NMR Spectroscopy Metabolomic Profiling 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, and Andrea Motta²

¹Department of Pharmacology, Catholic University of the Sacred Heart, Rome, Italy; ²Institute of Biomolecular Chemistry, National Research Council, Pozzuoli (Naples), Italy; ³Department of Paediatrics, Cystic Fibrosis Unit, Ospedale Pediatrico Bambino Gesù, Rome, Italy; ⁴Department of Drug Sciences, Faculty of Pharmacy, University G. D’Annunzio, Chieti, Italy; ⁵Department of Pediatrics and ⁶Department of Clinical and Experimental Medicine, University Federico II of Naples, Italy; ⁷Department of Paediatric Respiratory Medicine and ⁸Airway Disease Section, Imperial College School of Medicine at the National Heart and Lung Institute, London, United Kingdom

Address correspondence to:
Paolo Montuschi, M.D.
Department of Pharmacology, Faculty of Medicine, Catholic University of the Sacred Heart, Largo Francesco Vito 1, I-00168 Rome, Italy; e-mail: pmontuschi@rm.unicatt.it

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% (R²=0.84; Q²=0.79). Model validation with a testing sample set obtained from 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% (R²=0.82; Q²=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 ≥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°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 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 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) 1H-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 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 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.[18] 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).

Table 1. Subject characteristics*

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>Stable CF patients</th>
<th>Unstable CF patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>31</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td><strong>Age, yr</strong></td>
<td>14 ± 0.7</td>
<td>15 ± 0.9</td>
<td>15 ± 0.7</td>
</tr>
<tr>
<td><strong>Sex, F/M</strong></td>
<td>15/16</td>
<td>14/15</td>
<td>11/13</td>
</tr>
<tr>
<td><strong>FEV1, % predicted</strong></td>
<td>110.4 ± 2.5</td>
<td>86.6 ± 2.9*</td>
<td>75.7 ± 3.4*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td><strong>FVC, % predicted</strong></td>
<td>113.0 ± 2.2</td>
<td>89.1 ± 3.1*</td>
<td>86.1 ± 4.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td><strong>BMI (kg/m^2)</strong></td>
<td>20.3 ± 0.5</td>
<td>20.1 ± 0.6</td>
<td>19.3 ± 0.7</td>
</tr>
<tr>
<td><strong>CRP (mg/L)</strong></td>
<td>-</td>
<td>0.3 ± 0.1</td>
<td>2.0 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>
| WBC (x 10^9/L) | - | 8.2 ± 0.4 | 10.6 ± 0.6\(^\dagger\)  
| | | | p < 0.004  
| Bacterial infection | | |  
| Ps. Aeruginosa | - | 16 | 15  
| S. Aureus | - | 7 | 6  
| S. Aureus + Ps. Aeruginosa | - | 3 | -  
| S. Maltophilia | - | 1 | -  
| S. Aureus + S. Maltophilia | - | - | 1  
| S. Aureus + Ps. Aeruginosa + S. Maltophilia | - | - | 1  
| B. cepacia | - | - | 1  
| CFTR genotype\(^\star\) | | |  
| ΔF508/ΔF508 | - | 5 | 7  
| ΔF508/other | - | 15 | 11  
| Other/other | - | 8 | 4  
| ICS (y/n) | - | 23/6 | 24/0  
| Short-acting \(\beta\)2-agonists (y/n) | - | 25/4 | 23/1  
| Inhaled Tobramycin (y/n) | - | 17/12 | 17/7  
| Recombinant human DNase (y/n) | - | 18/11 | 22/2  

BMI, body mass index; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CRP, C reactive protein; FEV\textsubscript{1}, 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.

\(^\dagger\)Compared with healthy subjects; \(^\ddagger\)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.

Acknowledgements

We thank Matteo Stocchero, S-IN Soluzioni Informatiche (Vicenza, Italy) for helpful discussions on the merits of PLS-DA.

Competing interests: The authors have no competing interests with this manuscript.

Funding: Catholic University of the Sacred Heart, Academic Funds 2009-2011, Italian National Research Council (CNR)
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 ($R^2 = 0.82$ 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 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) ($R^2 = 0.81$ and $Q^2 = 0.73$), and stable from unstable CF patients (B) ($R^2 = 0.80$ and $Q^2 = 0.65$), 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 $^1$H-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).