Detection of obstructive sleep apnoea by an electronic nose

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ABSTRACT Diagnosis of obstructive sleep apnoea syndrome (OSAS) is technically demanding, cost-intensive and time-consuming. The measurement of volatile organic compounds by an electronic nose is an innovative method that determines distinct molecular patterns of exhaled breath in different patient groups. We addressed the following questions: What is the diagnostic accuracy of an electronic nose in the detection of OSAS and the ability to detect effects of standard therapy in patients with OSAS? Are these results related to changes in distinct markers of airway inflammation and extracellular remodelling?

We included 40 OSAS patients and 20 healthy controls. Exhaled breath of all participants was analysed using the Cyranose 320 electronic nose. Pharyngeal washings were performed to sample the upper airway compartment. For statistical analysis linear discriminant analysis was employed.

We identified a linear discriminant function separating OSAS from control ($p<0.0001$). The corresponding area under the receiver-operating curve was 0.85 (95% CI 0.75–0.96; sensitivity 0.93 and specificity 0.7). In pharyngeal washing fluids of OSAS patients, we observed higher levels of α_1-antitrypsin and markers of extracellular remodelling compared to controls.

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Introduction

Obstructive sleep apnoea syndrome (OSAS) is a common disease [1] associated with an increased risk for cardiovascular disorders [2, 3]. The current gold standard to confirm OSAS is multichannel polysomnography (PSG) [4]. This is technically demanding, time-consuming, and labour- and cost-intensive with limited availability.

Different screening tools have been developed to reduce the number of patients requiring PSG but most of them lack sensitivity and/or specificity. Clinical parameters such as the Epworth Sleepiness Scale, neck circumference and a composite clinical score show a large overlap between healthy controls and OSAS patients [5–7].

Technical tools sensing a change of body position as a surrogate for respiratory movements [8], sound analysis [9] and complex computerised analyses of ECG recordings [10] have been evaluated with different success rates. A novel screening tool predicting OSAS with acceptable accuracy and without overnight measurements could improve the OSAS screening algorithm.

Several studies have revealed that OSAS is associated with increased oxidative stress [11], as well as systemic and local inflammation [12], as indicated by increased concentrations of pro-inflammatory cytokines and other markers in the exhaled breath condensate (EBC) and serum [11]. The serum level of α_1-antitrypsin (α_1-AT), an acute phase reactant playing a major role in the control of inflammation [13], also seems to be elevated [14]. Other compounds such as matrix metalloproteases (MMPs) and tissue inhibitor of matrix metalloproteases (TIMPs) might also be involved in the disease process as mediators of the ongoing airway remodelling [15].

Exhaled breath contains hundreds of volatile organic compounds (VOCs), as demonstrated by mass spectrometry [16], and its analysis can provide information about systemic or local inflammation. Instead of identifying single compounds, the assessment of exhaled breath can also be performed by devices enabling the recognition of patterns of VOCs [17]. Indeed, such devices can distinguish between a number of diseases *via* their VOC profiles [18–20]. A recent review article summarising potential medical applications has been published elsewhere [21]. To our knowledge, VOC profiles have not been assessed in patients with OSAS.

Our hypothesis was that a pattern-recognising electronic nose is capable of distinguishing between OSAS patients and healthy controls. In addition, we assumed that therapy with continuous positive airway pressure (CPAP) has a detectable effect on airway inflammation and consequently the VOC profile. To substantiate the data by direct measurement of biochemical compounds, we assessed whether markers of inflammation and airway remodelling differ between healthy controls and OSAS before and/or after CPAP treatment. For this purpose we selected pH and conductivity of EBC, and MMP-9, TIMP-1 and α_1-AT in pharyngeal washing fluids.

Methods

Subjects and study design

20 healthy volunteers were recruited from the hospital staff and 40 OSAS patients from a sleep apnoea outpatient clinic before receiving CPAP therapy. Inclusion criteria for healthy controls were the ability and willingness to participate. Volunteers with any known chronic disease or any acute disease in the last 4 weeks before study entry or any medication taken on a regular basis were excluded. OSAS was defined as an apopnoea/hypopnoea index (AHI) >5 events·h^{-1} in combination with clinical signs of obstructive sleep apnoea. We excluded patients with any other chronic and/or acute respiratory disease of the upper and/or lower airways (*e.g.* asthma and chronic obstructive pulmonary disease (COPD)). These diseases were assessed by a questionnaire (patient-reported). In any case of doubt, lung function testing was performed. The following comorbid conditions were documented systematically: coronary heart disease, diabetes and arterial hypertension. Disease-specific medical therapy was allowed and left unaltered in the course of the study. The study was approved by the local ethics committee (Marburg Ethics Committee AZ 59/06 Amendment 3; Marburg, Germany) and written informed consent was obtained from each subject.

All participants had sleep studies and answered a questionnaire regarding symptoms, smoking habits, health status, medication and medical history. In addition, the following examinations were performed: collection of pharyngeal washing fluid, collection of EBC, measurement of exhaled breath with the Cyranose 320 electronic nose (Smiths Detection Group Ltd, Watford, UK). The first 20 of the 40 OSAS patients were examined again after 3 months of CPAP therapy.

Polysomnography/polygraphy

Patients with suspected OSAS underwent an overnight PSG (Embla N7000; TNI Medical AG, Bad Ems, Germany). Electroencephalogram, electrooculogram and electromyogram were measured using established

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procedures. Furthermore, thoracic and abdominal respiratory excursions, breath sounds, nasal airflow, ECG and oxygen saturation were recorded. In healthy controls, home polygraphy (SOMNOcheck2 R? Weinmann, Hamburg, Germany) was used to exclude OSAS. An AHI <5 events·h^{-1} was defined as the absence of OSAS.

Pharyngeal washing fluid

All participants had to have been fasting for at least 2 h, including no chewing gum or any kind of candy, and no tobacco smoking. They washed out their mouth with water before rinsing the throat with 25 mL of water by gargling. The fluid was stored at -80°C for ELISA-based analysis of TIMP-1 and MMP-9 (R&D Systems, Minneapolis, MN, USA), which were conducted according to the manufacturer's suggested routine procedure. α_1-AT was measured by ELISA as described previously [22]. The sensitivity of the ELISAs for TIMP-1, MMP-9 and α_1-AT was 30 pg·mL^{-1}, 30 pg·mL^{-1} and 40 pg·mL^{-1}, respectively.

EBC

EBC was collected by tidal breathing over 15 min using the ECoScreen Turbo (VIASYS; CareFusion Germany 234 GmbH, Höchberg, Germany) as described [23].

Electronic nose

The exhaled breath was assessed with the Cyranose 320 electronic nose. The participants breathed medicinal air (Aer medicinalis Linde; Linde Gas Therapeutics GmbH, Unterschleiβheim, Germany) and exhaled for 10 s at a flow rate of 100–200 mL·s^{-1} into a disposable collection bag, which then was assessed within 60 s. This medicinal air was also used as reference air for the 60-s baseline, followed by a 60-s sample draw from the collection bag, and completed by a 60-s purging of the electronic nose. These measurements were performed in triplicate [20].

Data analysis

The three data sets obtained by the electronic nose were averaged by taking their arithmetic mean for each individual. Principal component analysis was performed on these data. The resulting transformed data were fed into a linear discriminant analysis. The linear discriminant analysis results were then used for further analyses, including nonparametric statistical significance tests. The Mahalanobis distance between the groups was determined and a leave-one-out cross-validation of the data was performed to calculate the cross-validation value as described previously [24]. Additionally, a receiver operating characteristic (ROC) curve using the linear discriminant as a discriminative variable was constructed to determine the area under the curve (AUC). The values for sensitivity and specificity were derived from linear discriminant analysis "self-prediction" [25], meaning that the complete data set was used to calculate the values. To support this analysis we additionally performed a split-half analysis using the first 20 patients as the training set and the second half as the test set (and *vice versa*). Sensitivity and specificity were reported at the specific cut-off level, where the sum of the sensitivity and the specificity (Youden score) was highest [26].

Prior to the statistical comparisons, the data were checked for normal distribution by the Kolmogorov–Smirnov test. For data not normally distributed, nonparametric tests were used (Mann–Whitney U-test for unpaired and Wilcoxon matched-pairs signed-rank test for paired data), otherwise a t-test (unpaired or paired). To determine the correlation between the linear discriminant analysis and parameters of inflammation, the Spearman's rank correlation coefficient was calculated. Data are presented as mean $\pm$ SD unless stated otherwise. The software GraphPad Prism 5.00 (GraphPad Inc., San Diego, CA, USA) and SPSS Version 20 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Patients versus controls

Baseline characteristics of the 40 OSAS patients and 20 healthy volunteers are shown in table 1.

The linear discriminant analysis scores of OSAS patients and healthy controls differed statistically significantly from each other ($p<0.0001$; Mann–Whitney U-test) (fig. 1a). The Mahalanobis distance between the two groups was 1.88 and the cross-validation value 79.5%. When using a split-half analysis, 80% of the "second half" was correctly predicted to have OSAS. Conversely, when predicting the "first half" after having used the second half as training set, 85% were correctly predicted to suffer from OSAS. The corresponding area under the ROC curve was 0.85 (95% CI 0.745–0.960) (fig. 1b), indicating a sensitivity of 0.93 and a specificity of 0.70. Furthermore, the linear discriminant analysis was significantly correlated with the AHI (Spearman's $r=0.58$, $p<0.001$), indicating a "dose–response" relationship. There was no significant correlation between the linear discriminant analysis and the other measured markers (EBC pH, EBC conductivity and inflammatory markers in pharyngeal washings).

TABLE 1 Baseline characteristics of obstructive sleep apnoea syndrome (OSAS) patients and healthy controls

	OSAS patients	Healthy controls	p-value
Female/male n	3/37	8/12	<0.001[#]
Age years	55±10	40±8	<0.001[¶]
Height cm	175±7	175±8	0.888
Weight kg	99±15	78±14	<0.001[¶]
BMI kg·m^{-2}	32.00±4.5	25.4±4.1	<0.001[¶]
AHI events·h^{-1}	33.65±22.00	2.7±1.7	<0.001[¶]
AI events·h^{-1}	11.9±16.7	0.6±1.1	<0.001[+]
HI events·h^{-1}	22.6±19.5	2.1±1.5	<0.001[¶]
So_2 %	92.8±2.7	95.5±1.1	0.01[¶]

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index, AHI: apnoea/hypopnoea index; AI: apnoea index; HI: hypopnoea index; So_2: oxygen saturation. [#]: Fisher's exact test; [¶]: t-test; [+]: Mann–Whitney U-test.

EBC pH values (8.16±0.47 in OSAS *versus* 8.09±0.40 in healthy controls; p=0.27; t-test) and EBC conductivity (175.5±292.9 µS·cm^{-1} in OSAS *versus* 93±33.36 µS·cm^{-1} in healthy controls; p=0.17; t-test) of both groups did not differ significantly (fig. 2).

The α_1-AT concentrations in pharyngeal washing fluids were significantly higher in OSAS patients compared with healthy controls (60.6±52.0 µg·mL^{-1} *versus* 25.3±21.7 µg·mL^{-1}; p=0.007; t-test) (fig. 3a). In contrast,

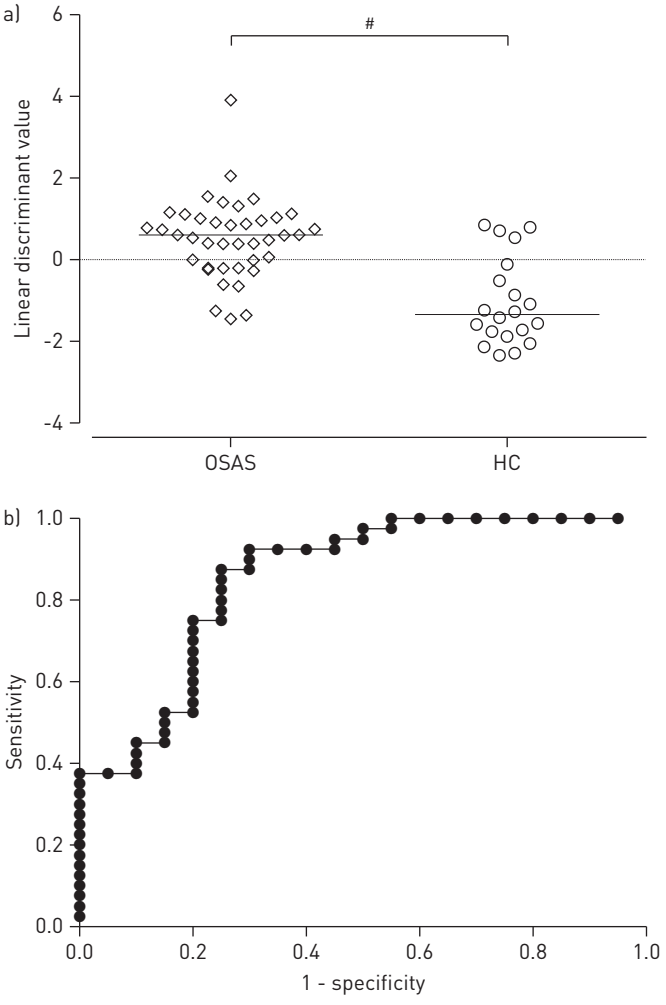

FIGURE 1 a) Linear discriminant analysis of sleep apnoea patients and healthy controls differ statistically significantly. b) The area under the receiver operating characteristic curve equals 0.85, resulting in a sensitivity of 0.93 and a specificity of 0.70. OSAS: obstructive sleep apnoea syndrome; HC: healthy control. [#]: p<0.0001, Mann–Whitney U-test.

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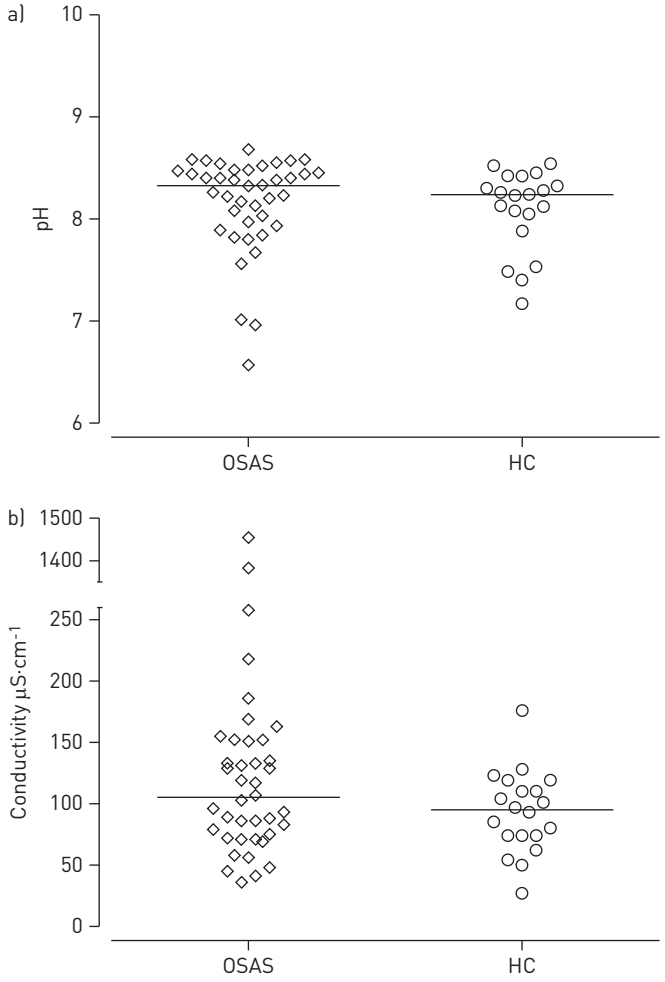

FIGURE 2 The exhaled breath condensate values of a) pH and b) conductivity do not differ statistically significantly (p=0.27 and p=0.17, respectively, t-test). OSAS: obstructive sleep apnoea syndrome; HC: healthy controls.

the difference in concentrations for MMP-9 (OSAS 5077.3±9104.5 pg·mL^{-1} *versus* healthy controls 1008.6±872.9 pg·mL^{-1}; p=0.06; t-test) and TIMP-1 (OSAS 7918.9±7075.7 pg·mL^{-1} *versus* healthy controls 6961.6±11 412 pg·mL^{-1}; p=0.16; Mann–Whitney U-test) did not reach statistical significance. However, the MMP-9/TIMP-1 ratio showed a significant difference between groups (OSAS 0.69±1.11 *versus* healthy controls 0.24±0.25; p=0.02; Mann–Whitney U-test) (fig. 3b). Compared with the VOC analysis, any other marker (EBC pH, EBC conductivity and any marker in pharyngeal washing fluid) was inferior in predicting OSAS (AUC of the ROC curves ranged from 0.59 to 0.71; data not shown). However, the combination of inflammatory markers in pharyngeal washings and EBC pH/conductivity with the linear discriminant analysis increased the diagnostic accuracy to 100% (AUC of the ROC 1, 95% CI 0.94–1.00).

Patients before and after 3 months of CPAP therapy

The characteristics of the first 20 OSAS patients measured before and after initiation of CPAP therapy are listed in table 2.

The linear discriminant analysis values of before and after initiation of standard CPAP therapy differed significantly (p=0.0003; Wilcoxon test) (fig. 4a). The Mahalanobis distance between the two groups was 1.83, the cross-validation value 63.1% and the corresponding area under the ROC curve 0.82 (95% CI 0.6825–0.9475) (fig. 4b), with a sensitivity of 0.80 and a specificity of 0.65.

EBC pH values of both visits were similar (pre- 8.08±0.52 *versus* post-initiation 8.05±0.85; p=0.63, Wilcoxon test) (fig. 5a); however, conductivity differed (pre- 186.7±303.6 µS·cm^{-1} *versus* post-initiation 97.9±59.35 µS·cm^{-1}; p<0.05, Wilcoxon test) (fig. 5b).

Moreover, the α_1-AT concentration decreased significantly after CPAP treatment (pre- 66.3±48.4 µg·mL^{-1} *versus* post-initiation 42.8±36.3 µg·mL^{-1}; p=0.017, paired t-test) (fig. 6a). For MMP-9 (pre- 7785.6±10 500 pg·mL^{-1}

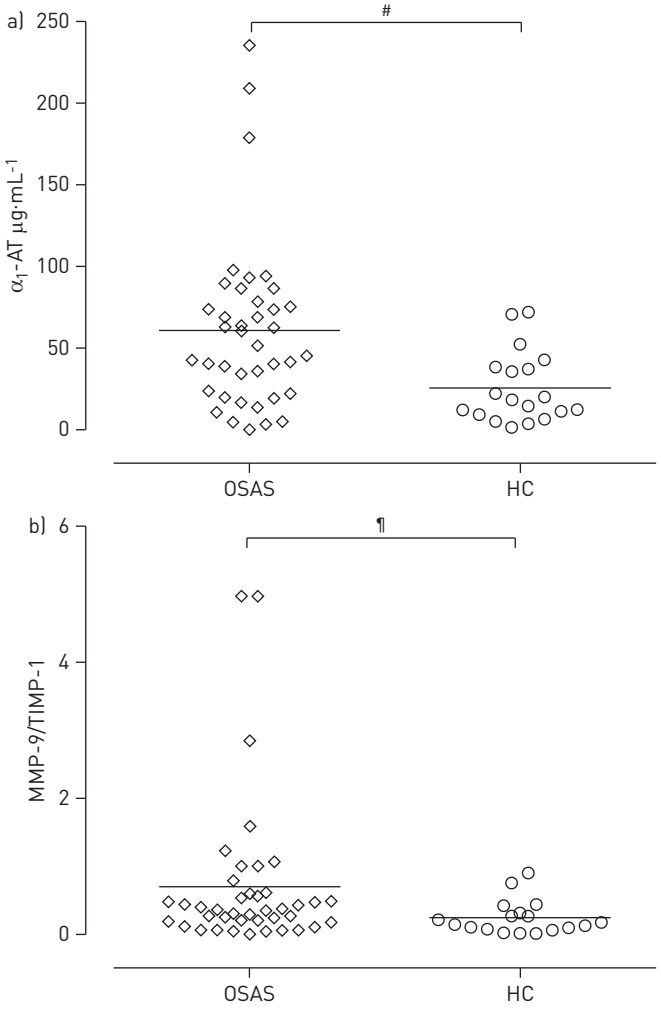

FIGURE 3 a) The α_1-antitrypsin (α_1-AT) concentrations and b) matrix metalloprotease (MMP)-9/tissue inhibitor of matrix metalloprotease (TIMP)-1 ratio in pharyngeal washings were significantly higher in obstructive sleep apnoea syndrome (OSAS) patients compared with healthy controls (HCs). #: p=0.007, t-test; ¶: p=0.02, Mann–Whitney U-test.

versus post-initiation 9185.8±11 003 pg·mL^{-1}; p=0.421, Wilcoxon test) and TIMP-1 (pre- 8344.7±7201.1 pg·mL^{-1} *versus* post-initiation 13 201±13 151 pg·mL^{-1}; p=0.314, Wilcoxon test) there was no significant difference. The same applied to the MMP-9/TIMP-1 ratio (pre- 0.87±1.2 *versus* post-initiation 0.98±0.97; p=0.37, Wilcoxon test) (fig. 6b).

TABLE 2 Characteristics of the subgroup of obstructive sleep apnoea syndrome (OSAS) patients with measurements at baseline and after 3 months of continuous positive airway pressure therapy

	OSAS patients	
	Pre-initiation	**After 3 months**
Female/male n	1/19	
Age years	57±9	
Height cm	175±7	
Weight kg	101±15	
BMI kg·m^{-2}	32.9±4.4	
AHI events·h^{-1}	32.0±22.8	2.9±3.4
AI events·h^{-1}	9.4±17.4	1.3±2.8
HI events·h^{1}	22.6±21.7	1.7±2.1
So_2 %	92.5±2.5	94.4±1.5

Data are presented as mean±SD. BMI: body mass index; AHI: apnoea/hypopnoea index; AI: apnoea index; HI: hypopnoea index; So_2: oxygen saturation.

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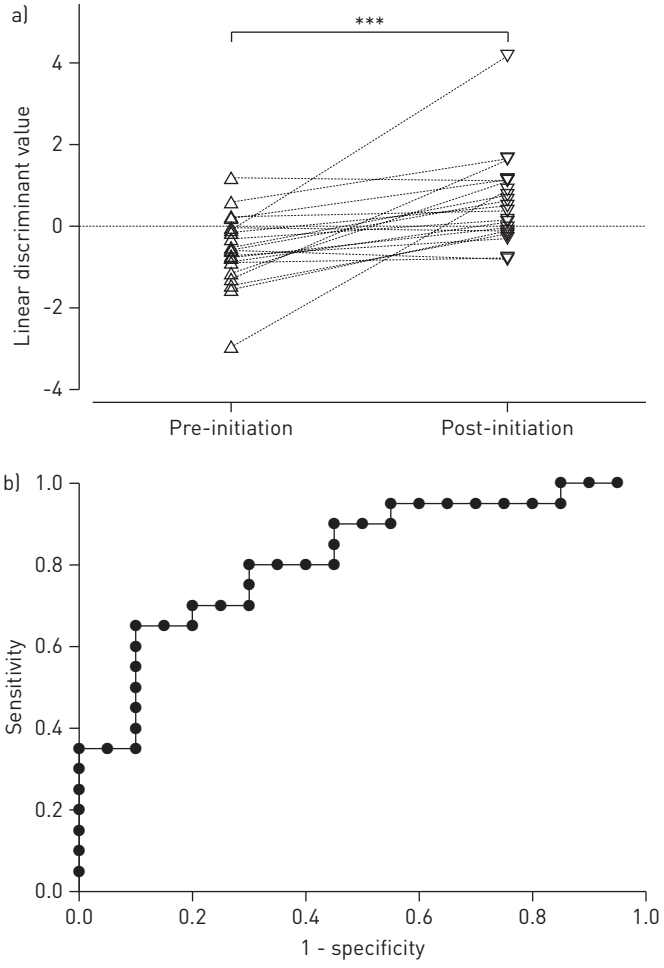

FIGURE 4 a) The (paired) values of the linear discriminant analysis of sleep apnoea patients before (pre-initiation) and 3 months after (post-initiation) initiation of continuous posititve airway pressure therapy differ statistical significantly. b) The area under the receiver operating characteristic curve equals 0.82, resulting in a sensitivity of 0.80 and a specificity of 0.65. ***: $p<0.001$, Wilcoxon signed-rank test.

Discussion

This study shows that the Cyranose 320 electronic nose can distinguish the pattern of VOCs present in the exhaled breath of patients with OSAS from that of healthy subjects. A significant correlation between the linear discriminant and the AHI could be observed. Furthermore, the electronic nose could discriminate the state before and after treatment with CPAP. This indicates that specific VOC patterns in exhaled breath are associated with untreated OSAS.

Exhaled breath analysis for VOCs is a relatively novel option to obtain information about diseases. Gas chromatography and mass spectrometry have been used in the past [16]. The simpler, pattern-recognising electronic noses allow the rapid recognition of VOC mixtures in terms of ''smellprints'' [27], but not the identification of individual molecular components [17].

Electronic noses of various sophistications have been tested in a variety of respiratory diseases. In principle, the diagnosis of ear, nose and throat infections [28] or pneumonia [29] is possible. Moreover, patients with lung cancer [30], asthma [31], COPD [18] or α_1-AT deficiency [20] could be recognised when compared with healthy controls or individuals with other respiratory diseases. In our study, we aimed to test the hypothesis that OSAS could be recognised by its VOC profile and that the profile would change after CPAP treatment. To our knowledge, this is the first study in which the smellprints of OSAS patients have been compared with healthy subjects.

Our results might be of interest, as nearly all screening tools for OSAS require overnight measurements. Although the available devices are easy to handle, some of the recordings exhibit poor quality, thus limiting their diagnostic value. Furthermore, some patients are unwilling to sleep while ''connected to an electronic device''. Conversely, overnight polygraphy with a limited number of channels does not require expensive medical staff, simply two patient visits to the clinic. Thus a diagnosis from other sources, *e.g.* ''within a breath'', would be desirable.

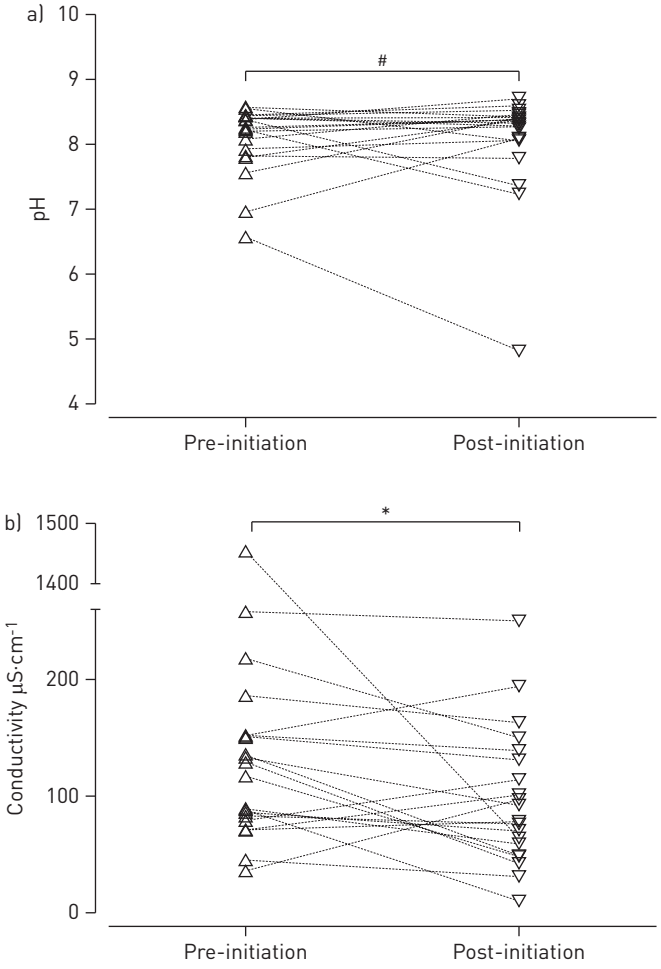

FIGURE 5 a) The pH values of the exhaled breath condensate (EBC) of both visits do not differ statistically significantly, but b) the conductivity values of the EBC of both visits do differ statistically significantly. #: p=0.63; *: p<0.05, both Wilcoxon signed-rank test.

One might wish that single substances could be described to predict differences in the exhaled breath profile of different patient groups. While gas chromatography and/or mass spectrometry could achieve that, due to the tremendous costs and time-consuming data analysis, these methods would not be feasible in a clinical setting. The idea to describe (on a computational level) patterns without knowing the disease-specific substance is a promising new attempt with a clinically directed on-site approach.

It could be argued that an AUC of 0.85 is not sufficient for a diagnostic tool. However, diagnostic tests are used in specific clinical situations. The electronic nose could be useful on two occasions. First, to rule out the disease in a low prevalence population, *e.g.* in a general practitioner's office where the prevalence of OSAS is ~2–4% [1]. A negative result would have a negative predictive value of 99.6% (95% CI 90.3–100%) and thus provide a high degree of certainty. Secondly, the device could be a decision aid with which to conduct overnight PSG. In a population with a high prevalence of OSAS (as high as 35% in obese, snoring subjects), a positive result would have a positive predictive value of 62.4% (95% CI 43.3–79.1%) and would pave the way towards overnight PSG. Conversely, a negative result would have a negative predictive value of 94.5% (95% CI 78.9–99.6%), thus drawing the attention to diagnoses other than OSAS. Furthermore, a value of 0.85 for an AUC is in the range of other diagnostic tests that are used in daily clinical practice, such as troponin for the diagnosis of myocardial infarction (AUC of 0.87 [32]).

To reveal whether there would be specific chemical or physicochemical alterations in OSAS patients, we also analysed EBC, focussing on easily accessible markers such as pH and electrical conductivity, which we have recently found to be robust and reproducible markers unaltered by respiratory manoeuvres [24]. EBC pH values were in the range of 8, which is comparable with what has been reported before [33–35]. However, we did not find major differences in these measures, which might be too unspecific for the disease.

In contrast, α_1-AT in pharyngeal washing fluid as a marker of inflammation showed elevated values in OSAS patients and these were reduced after only 3 months of CPAP treatment. This could reflect the response to the intermittent hypoxia and/or cyclic sheer forces that are alleviated by CPAP. Similar results

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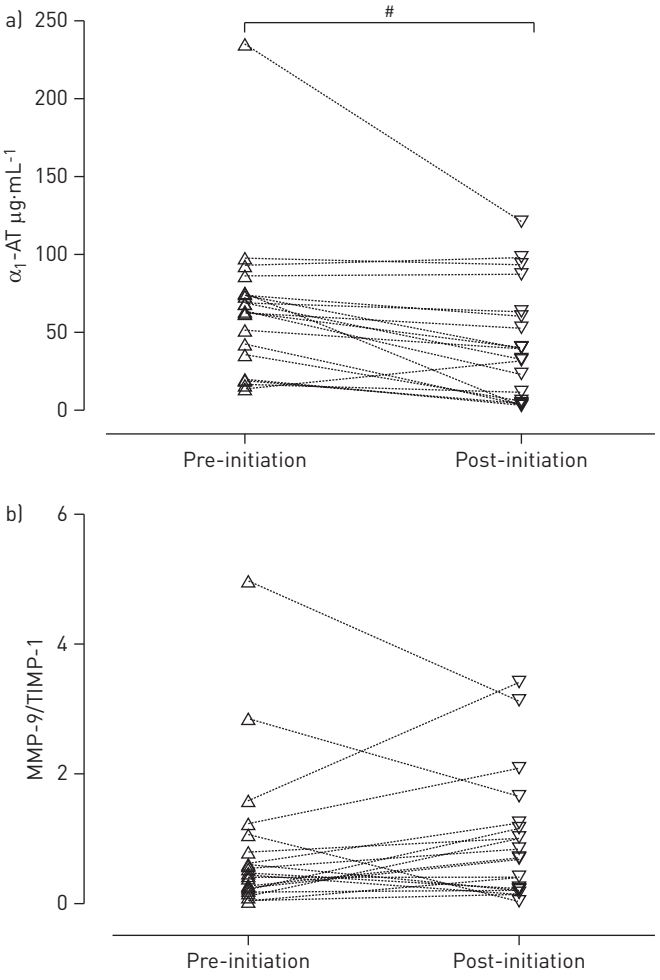

FIGURE 6 a) The α_1-antitrypsin (α_1-AT) concentration in pharyngeal washing fluids decreased significantly after 3 months of continuous positive airway pressure treatment. #: p=0.017, t-test for paired samples. b) Matrix metalloprotease (MMP)-9/tissue inhibitor of matrix metalloprotease (TIMP)-1 ratio did not change significantly in obstructive sleep apnoea syndrome patients before (pre-initiation) and after (post-initiation) treatment (p=0.37, Wilcoxon signed-rank test).

have been shown for local and systemic markers of inflammation and/or oxidative stress [36, 37]. We used MMP-9, TIMP-1 and their ratio MMP-9/TIMP-1 as further markers; the ratio showed elevated levels in OSAS. To our knowledge, this is the first time that the MMP-9/TIMP-1 ratio has been examined in a local compartment in OSAS. On a systemic level, increased MMP-9 concentration and activity have already been described in OSAS and these alterations could be reduced by CPAP therapy [38]. This leads us to believe that the increased ratio of MMP-9/TIMP-1 is an indicator of upper airway remodelling following chronic intermittent hypoxia and sheer forces.

This study has a number of limitations. The groups were not matched for age and body mass index (BMI), both of which are known to be potential confounders in exhaled breath analysis. To investigate whether age and BMI were confounders for the altered VOC mixture, we performed a logistic regression analysis with AHI as the dependent variable. The electronic nose-derived linear discriminant analysis was the best and solely significant predictor of AHI (OR 3.03, 95% CI 1.41–6.51) compared with BMI (OR 1.17, 95% CI 0.99–1.37) and age (OR 1.05, 95% CI 0.98–1.12). This was also reflected in a significant correlation between AHI and linear discriminant analysis (Spearman's r=0.58, p<0.001). Moreover, the significant change of the VOC profile before *versus* after CPAP treatment suggests that the differences between groups in the VOC profile were mainly due to the presence of OSAS. This represents a major advantage compared with previous publications where the VOC profile was assessed only once and significant correlations to other biological markers were not demonstrated.

Because of the high prevalence of comorbid conditions in OSAS patients, it could be argued that the altered VOC mixture was mainly due to comorbidities and/or associated medication. However, looking at the linear discriminant analysis graph (fig. 1a) and marking patients with coronary heart disease (n=3), diabetes (n=5) and arterial hypertension (n=27), there was no trend in the distribution of patients with comorbidities (see online supplementary material).

The data on inflammatory markers in pharyngeal washing are limited by missing standardised procedures to assess the absolute values of specific markers. HERR *et al.* [39] described defensin measurements in smokers, and pharyngeal washings have been used for the detection of potential respiratory pathogens [40]. Irrespective of this, the data for α_1-AT strengthen the assumption that α_1-AT is upregulated in untreated OSAS and pharyngeal washings are helpful to investigate this further.

Additionally, a sham CPAP and follow-up data would have strengthened our study and would have added data about the repeatability of the breathprint over time. However, the focus of this proof-of-concept study was on the diagnostic approach, with the goal of demonstrating the general possibility of the diagnostic potential of exhaled breath analysis regarding OSAS. We used the limited before/after comparison of exhaled breath to show that standard therapy changes the breathprint and to underline the assumption that the observed exhaled breath profile differences of OSAS *versus* healthy controls were mainly due to the presence/absence of obstructive episodes.

Most importantly, the results have to be validated in a separate cohort, possibly in an independent centre, in line with the Standards for the Reporting of Diagnostic accuracy studies (STARD) statement for the validation of diagnostic tests [41].

We conclude that the Cyranose 320 electronic nose is capable of distinguishing the exhaled breath of OSAS patients and control subjects with high accuracy.

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