study. No patient with CF had pancreatic insufficiency.

**Table E2. Concentrations of EBC metabolites responsible for between-group classification**

<table>
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<tr>
<th>EBC metabolite (µM)</th>
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<th>Stable cystic fibrosis patients (n = 29)</th>
<th>Unstable cystic fibrosis patients (n = 24)</th>
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<tr>
<td>Acetate</td>
<td>19.70 ± 10.33</td>
<td>7.50 ± 4.73</td>
<td>10.62 ± 5.82</td>
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<tr>
<td>Acetone</td>
<td>8.00 ± 3.85</td>
<td>25.33 ± 16.25</td>
<td>12.00 ± 7.55</td>
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<tr>
<td>Ethanol</td>
<td>10.17 ± 6.14</td>
<td>22.76 ± 10.54</td>
<td>13.80 ± 7.52</td>
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<tr>
<td>Methanol</td>
<td>4.00 ± 1.85</td>
<td>2.67 ± 1.82</td>
<td>3.33 ± 1.80</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>9.20 ± 4.83</td>
<td>15.17 ± 8.35</td>
<td>11.71 ± 5.91</td>
</tr>
</tbody>
</table>

*NMR signals were integrated and referred to the final TSP signal of known concentration (100 µM). EBC metabolite concentrations are expressed as mean ± SD. One-way ANOVA was used for comparing groups.
FIGURE LEGENDS

**Figure E1.** Metabolomic analysis of exhaled breath condensate (EBC) with $^1$H-nuclear magnetic resonance (NMR) spectroscopy: loading plot of data obtained from patients with cystic fibrosis (CF) vs healthy subjects (HS).

**Figure E2.** Metabolomic analysis of exhaled breath condensate (EBC) with $^1$H-nuclear magnetic resonance (NMR) spectroscopy: loading plot of data obtained from patients with stable cystic fibrosis (CF) vs unstable cystic fibrosis (CF).

**Figure E3.** Assessment of within-day repeatability of nuclear magnetic resonance (NMR) spectroscopy measures according to Bland and Altman. Two EBC samples were collected twice within the same day (at times 0 h and 12 h) from 16 subjects (5 healthy subjects, 5 patients with stable cystic fibrosis, and 6 patients with unstable cystic fibrosis). Each spectrum was subdivided in 6 regions (region 1: 8.6–6.6 ppm; region 2: 6.6–5.2 ppm; region 3: 4.4–3.4 ppm; region 4: 3.4–2.4 ppm; region 5: 2.4–1.4 ppm; region 6: 1.4–0.4 ppm), while region 4.40–5.20 ppm, containing the residual water resonance, was excluded. All regions were integrated and normalized to the total spectrum area. We obtained 6 parameters (the integrated fractional regions) for each spectrum obtained from 16 subjects so that 96 values were used to assess the within-repeatability of EBC metabolomics.
NMR Spectroscopy Metabolomic Profiling of Exhaled Breath Condensate in Patients with Stable and Unstable Cystic Fibrosis

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PM, DP, DM contributed equally to this work

Keywords: Metabolomics, Nuclear Magnetic Resonance Spectroscopy, Exhaled Breath Condensate, Cystic Fibrosis

Word Count for manuscript: 2991
What is the key question?

Does NMR spectroscopy of EBC discriminate between patients with unstable CF, stable CF, and healthy controls and are selected metabolites responsible for between-group differences?

What is the bottom line?

NMR spectroscopy of EBC is a reproducible technique which not only discriminates patients with unstable CF, stable CF and healthy subjects, but also unravels metabolic changes that characterize CF.

Why read on?

Learn how NMR-based metabolomics of EBC provides a unique opportunity for investigating the pathophysiology of CF and identifies a panel of unbiased potential biomarkers of CF. These markers may be useful end-points in future studies.
ABSTRACT

Background

Metabolomics could provide new insights into the pathophysiology of cystic fibrosis (CF) by identifying profiles of endogenous metabolites.

Objectives

We investigated whether metabolomics of exhaled breath condensate (EBC) could discriminate between patients with unstable CF, stable CF, and healthy subjects, and whether selected metabolites were responsible for between-group differences.

Methods

Twenty-nine stable CF patients, 24 unstable CF patients, and 31 healthy subjects (age 9-24 years) were studied. Study design was cross-sectional. Metabolomics was performed with high resolution Nuclear Magnetic Resonance (NMR) spectroscopy. Partial least squares-discriminant analysis was used as classifier. Results were validated in a second independent study.

Results

Intraclass correlation coefficients for between-day and technical repeatability were 0.93 and 0.96, respectively. Bland-Altman analysis showed good within-day repeatability. Correct classification rate of CF (n=53) vs healthy subjects (n=31) was 96% ($R^2=0.84$; $Q^2=0.79$). Model validation with a testing sample set obtained form subjects not included in the primary analysis (CF=23; healthy subjects=25) showed a sensitivity of 91% and a specificity of 96%. Classification rate of stable CF (n=29) vs unstable CF patients (n=24) was 95% ($R^2=0.82$; $Q^2=0.78$). Model external validation (stable CF=14; unstable CF=16) showed a sensitivity of 86% and a specificity of 94%. Ethanol, acetate, 2-propanol, and acetone are most discriminant between CF patients and healthy subjects, whereas acetate, ethanol, 2-propanol, and methanol...
are the most important metabolites for discriminating between stable and unstable CF patients.

**Conclusions**

NMR spectroscopy of EBC is reproducible; discriminates CF patients from healthy subjects and unstable from stable CF patients; identifies the metabolites responsible for between-group differences.
INTRODUCTION

Airway inflammation plays a central role in the pathophysiology of cystic fibrosis (CF).[1] It is not clear whether the exuberant airway inflammation in CF is due entirely to chronic bacterial infection, whether the CF airway epithelium is inherently pro-inflammatory in the absence of infection,[2] whether there is an excessive response to bacterial infection [3] or a defect in the resolution of inflammation.[4] Breath volatile compounds including hydrogen cyanide have been measured by mass spectrometry techniques in CF patients and compared with healthy subjects.[5, 6] Exhaled breath condensate (EBC) is a non-invasive technique for studying the composition of airway lining fluid.[7] Relatively few data on EBC metabolic profiles are available, and information on EBC metabolite composition is often obtained by analyzing single inflammatory molecules. Metabolomics involves the detection of metabolites in a biological fluid usually using high resolution Nuclear Magnetic Resonance (NMR) spectroscopy.[8] This approach enables a specific quantitative description of the low-molecular mass endogenous metabolites in a biological sample, provides a metabolic “fingerprint” which can be used for classification purposes, and focuses on the most important regions of the NMR spectrum for further analysis.[8] The identification of specific molecules and their relative variations might provide further insights into the pathophysiology of CF.

The presence of discriminating metabolites in a NMR spectrum can be tested with multivariate statistical algorithms, which allow a thorough comparison of datasets.[9] Metabolomics of EBC with NMR spectroscopy discriminates between children with mild-to-moderate persistent asthma and healthy children [10] and between patients with chronic obstructive pulmonary disease and healthy subjects.[11] Using NMR spectroscopy, chlorinated and brominated tyrosine residues were detected in sputum in CF patients, but not in healthy subjects.[12] Metabolomic profiles of bronchoalveolar lavage (BAL) fluid from CF children are correlated to the degree of airway inflammation.[13]
The primary objectives of the present study were to verify whether 1) EBC metabolomic analysis with NMR spectroscopy discriminates between patients with unstable CF, stable CF, and healthy controls and 2) selective metabolites are responsible for between-group differences.

METHODS

Subjects
Twenty-nine patients with stable CF, 24 patients with unstable CF and 31 age-matched healthy controls were studied. Healthy subjects had no history of respiratory or other diseases. They had no upper airway infections nor received any medication in the previous four weeks. The diagnosis of CF was based on clinical, radiological and genotypic characteristics and an abnormal sweat test (sweat chloride \( \geq 60 \) mmol/L).[14] Stable CF patients had no change in cough or shortness of breath, no requirement for oral or intravenous antibiotics, and no significant spirometry change in the previous 4 weeks. Unstable CF patients were assessed before treatment for pulmonary exacerbation, defined by the presence of at least two of the following signs: oral temperature \( >38^\circ\text{C} \), more frequent coughing, increased sputum volume, appetite or weight loss, absence from school or work due to illness (at least 3 of preceding 7 days), and symptoms of upper respiratory tract infection, associated with at least one of the following: decrease in forced vital capacity (FVC) of at least 10%; increase in respiratory rate of at least 10 breath per minute; peripheral blood neutrophil count of 15000 per cubic millimetre or more.[15] All subjects were nonsmokers (see also online supplement).
Study design

The study was cross-sectional. Subjects attended the CF Unit outpatient clinic of Ospedale Pediatrico Bambino Gesù, Rome, Italy, on one occasion for clinical examination, EBC collection, spirometry, chest X ray, and blood sampling. Between-day repeatability was assessed in 5 healthy and 11 CF subjects, among those who participated in the principal study, by collecting three EBC samples within 7 days of the first. From the same subjects, separate EBC samples were collected twice within the same day for assessing within-day repeatability. External validation of the classification model was performed by testing EBC samples obtained from subjects not included in the primary analysis and collected in a different centre under similar experimental conditions (CF Unit, University of Naples Federico II, Italy) (Table E1). Informed consent was obtained from parents and adult subjects, and age-appropriate assent from children. The study was approved by the local Ethics Committees.

EBC sampling

Before EBC collection, subjects refrained from eating for at least 3 hours. EBC was collected using a condenser (Ecoscreen, Jaeger, Hoechberg, Germany), which has a saliva trap to reduce the chance of salivary contamination,[11] in a windowless clinic facility without disinfectant dispensers; CF patients had not used wipes prior to the study. Saliva was also obtained. EBC was immediately transferred into 10 mL glass vials, closed with 20 mm butyl rubber lined with polytetrafluoroethylene septa and crimped with perforated aluminium seals. Before sealing, volatile substances were removed from samples by a gentle nitrogen gas flow for 3 minutes.[11] (see online supplement) Samples were then frozen in liquid nitrogen to immediately “quench” metabolism and preserve the metabolite concentrations.
NMR sample preparation

EBC and saliva samples were rapidly defrosted. 70 μL of reference standard D$_2$O solution [containing 0.1 mM sodium 3-trimethylsilyl [2,2,3,3-$^{2}$H$_4$] propionate (TSP) and sodium azide at 3 mM] were added to 630 μL of sample.

NMR spectroscopy measurements

Spectra were recorded on a 600 MHz Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a CryoProbe™. One-dimensional (1D) $^1$H-NMR and two-dimensional (2D) clean total correlation spectroscopy (TOCSY) spectra were recorded using a standard pulse sequence.[16] Detection limit was 0.14 μM. Full details are given in the online supplement.

Spectral and statistical analysis

There are no data in the literature for a sample size calculation in a NMR-based metabolomic study. The spectral 0.12–8.60 ppm region of the high-resolution $^1$H-NMR spectra was automatically data reduced to integrated regions (buckets) of 0.02 ppm each using the AMIX 3.6 package (Bruker Biospin GmbH, Rheinstetten, Germany). To account for possible metabolite concentration variations due to dilution, each bucket was normalized to the corresponding total spectral area or spectra were referenced to a known TSP concentration (0.1 mM) added to the samples. No difference was observed between these approaches.

To examine EBC metabolite clustering in an unsupervised manner, we applied the spectral filtering orthogonal signal correction routine [17] with the partial least squares-discriminant analysis (PLS-DA).
Within-day repeatability of NMR spectroscopy was assessed according to Bland-Altman. Between-day and technical repeatability were assessed with ANOVA for repeated measures and expressed as intraclass correlation coefficient (ICC). Data were expressed as mean±SEM after assessing for normality with D’Agostino-Pearson omnibus normality test. One-way ANOVA and unpaired t test were used for comparing groups. Significance was defined as a value of p<0.05.

RESULTS

Patient characteristics

Subject characteristics are provided in Table 1. Forced expiratory volume in one second (FEV1) and FVC were reduced in CF patients compared with healthy subjects (Table 1). Unstable CF patients had lower FEV1 than stable CF patients (Table 1). Unstable CF patients had higher systemic inflammation than stable CF patients as reflected by serum C-reactive protein and white blood cell counts (Table 1).

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<tr>
<th>Table 1. Subject characteristics*</th>
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<td>Recombinant human DNase (y/n)</td>
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BMI, body mass index; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CRP, C reactive protein; FEV₁, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroids; WBC, peripheral white blood cell counts.

*Data are expressed as n or mean ± SEM. One-way ANOVA and unpaired t test were used for comparing groups. Significance was defined as a value of p < 0.05.

#Compared with healthy subjects; §compared with stable CF patients.
Genotype was not determined in one patient with stable CF and in two patients with unstable CF.

Age range: healthy subjects: 9-23 years; stable CF patients: 9-24 years; unstable CF years: 9-23 years. Patients with CF with concomitant allergy and/or asthma were excluded from the study. No patient with CF, except one patient with stable CF, had pancreatic insufficiency. Full details about treatment are given in the online supplement material.

**NMR spectroscopy of EBC discriminates between patients with CF and healthy controls**

The EBC NMR profiling of patients with stable and unstable CF was compared to that in healthy controls. Figure 1 shows representative EBC 1D spectra of a healthy subject (Figure 1A), a patient with stable CF (Figure 1B), and a patient with unstable CF (Figure 1C). Spectra resonances were assigned to single metabolites by resorting to 2D $^1$H-$^1$H TOCSY and $^1$H-$^{13}$C Heteronuclear Single Quantum Coherence experiments (not shown), and compared with the Human Metabolome Database [19, http://www.hmdb.ca/] and published chemical-shift data.[20]

PLS-DA showed a strong regression model (96%) between healthy and CF patients (Figure 2A) in which data variation is well explained by the model ($R^2$), with a very good predictivity ($Q^2$) ($R^2=0.84$ and $Q^2=0.79$). To validate this model, a sample set obtained from subjects not included in the primary analysis (23 CF patients, 25 healthy subjects) (Table E1) was tested blindly. The model correctly identified 21 out of 23 patients with CF (91% accuracy) and 24 out of 25 healthy subjects (96% accuracy, and 4.0% false-positives) with a sensitivity of 91%, a specificity of 96%, a positive likelihood ratio (LR+) of 23, and a negative likelihood ratio (LR−) of 0.08.
NMR profiling of EBC discriminates between stable and unstable CF patients

PLS-DA yielded a model of stable (n=29) vs unstable (n=24) CF patients, which resulted in three predictive and three orthogonal components (R²=0.82 and Q²=0.78) (Figure 3A).

To validate this model, a sample set obtained from subjects not included in the primary analysis (14 stable and 16 unstable CF patients) (Table E1) was tested blindly. The model correctly identified 12 out of 14 patients with stable CF (86% accuracy), and 15 out of 16 patients with unstable CF (94% accuracy), with a sensitivity of 86%, a specificity of 94%, LR+ of 15, and LR- of 0.14.

Four metabolites differentiate classes

The variables responsible for between-class differences observed in the score plot can be identified from the loading plot (Figure E1), which reports the spectral “buckets” containing metabolite resonances. The Variables of Importance Plot (VIP) describes the most important metabolites in making accurate the model. The first 14 buckets (corresponding to 11 metabolites) separate CF patients (n=53) from healthy subjects (n=31) (Figure 2B). Ethanol and 2-propanol appear more than once as their different chemical groups resonate at different frequencies (Figure 2B).

Differences between stable and unstable CF patients are based upon the spectral “buckets” of the corresponding loading plot (Figure E2). The 9 most relevant buckets (7 metabolites) generating the model are shown in the VIP (Figure 3B). Ethanol and 2-propanol are reported twice as their different chemical groups resonate at different frequencies (Figure 3B).

Not all metabolites shown in Figure 2B and 3B are required for between-group classification. The first four metabolites, namely acetate, ethanol, 2-propanol and acetone signals at 1.93, 1.19, 1.17 and 2.23 ppm, respectively, discriminate between CF patients and healthy subjects.
(R²=0.81 and Q²=0.73) (Figure 2B). Likewise, ethanol, acetate, 2-propanol and methanol signals at 1.19, 1.93, 1.17 and 3.37 ppm, respectively, discriminate between patients with stable and unstable CF (R²=0.80 and Q²=0.65) (Figure 3B). These data suggest that a limited number of metabolites can be used to pinpoint some of the metabolic changes in CF.

Figure 4 represents the difference between the coefficients of variation plots of the four selected metabolites between healthy subjects and CF patients (i.e., healthy subjects minus CF patients) (Figure 4A), and the difference between stable vs unstable CF patients (Figure 4B). Acetate is higher in healthy subjects than in CF patients, whereas ethanol, 2-propanol and, to a much lesser extent, acetone are higher in CF patients than in healthy subjects (Figure 4A). Within CF patients, ethanol and 2-propanol are elevated in stable CF patients, whereas acetate and methanol are elevated in unstable CF patients (Figure 4B). These data indicate that selective profiles of EBC metabolites might be useful for identifying biomarkers of CF. Estimated metabolite concentrations are reported in Table E2.

**NMR spectra of EBC are reproducible**

All peaks were used for assessing within-day repeatability. Each spectrum was subdivided in 6 regions which were integrated and normalized to the total spectrum area (see online supplement). The SD was within ±1.96 SD in 94 out of 96 samples (Figure E3), indicating a good within-day repeatability.

Between-day repeatability was expressed as ICC of the 4.4–0.4 ppm spectral region which contains all the relevant signals shown in Figure 2B and 3B. The ICC was 0.93. Technical repeatability was assessed repeating NMR spectroscopy on three different samples (1 from a healthy subject, 1 from a patient with stable CF, and 1 from a patient with unstable CF) 10 times consecutively. The ICC for the 4.4–0.4 ppm spectral region was 0.96.
No effect of salivary contamination or cleaning solution on EBC profiles

Saliva and EBC spectra were different indicating no significant salivary contamination of EBC (Figure 5). No peak was observed in the spectrum of the sodium hypochlorite solution (3.55 mM) used for disinfection of EBC collection setup (not shown), indicating that the cleaning procedure does not affect results.

Correlations

There was no correlation between metabolomic data and spirometry in any study group. In the CF groups, there was no correlation between EBC metabolites and type of bacterial infection or genotypes. In stable CF patients, there was no correlation between metabolomic profiles and tobramycin or human recombinant DNase treatment. Assessing other correlations was difficult due to the small numbers of patients in some subgroups (Table 1).

DISCUSSION

The novel features of our study are: 1) the demonstration that NMR-based metabolomics can be applied to EBC to discriminate between persons with CF and age-matched healthy control subjects and, more interestingly, between stable and unstable CF patients; 2) the use of high resolution 2D-NMR spectroscopy that enables the highly specific identification of the EBC metabolites that account for between-group differences; 3) the external validation of these results in independent datasets; 4) the largest application of NMR spectroscopy to CF to date. The technique has good within-day, between-day (ICC=0.93) and technical repeatability (ICC=0.96). A detection limit of 0.14 μM, notably low for NMR-based metabolomics,[21] indicates that it can be exploited for a more complete profiling of EBC metabolites.

Unsurprisingly given the complexity of CF,[1] profiles of metabolites rather than a single metabolite are needed to enable between-group discrimination. In previous studies,
several metabolites have been identified in the gas phase of the exhaled breath in healthy subjects [22-24] and in CF patients [24-28]. In the present study in EBC, most of the variance between CF patients and healthy subjects depend upon ethanol, acetate, 2-propanol, and acetone, whereas acetate, ethanol, 2-propanol, and methanol are the most important metabolites for discriminating between stable and unstable CF patients. Although similar metabolites are present in all study groups, their different distribution allows for this classification. Compared to healthy subjects, ethanol, 2-propanol and, to a much lesser extent, acetone concentrations in EBC were increased in patients with CF (stable and unstable), whereas acetate was reduced. Unstable CF patients had higher methanol and acetate concentrations in EBC and lower ethanol and 2-propanol than stable CF patients. Metabolite differences in opposite directions in CF patients and healthy subjects suggest that the differences are not a dilutional artifact. Moreover, referencing each bucket to the corresponding total spectral area and the spectra to a known concentration of TSP added to the samples normalizes for possible signal variation due to EBC dilution.

It is possible that differences in EBC metabolites between CF and healthy subjects might be partially due to medications (healthy subjects received no treatment). It is not ethical to stop the CF patients’ treatment. Pharmacological treatment is less likely to be responsible for the differences in EBC metabolites between unstable and stable CF patients observed in our study as numbers treated with ICS and SABA were similar in the two groups (Table 1) indicating that ICS/SABA treatment has little effect on the results. There were no differences in a subgroup of stable CF patients between those who were and were not being treated with either inhaled tobramycin or recombinant human DNase (online supplement, Results, Subgroup analysis). However, the cross-sectional design of this study precludes definitive conclusions on the effects of these drugs in patients with CF. Large prospective controlled studies to
establish the effect of pharmacological treatment on EBC metabolites in CF patients are required.

Acetic acid, acetone, ethanol, 2-propanol, and methanol have previously been detected in exhaled breath in healthy subjects.[23, 29, 30] Using $^1$H-NMR, acetone was detected in the BAL fluid of pediatric CF patients with varying levels of inflammation.[13] 2-Propanol, an enzyme-mediated product of reduction of acetone, was demonstrated in a breath sample from one CF patient infected with *Pseudomonas aeruginosa*.[31] We speculate that the elevated EBC ethanol concentrations in CF patients may be related to the reduced capacity of *Pseudomonas aeruginosa* to oxidize ethanol to acetate,[32] whereas the elevated EBC 2-propanol might be due to bacterial metabolism and/or increased lipolysis and lipid peroxidation.[31] Elevated EBC acetate in healthy subjects could reflect metabolism of oral resident bacteria, such as *Streptococcus mutans*, which degrades pyruvate into end products of metabolism including acetate and lactate.[33]

There are other factors to be considered in the interpretation of the results. NMR-based metabolomics is not affected by the cleaning procedure used in this study.[34]. We cannot ascertain the source(s) of EBC metabolites. Salivary and EBC NMR spectra are completely different, suggesting salivary contamination is unlikely. Furthermore, EBC metabolite signals are obtainable in tracheostomized patients in whom there can be no salivary contamination.[11] However, normal bacterial flora, or the pathogens characteristic of CF [35] may have been the source of some of the signals. Determining whether EBC metabolites are of host or microbial origin is difficult due to the heterogeneous population of CF pathogens, the lack of specificity of many metabolites to either the host or microbial metabolome, and the complexity of the host-microbial interactions in CF. Further studies are required to establish the specific origin of EBC metabolites, including the effect of anaerobic, fungal or mycobacterial infections. Nonetheless, the point of the manuscript is to determine
whether disease states could be differentiated, not the site of origin of the metabolites. Indeed, if the metabolites are discriminatory, their precise site of origin is less important to the clinician.

NMR-based metabolomics of EBC may be a useful contribution to the definition and management of a CF infective exacerbation, although longitudinal studies are required to confirm this. Ascertaining whether, and after how long, intravenous antibiotic treatment in unstable CF patients reverts EBC metabolic profiles to those observed in stable CF patients was beyond the scope of the present study, but would clearly need to be performed to evaluate the clinical utility of the measurements. We plan to perform a study to address this issue.

In conclusion, NMR spectroscopy of EBC can be used to discriminate CF patients from healthy subjects and between patients with unstable and stable CF. This technique allows identification of unbiased potential biomarkers of CF, some of which may be useful surrogate end-points in clinical trials, and also in unraveling metabolic changes that characterize this disease.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1. NMR spectra of EBC samples.** Representative one-dimensional $^1$H spectra of a healthy subject (A), a patient with stable CF (B) and a patient with unstable CF (C). All signals were assigned to single metabolites by resorting to two-dimensional NMR experiments and referring to published data on metabolite chemical shifts. Absorption (related to the intensity) is plotted on the y-axis and magnetic field strength is plotted on the x-axis (scale is in parts per million and usually goes from 0-12 ppm).

**Figure 2. Cystic Fibrosis vs Control Subjects.** Partial least squares-discriminant analysis (PLS-DA) with the spectral filtering orthogonal signal correction (OSC) of EBC metabolites in CF patients compared to healthy control subjects. A) Score plot showing the model’s degree of separation between healthy subjects (filled circles) and CF patients (empty circles). In the score plot, $t[1]$ and $t[2]$ along the axes represent the scores (the “first two partial least squares components”) of the model, which are sufficient to build a satisfactory classification model. The model showed a strong regression (96%). B) Variables of Importance Plot (VIP) that represents the 14 most important buckets (11 metabolites) generating this model ($R^2 = 0.84$ and $Q^2 = 0.79$). Metabolite assignments are indicated together with the corresponding chemical shift. Ethanol and 2-propanol are reported more than once as their different chemical groups resonate at different frequencies. The x-axis reports the buckets, identified with chemical shift (in ppm) and metabolite name, and is labeled “VAR-ID” (variable identity); the y-axis, labeled “VIP[2]”, shows the strength of the labeled metabolites in the classification between CF patients vs healthy subjects. Error bars represent 95% confidence intervals.
**Figure 3. Stable vs Unstable Cystic Fibrosis.** A) Partial least squares-discriminant analysis (PLS-DA) with the spectral filtering orthogonal signal correction (OSC) of EBC metabolites in stable (blue dots) compared to unstable (red dots) CF subjects. In the score plot, t[1], t[2] and t[3] along the axes represent the scores (the “first three partial least squares components”) of the model, which are sufficient to build a satisfactory classification model. B) Variables of Importance Plot that represents the 9 most important metabolites generating this model (R² = 0.82 and Q² = 0.78). Metabolite assignments are indicated together with the corresponding chemical shift. Ethanol and 2-propanol are reported more than once as their different chemical groups resonate at different frequencies. The x-axis reports the buckets, identified with chemical shift (in ppm) and metabolite name, and is labeled “VAR-ID” (variable identity); the y-axis, labeled “VIP[2]”, shows the strength of the labeled metabolites in the classification between stable vs unstable CF patients. Error bars represent 95% confidence intervals.

**Figure 4. A Panel of Four Metabolites Differentiates EBC Classes.** The four selected metabolites, which differentiate healthy subjects from CF patients (A) (R² = 0.81 and Q² = 0.73), and stable from unstable CF patients (B) (R² = 0.80 and Q² = 0.65), are reported as differences of coefficients of variation plots (i.e., healthy subjects minus CF, and stable minus unstable CF). Metabolite assignments are indicated together with the corresponding chemical shift. Error bars represent 95% confidence intervals.

**Figure 5. Nuclear magnetic resonance (NMR) spectroscopy of exhaled breath condensate (EBC) and saliva.** Representative one-dimensional ¹H-NMR spectra of EBC spiked with saliva (A), saliva (B), and EBC (C) samples from a CF patient. The saliva sample shows signals between 3.3 and 4.5 ppm originating from carbohydrates (B), which are virtually absent in the EBC spectrum (C). In the EBC spectrum of a CF patient spiked with...
his saliva (A), the overall resonance distribution indicates the presence of saliva. Absorption (related to the intensity) is plotted on the y-axis and magnetic field strength is plotted on the x-axis (scale is in parts per million and usually goes from 0-12 ppm).