Online Supplement

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

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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 D_2O solution [containing 1 mM sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] 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 $\delta = 0.00$ ppm. Spectra were referred to the lactate doublet (βCH_3) resonating at 1.33 ppm for ^1H, and 20.76 ppm for ^{13}C.

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 (FEV_1) 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, Umea, 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 beclomethasone 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 (FEV_1), 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-C**H** of 2-propanol, while the signal at 3.58 ppm stems from the O-C$\mathbf{H}_2$ 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.

Subroup 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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Table E1. Validation subjects characteristics*

	Healthy subjects	Stable CF patients	Unstable CF patients
n	25	14	16
Age, yr	15 ± 0.8	14 ± 0.9	15 ± 0.7
Sex, F/M	12/13	7/7	8/8
FEV_1, % predicted	111.8 ± 2.7	107.1 ± 3.2[#] p < 0.001	54.2 ± 1.9[§] p < 0.014
FVC, % predicted	115.3 ± 3.5	109.0 ± 2.0[#] p < 0.017	70.4 ± 1.7[§] p < 0.013
BMI (kg/m^2)	20.9 ± 0.6	20.0 ± 0.4	19.0 ± 0.8
CRP (mg/L)	-	0.4 ± 0.1	2.8 ± 0.3[§] p < 0.0001
WBC ($\times 10^9$/L)	-	7.1 ± 0.6	11.7 ± 0.5[§] p < 0.004
Bacterial colonization			
Ps. Aeruginosa	-	1	10
S. Aureus	-	13	14
S. Aureus + Ps. Aeruginosa	-	1	8
S. Maltophilia	-	3	0
S. Aureus + S. Maltophilia	-	3	0
S. Aureus + Ps. Aeruginosa + S. Maltophilia	-	0	0
B. cepacia	-	0	0
CFTR genotype[^]			
ΔF508/ΔF508	-	6	4
ΔF508/other	-	2	6
other/other	-	5	4
ICS (y/n)	-	10/4	12/4
Short-acting β_2-agonists (y/n)	-	12/2	14/2
Inhaled tobramycin and/or colistin (y/n)	-	4/10	10/6
Recombinant human DNase	-	7/7	11/5

(y/n)

BMI, body mass index; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CRP, C reactive protein; FEV_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 $p < 0.05$.

[#]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*

	Healthy subjects (n = 31)	Stable cystic fibrosis patients (n = 29)	Unstable cystic fibrosis patients (n = 24)
EBC metabolite (μM)			
Acetate	19.70 ± 10.33	7.50 ± 4.73	10.62 ± 5.82
Acetone	8.00 ± 3.85	25.33 ± 16.25	12.00 ± 7.55
Ethanol	10.17 ± 6.14	22.76 ± 10.54	13.80 ± 7.52
Methanol	4.00 ± 1.85	2.67 ± 1.82	3.33 ± 1.80
2-Propanol	9.20 ± 4.83	15.17 ± 8.35	11.71 ± 5.91

*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 form 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.