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Volatile organic compounds in the exhaled breath of young patients with cystic fibrosis

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ABSTRACT: Inflammatory mediators in the exhaled breath are receiving growing medical interest as noninvasive disease markers. Volatile organic compounds have been investigated in this context, but clinical information and methodological standards are limited.

The levels of ethane, propane, *n*-pentane, methanol, ethanol, 2-propanol, acetone, isoprene, benzene, toluene, dimethyl sulphide (DMS) and limonene were measured in repeated breath samples from 20 cystic fibrosis patients and 20 healthy controls (aged 8–29 yrs). Three end-exhaled and one ambient air sample were collected per person and analysed on a customised gas chromatography system.

Intra-subject coefficients of variation ranged between 9 and 34%, and hydrocarbon breath levels were influenced by their inspired concentrations. The alveolar gradient for pentane was higher in cystic fibrosis patients than in healthy controls (0.36 *versus* 0.21 ppb) and inversely proportional to forced expiratory volume in one second; highest values were observed in patients with pulmonary exacerbations (0.73 *versus* 0.24 ppb). Cystic fibrosis patients also exhibited a lower output of DMS (3.9 *versus* 7.6 ppb). Group differences were not significant for ethane and the remaining substances.

It was concluded that chemical breath analysis for volatile organic compounds is feasible and may hold potential for the noninvasive diagnosis and follow-up of inflammatory processes in cystic fibrosis lung disease.

KEYWORDS: Biological markers, breath tests, cystic fibrosis, gas chromatography

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More than 30 yrs after PAULING *et al.* [1] described the abundance of volatile organic compounds (VOC) in human breath, regular automated measurement has only been realised for ethanol in the context of traffic-related toxicology. Other exhaled compounds have attracted attention in a medical context: Ethane and *n*-pentane were linked to the *in vivo* level of lipid peroxidation and oxidative stress [2–4], breath acetone was shown to correlate with the metabolic state of diabetic patients [5] or mice on a ketogenic diet [6], and a decrease in exhaled isoprene was reported shortly after ozone exposure [7] and in acute pulmonary exacerbations of cystic fibrosis (CF) [8]. Yet, despite a growing scientific focus on nitric oxide and other exhaled biomarkers of pulmonary disease [9, 10], data on breath VOC remain scarce and important methodological questions unanswered, such as the standardised collection, handling and analysis of human breath samples. Gas chromatography as the most appropriate method can be affected by the high water and carbon dioxide content of breath samples, and no standard has been established regarding the choice of pre-concentration procedures, temperature programmes, column and detector [11]. This has impeded the development of biochemical breath analysis as a clinical tool.

Lung disease in CF is characterised by chronic airway inflammation, retention of viscous secretions, bronchiectasis and, often, bacterial infection [12, 13]. These factors contribute to a variable clinical course with progressive bronchial obstruction and hyperinflation. Scoring systems based on the level of symptoms and findings from radiography, microbiology and pulmonary function tests may be helpful in a research setting and for defining a pulmonary exacerbation. Bronchoscopic lavage or biopsy can be applied for the objective assessment of inflammatory processes, but the procedural risks are often not justified. Hence, most treatment decisions remain based on clinical judgement and secondary parameters derived from pulmonary function testing, chest radiography or blood analysis [14].

It was hypothesised that exhaled VOC can be accurately and reproducibly determined in breath samples from CF patients and matched healthy controls, and that the pulmonary exchange rate of

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these potential biochemical markers is related to other descriptors of CF lung disease.

METHODS

Subjects

Fifteen patients were recruited in a stable state during scheduled outpatient visits to Aachen CF centre, and five hospitalised patients were included during intravenous antibiotic treatment for pulmonary exacerbations. Details on their medical history, current status and environmental tobacco smoke exposure were gathered from chart records and a structured interview. Long-term medication was unchanged over the previous month and included inhaled antibiotics in five, DNase in four and inhaled steroids in seven patients; six subjects received insulin due to CF-related diabetes.

A total of 20 healthy subjects free from chronic lung disease or respiratory tract infection in the preceding month served as control group. All subjects were nonsmokers, and distribution of age (8–29 yrs), sex (11 females in each group) and height were comparable between groups. However, CF patients had significantly lower body mass index Z-scores (-0.8 *versus* +0.3 [15]). Spirometry was normal in all control subjects (median forced expiratory volume in one second (FEV_1) 108% predicted (% pred), range 85–138), but revealed a varying degree of bronchial obstruction in the CF group (median FEV_1 65%, range 21–105).

All test persons (and legal guardians, if applicable) gave their written informed consent, and the study protocol had been approved by the local ethics committee.

Breath sampling

Prior to the investigation, subjects were asked to refrain from eating and strenuous physical activity for at least 3 h. After 10 min of rest in the study room, they were instructed to deliver their exhaled breath through a steel mouthpiece into 6-L canisters of electropolished stainless steel with a fused-silica inner lining (SilcoSteel®, Supelco Co., Bellefonte, PA, USA). These had been pre-conditioned by four-fold evacuation to <500 Pa and subsequent pressurisation with pure synthetic air to 300 kPa; followed by a final evacuation. Residual VOC content was assessed by gas chromatography, and canisters were only released if the integrated peak areas of all remaining signals corresponded to a total VOC volume mixing ratio <20 ppt.

The target breathing manoeuvre consisted of a deep inspiration and a 5-s breath-hold, followed by slow and complete exhalation over 10 s. Breath air was discarded during the first 2 s of exhalation and then directed into the canister by a magnetic valve. This was repeated for >1–3 breaths, typically producing final pressures of 30–40 kPa in the canister. Three breath samples were collected per session at 15-min intervals together with one sample of room air. All measurements were performed in a windowless conference facility inside Aachen University Medical Center, controlled by a central ventilation system and without disinfectant dispensers or frequent person traffic. Gas samples were transported to Research Center Jülich within 24 h of collection for the determination of VOC content.

Analytical procedure

After measuring their initial gas pressure, canisters were pressurised to 300 kPa with pure synthetic air (quality 5.0=99.999%) in order to further reduce the relative humidity of the sample. Gas specimens were then analysed using a gas chromatograph (HP 6890; Hewlett Packard Co., Palo Alto, CA, USA) and a specially designed sampling manifold, as previously described [16] and illustrated in figure 1. Trace species from a sample volume of 800 cm^3 were pre-concentrated at a flow rate of 80 $mL \cdot min^{-1}$ on a sample loop (20 cm length, internal diameter (ID) 2 mm) packed with glass beads of 0.25 mm diameter at liquid nitrogen temperature. Subsequently, the sample was thermally desorbed at 120°C and injected on a capillary column (DB-1, 120 m × 0.32 mm ID, 3 μm film thickness). After injection, the column was kept isothermal at -60°C for 5 min, then heated to 200°C at a rate of $5°C \cdot min^{-1}$ and finally maintained at 200°C for 15 min. Signals were gathered from a flame ionisation detector, which received 98% of the column output through a split valve. The remaining

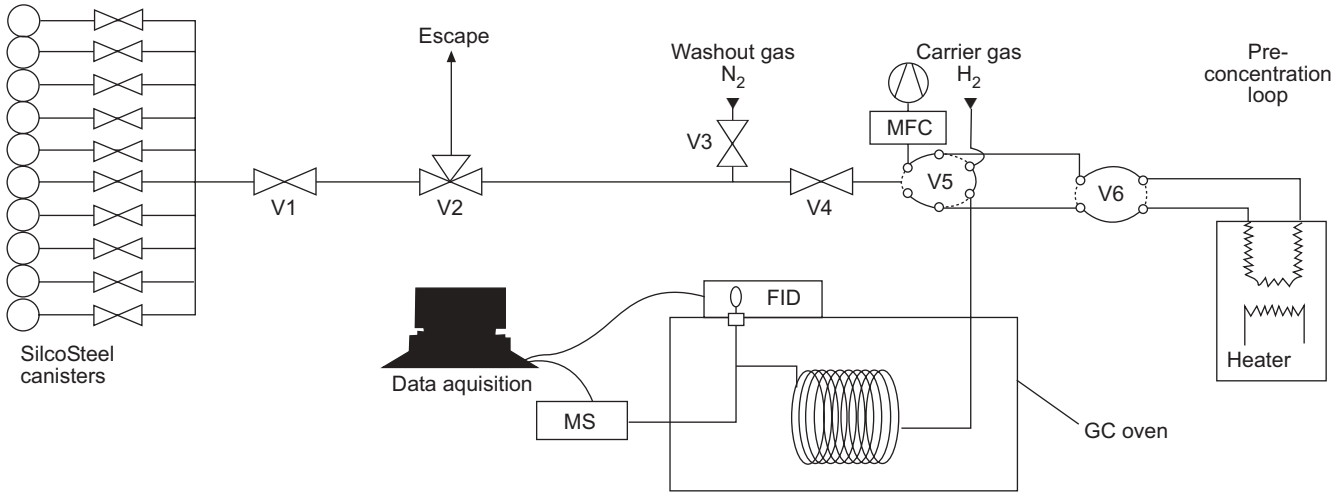

FIGURE 1. Experimental set-up of the gas chromatography (GC) system with canister manifold, pneumatic valves (V 1–6), mass flow controller (MFC), pre-concentration loop, capillary column, oven, flame ionisation detector (FID) and mass spectrometer (MS). A set of 10 individual 6-L canisters can be analysed in unattended operation.

flow was directed to an ion trap mass spectrometer (Saturn 9000; Varian Inc., Palo Alto, CA, USA) used for the identification of specific peaks. In the order of elution from the column, signals for ethane, propane, methanol, ethanol, *n*-pentane, acetone, 2-propanol, isoprene, dimethyl sulphide (DMS), benzene, toluene and limonene were identified and the corresponding peak areas were determined by semi-automated integration. All readings were quality controlled by the same experienced investigator (B. Mittermaier); figure 2 shows a typical breath chromatogram. Analysis of one sample lasted for about 90 min, and sets of 10 canisters could be analysed on the system in unattended operation.

VOC volume mixing ratios were finally calculated from the product of their peak area and the respective mass response factor. These factors were derived from analysis of a 74-compound high-precision mixture containing alkanes, aromatics, terpenes, aldehydes and ketones at known mixing ratios between 1 and 5 ppb (Cylinder CC169190; Apel-Riemer Environmental Inc., Denver, CO, USA). Calibration measurements were performed every 1–2 weeks and showed extremely stable retention times and mass response factors with negligible variation and no drift across the study period.

Experimental validation

Before engaging in the clinical protocol, the potential impact of humidity in the exhaled breath on the system's analytical performance was studied. To this end, 50 μL of ultrapure water was added to 50 kPa of a calibration gas sample; the pairs of humidified and dry standard mixtures were then stored for 1 day and then measured on the gas chromatography system as described above. The resulting chromatograms (fig. 3) showed identical retention times; it should be noted that the few peaks with apparent reduction in the humidified mixture belong to substances that were not analysed in the exhaled samples. Furthermore, only minimal variation in peak amplitude was observed for the compounds under investigation (as shown in fig. 4). Slightly higher relative errors for methanol may be attributed to its low relative content in the standard mixture (5 ppb or ~3% of exhaled concentrations).

Error estimation

Experimental uncertainties can be introduced by a number of factors and are an important consideration. The impact of canister transport and storage was assessed by analysing the VOC content in parallel samples of either dry standard gas

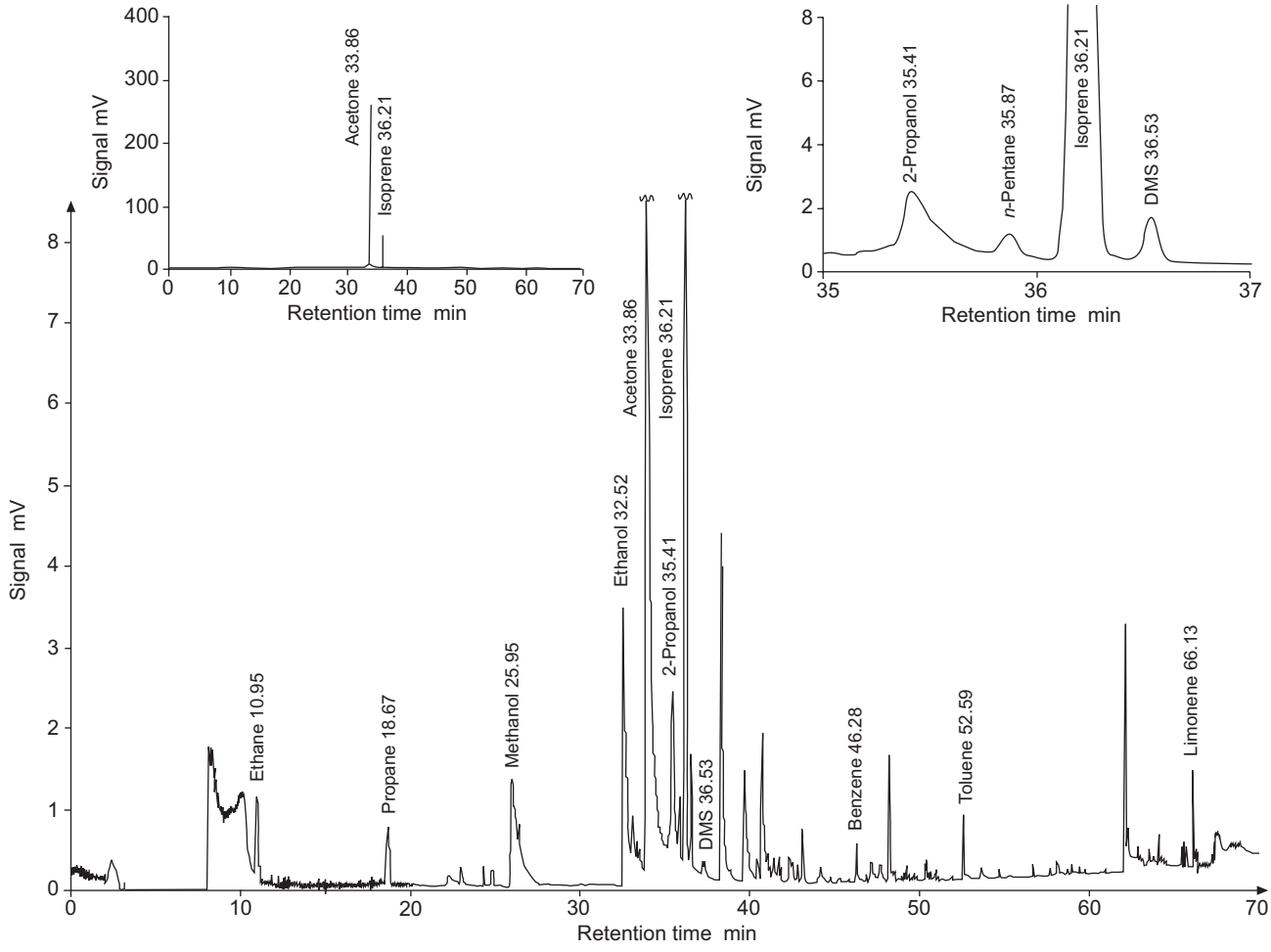

FIGURE 2. Original chromatogram of a breath sample; investigated compounds are labelled with names and retention times above the respective peaks. The left insertion shows the complete chromatogram demonstrating that acetone and isoprene are the dominating compounds in exhaled breath air, the right insertion shows a blow-up of the region adjacent to the isoprene peak.

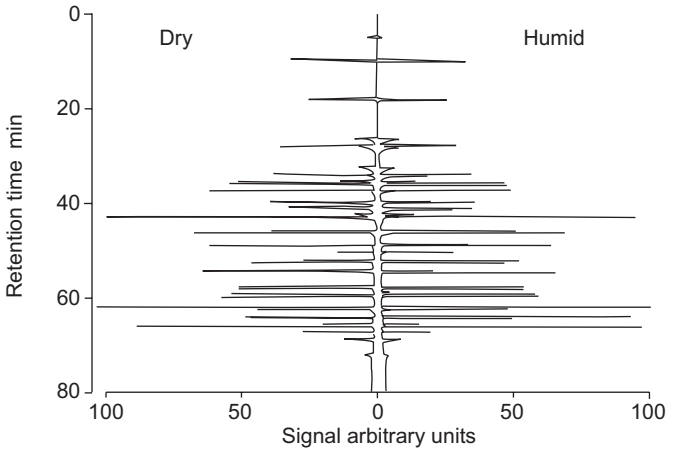

FIGURE 3. Chromatograms obtained from dry (left-hand side) and humidified (right-hand side) samples of calibration gas.

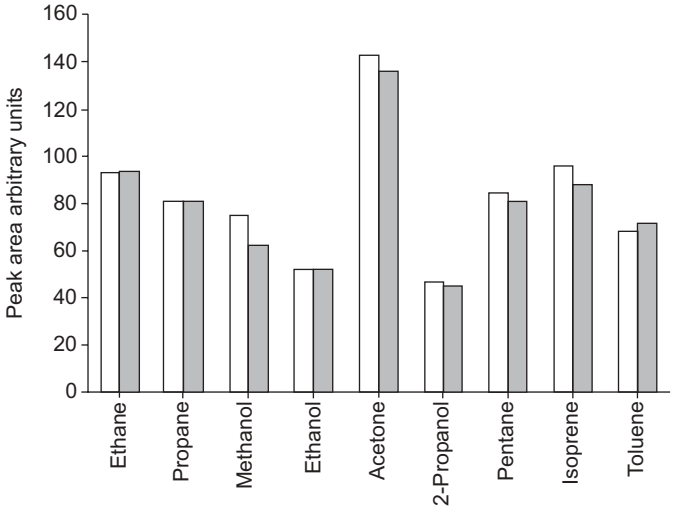

FIGURE 4. Differences in peak areas for specific volatile organic compounds (normalised to the peak area of propane, which is not affected by water) between dry (□) and humidified standard gas mixtures (■).

mixtures, ambient or exhaled air after 0, 1, 2 and 3 days. Results from a total of 30 measurements consistently showed that the mixing ratios of non-methane hydrocarbons, aromatic compounds, DMS and terpenes were stable within 5% over 2 days compared with an instantaneously analysed sample. Alcohol concentrations differed by up to 10% in breath samples.

In addition, measurement accuracy depends on the quality of the calibration standard ($\leqslant$5% between true and declared gas concentrations, Apel-Riemer Environmental Inc.) and of the mass flow controller used for diluting the calibration gas ($\leqslant$2% deviation, MKS Instruments, Wilmington, MA, USA). Analytical accuracy is affected by uncertainties of the calibration function and the graphical analysis. The linear regression calculated across a number of dilution steps of the standard mixture carries a relative uncertainty of <5% for the individual species. Uncertainties of the peak areas are caused by their reproducibility (known to lie $\leqslant$4%), and by an integration uncertainty estimated at <6%. The integration uncertainty is highest at low concentration levels. Geometric addition of all these factors yielded an overall experimental uncertainty of 12%.

Statistics

Reproducibility of breath measurements was assessed by calculating intra-subject coefficients of variation across the three successive samples. Group comparison was performed by Mann–Whitney U-test, and associations between parameters were investigated by linear regression analysis. A p-value <0.05 was considered statistically significant.

RESULTS

Breath sampling proved to be feasible in all subjects without discomfort or adverse events. Due to chromatographic interference, exhaled concentrations were unavailable for 2-propanol in four samples and limonene in one sample. DMS could not be detected in all but one ambient probe. On all other occasions, discrete volume mixing ratios were determined for the compounds specified above. Their short-term reproducibility was generally good, and average intra-subject coefficients of variance across the three consecutive measurements ranged from 9% for acetone to 34% for ethanol (table 1). A significant and linear influence of ambient conditions on exhaled concentrations was demonstrated for ethane ($r=0.63$), propane ($r=0.72$), pentane ($r=0.6$) and methanol ($r=0.59$, all with $p<0.001$). Therefore, subsequent analyses were based on the alveolar gradient of a specific compound: $\Delta[VOCi]=[VOCi]_{breath}-[VOCi]_{room}$, where [VOCi] is the mixing ratio of a given VOC in the respective sample type. These differences are equivalent to the endogenous rate of production or absorption of a specific compound per unit ventilation rate, assuming a steady state equilibrium between body tissues, lungs and expired gas. Values for exhaled, ambient and Δ[VOCi] are given in table 2.

TABLE 1 Short-term variability of exhaled volatile organic compound (VOC) in cystic fibrosis patients (CF) and healthy controls. Both groups as expressed by mean, and minimal and maximal intra-subject coefficients of variation

VOC	CF	Controls	All	Range
Ethane	16	8	12	0–38
Propane	11	13	12	1–50
Pentane	22	19	20	1–66
Methanol	14	11	13	1–77
Ethanol	38	29	34	4–94
2-Propanol	25	22	23	3–64
Acetone	9	8	9	1–41
Isoprene	21	22	21	3–77
Benzene	28	12	20	3–68
Toluene	23	12	18	2–66
DMS	14	14	14	0–60
Limonene	34	27	30	2–116

Date are presented as %. DMS: dimethyl sulphide.

TABLE 2 Mean exhaled and ambient volatile organic compound (VOC) concentrations (in ppb) with their mean difference (Δ) and 95% confidence intervals (CI) in cystic fibrosis patients (CF) and healthy controls

VOC	CF				Controls			
	Breath	Room	Δ	CI	Breath	Room	Δ	CI
Ethane	2.76	2.38	0.39	-0.04–0.82	2.47	2.37	0.10	-0.25–0.44
Propane	1.95	1.42	0.53	0.31–0.75	1.95	1.38	0.58	0.08–1.08
Pentane	0.55	0.19	0.36	0.24–0.48	0.43	0.22	0.21	0.13–0.29*
Methanol	200	7.30	193	125–261	272	7.88	265	198–331
Ethanol	157	13.3	144	-15–302	195	30.0	165	87–243
2-Propanol	6.99	21.9	-14.9	-22.8– -6.94	9.96	15.7	-5.76	-11.7–0.16#
Acetone	402	2.39	400	322–478	469	1.74	467	383–551
Isoprene	106	0.80	105	83–127	115	0.45	114	88–140
Benzene	0.16	0.19	-0.04	-0.13–0.05	0.13	0.21	-0.08	-0.15– -0.01
Toluene	0.43	0.68	-0.25	-0.53–0.03	0.29	0.80	-0.51	-0.82– -0.20
DMS	3.89	0.00	3.89	2.24–5.54	7.58	0.00	7.58	5.73–9.43#
Limonene	2.42	0.14	2.28	0.53–4.02	2.30	0.09	2.21	0.83–3.59

For comparison of Δ between groups: *: $p<0.05$; and #: $p<0.005$. DMS: dimethyl sulphide.

Group comparison showed a significantly higher pentane output ($p=0.04$), lower DMS production ($p=0.001$) and higher 2-propanol uptake ($p=0.003$) in CF patients *versus* healthy controls. No significant differences were observed for ethane, propane, methanol, ethanol, acetone, isoprene, benzene, toluene or limonene (table 2). Among CF patients only, correlation with FEV_1 (expressed as % pred) was demonstrated for breath toluene ($r=0.72$, $p<0.005$) and Δ[pentane] ($r= -0.62$ and $p<0.005$; fig. 5). Comparison between CF subjects on *i.v.* antibiotics and stable outpatients revealed significantly higher values for Δ[pentane] in the former group (0.73 *versus* 0.26 ppb, $p=0.007$). Furthermore, Δ[pentane] was higher in patients with malnutrition ($r= -0.46$, $p=0.04$ for linear regression to body mass index Z-score) or chronic Pseudomonas infection ($n=16$, $p<0.001$). The breath of diabetic patients contained more isoprene (141 *versus* 89 ppb, $p=0.003$) and less DMS (1.43 *versus* 4.95 ppb, $p=0.04$) and methanol (109 *versus* 229 ppb, $p=0.04$) than CF subjects with normal glucose tolerance. Inhaled antibiotic treatment was associated with lower Δ[ethane] (-0.49 *versus* 0.68 ppb, $p=0.001$), and patients with domestic tobacco-smoke exposure ($n=9$) exhibited higher values for Δ[methanol] (281 *versus* 120 ppb, $p<0.01$) and exhaled or Δ[DMS] (6.1 *versus* 2.1 ppb, $p<0.01$). Exhaled VOC were not significantly associated with atopic status, treatment with inhaled steroids and DNase, or with CF genotype (stratified according to the number of delF508 alleles).

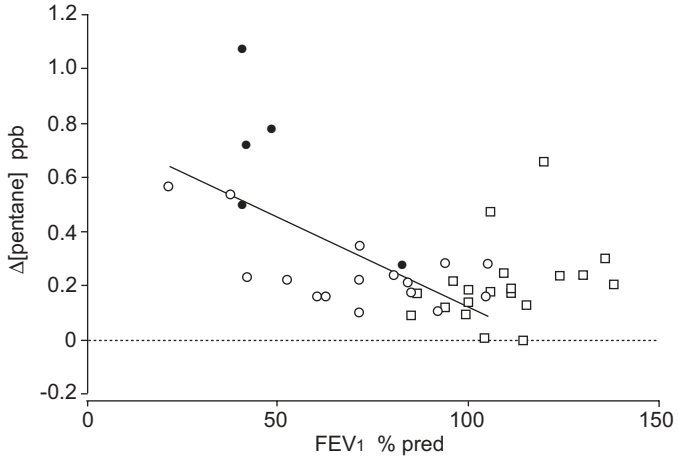

FIGURE 5. Relation between exhaled pentane output and forced expiratory volume on one second (FEV_1) in healthy controls (□), stable cystic fibrosis (CF) patients (○) and CF patients during exacerbation (●), including regression line for the combined CF group ($r= -0.62$, $p=0.004$).

DISCUSSION

In this study, ambient and exhaled breath concentrations of 12 volatile trace gases were measured in children, adolescents and young adults who were either healthy or affected by chronic airway inflammation due to CF. Based on the assumption that different breathing patterns might affect exhaled parameters, as recently demonstrated by COPE *et al.* [17], a standardised breathing manoeuvre was developed to define the degree of inspiratory and expiratory gas mixing and to ensure an air sample of alveolar origin.

Exhaled gas was delivered into chemically inert containers through a custom-built device. Sampling and analytical technology with a single column-two detector gas chromatography system were adapted from atmospheric chemistry [16]; they had not previously been applied to human breath measurements. Partial canister filling and subsequent dilution contributed to a lower water content of the breath sample that was finally transferred to the gas chromatograph. Since this is a critical point in the analysis of exhaled air, the effects of humidity had been thoroughly assessed beforehand. Interferences might be expected at three different levels: 1) condensation on the canister surface with adsorption of compounds, 2) blockage of the pre-concentration loop by ice, and 3) alteration of the chromatographic signal. The validation experiments carried out gave no evidence for any of these problems, but

demonstrated high storage stability and no relevant water effect on retention times and peak amplitudes. Therefore, the present authors are confident that the employed method of gas collection, separation and VOC detection is robust and equally applicable to dry gas standards, partly humid ambient air and water-saturated human breath samples.

Analytical performance for exhaled VOC then proved to be very good, with lower limits of detection in the range of 10–20 ppt. Ambient levels were not negligible for a number of compounds, therefore the present authors chose to determine the pulmonary exchange rate of a specific gas from the difference between inspired and expired concentration. The results showed substantial inter-subject variability within both groups, with significant differences for pentane, DMS and 2-propanol. Pentane and toluene were linearly correlated to lung function in CF patients only, and ethane levels did not discriminate CF patients from healthy controls.

Inflammatory processes have been recognised as central in the pathophysiology of many chronic lung diseases, and numerous research groups are committed to the development of sensitive markers and methods. Apparent advantages of biochemical breath analysis are its intrinsically noninvasive nature, as well as the close physiological proximity between target tissue and analytical substrate. In the context of CF, noninvasive markers of pulmonary inflammation could contribute to an individual tailoring of treatment through monitoring of oxidative stress and disease progression [14, 18]. Exhaled pentane has been proposed as such a marker for airway pathology [10], and available evidence suggests that peroxidation of polyunsaturated fatty acids in the liver is its major source. Increased pentane concentrations were reported in the breath of patients with acute asthma [19], pulmonary infections [20] and after acute cigarette smoke exposure [21]. A single observation in adult CF patients has previously been reported in abstract form [22] and as letter to the editor [23], apparently without a subsequent original contribution. The authors collected tidal air over 2 min after a 10-min washout and found substantially higher breath pentane output in CF patients compared with healthy controls, but details on patient characteristics, analytical methods and results are not available. The present investigation demonstrated that Δ[pentane] was significantly higher in patients than in healthy controls and inversely correlated with lung function and nutritional status. Moreover, even higher pentane gradients were detected during pulmonary exacerbations and in the presence of chronic Pseudomonas infection, which is a well-known stimulant of neutrophilic airway inflammation. All of these findings support the hypothesis that exhaled pentane reflects *in vivo* oxidative processes in CF patients taking part in the present study. For individual subjects, however, there was wide scatter (fig. 5), with substantial overlap between results from CF patients and controls. So far, the long-term variability and discriminative power of pentane in specific clinical situations (such as the diagnosis of a pulmonary exacerbation) have not yet been assessed. As proposed by SPRINGFIELD and LEVITT [24], chromatographic analysis should be performed with diligence and ambient sources taken into account.

Ethane is another major product of lipid peroxidation, and increased breath levels have been reported in patients with asthma or CF [25, 26]. This finding was not reproduced in the present study; this may be due to different methodology and to the correction for ambient ethane, which is not mentioned by PAREDI *et al.* [26] in their study of exhaled ethane in CF. However, inhaled antibiotic treatment was associated with lower ethane output in patients in the present study, raising the possibility of an additional source from bacterial production. Similarly, propane was emitted at comparable levels in patients and controls. This C_3-hydrocarbon is probably derived mainly from protein degradation and faecal flora and is of doubtful use as a marker for lipid peroxidation [10].

Decreased pulmonary output of isoprene has been described in acute exacerbations of CF, with a return to normal values after treatment [9]. Again, this finding was not reproducible in the present cross-sectional study. Exhaled isoprene did not differ between stable and exacerbated CF patients and between CF patients and healthy controls. The implication of higher isoprene levels in diabetic patients remains unclear; a previous investigation failed to show a difference between schoolchildren with type I diabetes and healthy peers [27].

Volatile sulphur compounds in the breath have previously been assessed in the context of oral malodour [28], occupational exposure [29], congenital enzyme deficiency [30] and liver cirrhosis [31]. While elevation of DMS was found in all of these conditions, a markedly decreased breath output was measured in the CF population. Although progressive cholestasis and hepatic fibrosis occur regularly in the course of CF, none of the present study patients had severe liver involvement. Furthermore, exhaled DMS levels were significantly higher in patients with environmental tobacco smoke exposure and lower in those on insulin or inhaled antibiotics. Due to the complexity of biochemical pathways and the potential number of unknown confounding factors, these interesting results remain descriptive at present.

Limonene has been described as elevated in the breath of patients with liver disease [32]. Monoterpenes have been identified as potential antioxidants with a primarily nutritional source, although their exact role in the human metabolism is unclear. Limonene was detected at mixing ratios between 0.1 and 15 ppb in the exhaled breath of the present study's CF patients and healthy controls without a systematic group difference.

Among the remaining compounds, 2-propanol was actually taken up from the ambient air in significantly higher quantities by CF patients than controls. Ethanol release exhibited the highest variability, while methanol output was higher in the presence of tobacco smoke exposure and lower in chronically Pseudomonas infected patients. However, no differences between CF patients and healthy controls were observed for these alcohols. Likewise, a net uptake was described for the aromatic compounds benzene and toluene without a significant group difference. Acetone proved to be the most abundant VOC in human breath (fig. 2) with high repeatability, but was not associated with diabetes and again not discriminating between CF patients and healthy controls.

In conclusion, young patients with CF showed a higher pentane and lower DMS output in their breath compared with normal controls. Their alveolar gradient of *n*-pentane was related to pulmonary function, nutritional status,

Pseudomonas infection and the presence of a pulmonary exacerbation. Additional evidence on the prognostic value of exhaled pentane as an inflammatory marker might be gathered by serial measurements in a patient cohort before and after antibiotic treatment of exacerbations. This was beyond the scope of the present study, but should be addressed in the near future. Group comparison yielded no differences for ethane, isoprene, limonene and several other VOC.

The present authors admit that some of these observations result in more new questions than answers. Long-term variability of breath volatile organic compounds has not been assessed in either healthy people or patients with respiratory disease. Today's equipment and procedures for sampling and analysis are fairly complex and unstandardised, not to mention the lack of established reference values. Therefore, with knowledge currently available, it is still of ambiguous practical value to determine the concentration of a specific volatile organic compounds in the exhaled breath of a patient. Nevertheless, the present investigation may add strength to the evolving concept that chemical breath analysis has the potential to open a new diagnostic window on important aspects of pulmonary physiology and pathology in cystic fibrosis and other chronic inflammatory conditions.

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