

Exhaled air molecular profiling in relation to inflammatory subtype and activity in COPD

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Date: 26 April 2011

Word count: 3626

Key words: airway inflammation, COPD, exhaled breath analysis, induced sputum, metabolomics, volatile organic compounds

ABSTRACT

Background Eosinophilic inflammation in COPD is predictive for responses to inhaled steroids. We hypothesized that inflammatory subtype in mild and moderate COPD can be assessed by exhaled breath metabolomics.

Methods Exhaled compounds were analyzed using gas-chromatography and mass-spectrometry (GC-MS) and electronic nose (eNose) in 28 COPD patients (12/16 GOLD I/II). Differential cell counts, ECP and MPO were measured in induced sputum. Relationships between exhaled compounds, eNose breathprints and sputum inflammatory markers were analyzed and ROC curves were constructed.

Results Exhaled compounds were highly associated with sputum cell counts (8 compounds with eosinophils, 17 with neutrophils: $p < 0.01$). Only one compound (alkylated benzene) overlapped between eosinophilic and neutrophilic profiles. GC-MS and eNose breathprints were associated with markers of inflammatory activity in GOLD stage I (ECP: 19 compounds, $p < 0.01$; eNose breathprint $r = 0.84$, $p = 0.002$) (MPO: 4 compounds $p < 0.01$; eNose $r = 0.72$, $p = 0.008$). ROC analysis for eNose showed high sensitivity and specificity for inflammatory activity in mild COPD (ECP: AUC=1.00) (MPO: AUC=0.96) but not for moderate COPD.

Conclusions Exhaled molecular profiles are closely associated with the type of inflammatory cell and their activation status in mild and moderate COPD. This suggests that breath analysis may be used for assessment and monitoring of airways inflammation in COPD.

Word count: 200

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a disease characterized by not completely reversible, progressive airflow limitation [1]. Several clinical features can be observed within the broad spectrum of COPD, including emphysema, chronic bronchitis, small airways disease and asthma-like characteristics such as airway hyperresponsiveness [2-4]. The inflammatory response in COPD is predominantly neutrophilic, but also comprises involvement of eosinophils [5,6]. The presence and activation status of these cells varies between subgroups of patients and may reflect distinct pathophysiological mechanisms within the disease [7,8]. It is increasingly acknowledged that inflammatory phenotyping of COPD patients can lead to a more effective tailored treatment. Indeed, several studies have shown a greater response to corticosteroid treatment in eosinophil predominant COPD [9-11].

Sputum induction is a safe and reliable method to provide information about inflammatory cells and mediators in COPD [12]. However, it is a time consuming procedure and patients may find it unpleasant. The different clinical, immunological and oxidative mechanisms of COPD emphasize the need to provide composite profiles of biomarkers of the disease [13]. Integrative metabolomic assessment of molecular signatures by high-dimensional diagnostic techniques can provide fingerprints of disease. In COPD, this has been done at the gene expression and protein level using blood and broncho-alveolar lavage fluid (BAL) [13,14].

Exhaled air contains mixtures of gases including volatile organic compounds (VOCs) and nitric oxide (FeNO). Assessment of the profile of these volatiles

by gas-chromatography and mass-spectrometry (GC-MS), nano-sensors of an electronic nose (eNose) and an NO-analyzer might lead to the discovery of novel disease specific patterns of molecular biomarkers [15]. We have recently shown that exhaled breathprints by eNose can distinguish well-defined COPD from asthma [16]. It is unknown whether exhaled breathprints in COPD are related to the underlying inflammatory phenotype. If so, breathprints may reflect the type and activity of the predominant inflammatory pathways thereby facilitating management of the disease. Since there is evidence that *e.g.* oxidative stress responses are markedly different in COPD GOLD stage I as compared to later stages of the disease [17-19], the underlying mechanistic profile is likely to depend on GOLD stage. Therefore, non-invasive assessment of exhaled volatiles may also provide new insights into inflammatory pathways in relation to disease stages in COPD.

In this study, we hypothesized that metabolomic exhaled breath profiling in COPD captures the cell type and activation status as reflected by differential cell counts, eosinophil cationic protein (ECP) and myeloperoxidase (MPO) in induced sputum. Furthermore, we have examined whether the association between exhaled breathprints and inflammatory markers is different between COPD GOLD stages I and II. We aimed to test this hypothesis by examining the relationship of exhaled molecular profiles measured by GC-MS, eNose and FeNO-analyzer, with markers of inflammation from induced sputum in COPD patients with mild and moderately severe disease.

METHODS

Subjects

Thirty-two patients with mild to moderately severe COPD (GOLD stages I and II) were recruited in the outpatient clinics of two participating centers in The Netherlands [1]. Inclusion criteria were symptoms of dyspnea, chronic cough or sputum production, current or ex-smoking, a smoking history of ≥ 20 packyears, postbronchodilator $FEV_1 > 1.5$ liter and $> 50\%$ of predicted value, and FEV_1/FVC ratio < 0.70 . Patients were excluded in case of exacerbation or respiratory infection ≤ 4 weeks prior to recruitment, and any other pulmonary disease other than COPD including known asthma. In case of a respiratory infection, a 4 weeks recovery period was taken into account before re-evaluation for inclusion. Predefined subgroups were made with respect to GOLD stages I and II. Before the study, 14 of 32 patients were on inhaled corticosteroid treatment and stopped this medication at least 4 weeks prior to the study.

The study was approved by the Hospital Medical Ethics Committee and all patients gave their written informed consent. The study was registered in the Netherlands trial register, www.trialregister.nl under NTR 1283.

Design

The study had a cross-sectional design and was comprised of two study days. At the screening visit, inclusion and exclusion criteria were examined, and lung function tests were performed.

At the second visit, exhaled breath was captured for analysis by GC-MS, eNose and FeNO-analyzer, sputum was induced by hypertonic saline, atopy was assessed and a venous blood sample was obtained. Before sputum induction and exhaled breath analysis, patients were asked to refrain from eating, drinking and smoking for 2 hours, short-acting bronchodilators for 12 hours, and long-acting bronchodilators, anti-cholinergics, leukotriene antagonists, and anti-histamines for 24 hours.

The sample size was based on the following consideration: if molecular compounds or eNose breathprints are to be used in clinical practice to identify involvement of inflammatory pathways, their association should be considerable. We defined the association to be potentially useful if the correlation coefficient was larger than 0.6/0.7. To find such correlation statistically significant ($\alpha = 0.05$) with 80% power 17/11 patients were required, respectively.

Measurements

Lung function and allergy testing

Spirometry (MasterscreenPneumo; Jaeger; Würzburg, Germany) was performed according to the latest ERS recommendations [20]. Diffusion capacity for carbon monoxide ($D_{L,CO}$) was measured according to the recommendations using the single breath method and was corrected for hemoglobin level [21]. FeNO was measured with a portable analyzer (flow rate 50mL/s; NIOX Mino System, Aerocrine, Sweden) according to the ATS guidelines [22].

Allergy testing was performed using a skin prick test to 12 common airborne allergens (ALK-Abello; Benelux) or RAST. Atopy was indicated by positivity (> 3 mm wheal) to one or more allergens, or positive RAST.

Sputum induction and processing

Sputum was induced by inhalation of sodium chloride aerosols 3 times at intervals of 5 min duration, according to the ERS recommendations [23]. Prior to each sputum induction, 200 µg salbutamol was inhaled. All sputum samples were collected in sterile containers and processed within 2 hours.

Sputum processing was done by the validated, so-called full-sample technique using DTT [24]. Differential cell counts were based on 500 non-squamous cells and expressed as percentage of non-squamous cells. Absolute cell numbers were calculated as (% cell x total cell count)/sputum weight. Sputum samples containing > 20% squamous cells were excluded from analysis. All sputum and blood cell counts were performed by experienced and qualified lab technicians who were blinded to the clinical details.

As biomarkers of local activation of eosinophils and neutrophils, respectively, levels of eosinophil cationic protein (ECP; detection limit > 60 pg/ml) and myeloperoxidase (MPO; detection limit > 1.5 ng/ml) were measured by enzyme-linked immunosorbent assays (ELISA) in sputum supernatant [25].

Exhaled breath collection

Exhaled breath collection was done as previously described [16,26]. Patients breathed normally for 5 min with the nose clipped through a mouthpiece,

connected to a three-way non re-breathing valve, an inspiratory VOC-filter (A2, North Safety, NL) and an expiratory silica reservoir for conditioning of the humidity level. After a single deep maximal inspiration the patient exhaled one vital capacity volume into a 10 L Tedlar bag (SKC Inc., Eighty Four, PA, USA) connected to the expiratory port and the silica reservoir. In addition, a Tedlar bag was filled with VOC-filtered room air for comparison (background air sample).

Breath analysis by gas chromatography and mass spectrometry (GC-MS) was performed as previously described [16,26]. The content of the Tedlar bags was transported by a vacuum pump (0.5 l at a flow rate 200 ml/sec) into stainless steel adsorption tubes (Supelco, Zwijndrecht, The Netherlands), filled with Tenax GR (Varian Chrompack, Middelburg, The Netherlands). Tubes were placed inside the thermal desorption unit (TDSA, Gerstel, Mülheim an der Ruhr, Germany) and were thermally desorbed for 10 minutes at 200 °C using helium (temperature gradient of 60 °C/min) with a gas flow rate of 15 ml/min in order to transfer the VOCs into the GC capillary column. Solvent venting mode was used to transfer the entire sample to the packed liner (filled with Tenax TA), held at 0 °C. After collection of the VOCs in the packed liner, it was heated to 300°C. To minimize band broadening caused by the split less injection from the packed liner, a cold trap (CTS2, Gerstel, Mülheim an der Ruhr, Germany) was used with the initial temperature set at -150°C. After 2 minutes the trap was heated to 280°C at 20°C/sec. The VOCs were separated by capillary gas chromatography using helium as the carrier gas at 1.2 ml/min (6890N GC, Agilent, Santa Clara, CA, USA) on a VF1-MS

column (30 m*0.25 mm, film thickness 1 μ m, 100% dimethylpolysiloxane, Varian Chrompack, Middelburg, The Netherlands). The following temperature program was applied for the gas chromatograph: 40 °C isothermal during 5 minutes, then raised at 10 °C/minute until 300 °C, held isothermal for 5 minutes. For detection and identification of the separated compounds, a quadrupole mass spectrometer (5975 MSD, Agilent, Santa Clara, CA, USA) was used in electron ionization mode at 70eV, with a scan range of 14-300 Da.

Gaseous calibration standards using 10 ppmv toluene in nitrogen (Air Products, Amsterdam, The Netherlands) were made by use of a home built dilution system and loaded onto adsorption tubes to check the sensitivity of the system. Reproducibility of exhaled breath samples analyzed by GC-MS has been shown to be highly adequate [27].

Breath analysis by electronic nose

Within 10 minutes after breath collection the Cyranose 320 electronic nose (Smiths Detection, Pasadena, Ca, USA) was connected to the Tedlar bag, followed by 1 minute sampling of the exhaled air, in parallel to sampling a Tedlar bag filled with VOC-filtered room air (background air) for comparison [16,26]. The changes in electrical resistance of each of the 32 polymer sensors that constitute the raw eNose data [28] were used for further analysis with offline pattern-recognition software. All measurements were performed in duplicate and every very first analysis by the eNose during each session was excluded from analysis as was recommended by the manufacturer ('first sniff

effect') due to deviant sensor deflections. Measurements of breath samples by electronic nose have been shown to be highly reproducible [16].

Statistical analysis

SPSS (version 17.0), Graphpad Prism (version 5.01) and MatLab® were used for data analysis. Results were expressed as mean and standard deviation if data were normally distributed and as median and interquartile range if data were non-normally distributed. Non-normally distributed data were log-transformed for further analysis. If no cells were counted, a value of 0.1 was taken before log-transformation.

The GC-MS chromatograms were aligned using MetAlign software [29]. Details on the software are described elsewhere [30]. Noise-values were replaced by zeros and background air chromatograms were subtracted from breath samples. Duplicate breath samples were averaged. The output of the software is a peak table that was exported into Matlab®. The correlation coefficient and the corresponding p-value between the peaks and the measured inflammatory parameter were calculated. In view of multiple testing, a p-value of <0.01 was considered significant (Šidák correction based on correlation coefficient > 0.6). Compounds representing significantly correlated peaks were identified by comparison of mass spectra to the NIST library and checked manually by an experienced mass spectrometrist.

eNose raw data (change in resistance of sensors) were restructured by principal component analysis from the original 32 sensors to 4 principal

components (PC) that captured 96.9% of the variance within the dataset. The relationship between the markers of airway inflammation and the principal components was analyzed using Pearson's correlation coefficient (r). The principal components (PC 1, 2 and 3) showing the most significant correlations were selected for further analysis.

For ECP and MPO, receiver operating characteristic (ROC) analysis was performed to assess the detection rate (sensitivity) and false-positive rate (1-specificity) of eNose principal components for airway inflammation. As there is no consensus about cut-off points for ECP and MPO in inflammatory subtypes in COPD, quartiles were taken as cut-off points for absence or presence of eosinophilic and neutrophilic inflammation. For each of the quartile cut-off points (25, 50, 75 %) the Youden index was calculated, and the quartile with the highest Youden index was selected as the best fitting model. The breathprint principal component values with the best combination of detection and false-positive rate were considered as the definite eNose principal component cut-off points for prediction of ECP and MPO by ROC analysis.

RESULTS

Subjects

Twenty-eight of 32 patients completed the study. Four patients dropped out for reasons of: non-compliance with medication restrictions (n=1), lost to follow up (n=1), FEV₁ < 1.2 liter prior to sputum induction (n=1) and inability to perform techniques necessary to measure lung function (n=1). Five hypertonic saline-induced sputum samples were excluded from analysis because of the presence of >80% non-squamous cells. Baseline characteristics are summarized in Table 1. Apart from significant differences in postbronchodilator FEV₁ (L and %pred) (p=0.001), only the use of inhaled corticosteroids (ICS) 4 weeks prior to the study differed between GOLD stages I and II (p=0.022). Cellular and soluble markers of airway inflammation in exhaled air, sputum and in blood (Table 2) showed no significant differences between GOLD stages I and II.

Relationship between exhaled compounds identified by GC-MS and inflammatory cell counts

Twenty-six different VOCs in the exhaled breath samples of the study population were found to be significantly correlated to markers of airway inflammation (Table 3). Figure 1 shows typical GC-MS chromatograms of GOLD stage I and II patients. Only one compound (alkylated benzene; Table 4) was indicative for both cell types and more compounds correlated strongly with sputum neutrophils than with eosinophils. The exact molecular identity of the alkylated benzene could not be determined due to the high similarity of the

mass spectra of homologues in this class of compounds. Between the two disease stages, more compounds were found to correlate with cell counts for GOLD stage II patients (Table 3). Compounds that were correlated ($p < 0.01$) with sputum cell counts were mostly larger hydrocarbons (alkanes, methyl-branched alkanes, aromatic compounds).

Relationship between exhaled compounds identified by GC-MS and inflammatory cell activation

Exhaled compounds were also found to be associated with markers of inflammatory cell activation: ECP for eosinophils and MPO for neutrophils (Table 3). Notably, correlated compounds were largely different between the GOLD stages. For GOLD stage I, 18 compounds were identified that significantly correlated to ECP and 4 to MPO. For GOLD stage II and ECP, 9 different compounds and 1 identical compound were found as compared to GOLD stage I. For GOLD stage II and MPO, 1 different compound and 2 identical compounds were found as compared to GOLD stage I (Table 3).

Relationship between electronic nose exhaled breathprints and inflammatory cell counts and activation

The associations of eNose breathprints with ECP and MPO were consistent to those observed with GC-MS, showing more prominent correlations in mild as compared to moderate disease (Figure 2). For GOLD stage I, there was a strong correlation between exhaled breathprint PC 2 and ECP ($r = 0.84$, $p = 0.002$; Figure 2b) and MPO ($r = 0.72$, $p = 0.008$; Figure 2e).

For GOLD stage II, none of the correlations found in GOLD stage I could be observed. There were no significant correlations between breathprints and levels of ECP (Figure 2c) and MPO (Figure 2f).

No significant correlations were found between breathprints and sputum differential cell counts, and between FeNO and cell counts or markers of cell activation.

Performance estimation of exhaled breathprints for inflammatory activity

The detection (sensitivity) and false-positive rates (1-specificity) of exhaled breathprints for sputum ECP and MPO are listed in Table 5. For sputum ECP, the 50% percentile (cutoff at 152 ng/ml) showed an AUC of the ROC curve of 0.86 for the total COPD group (Figure 3a). Split analysis for GOLD stages I and II showed a good performance of breathprint PC 2 for sputum ECP in mild COPD (AUC=1.00; cutoff 75% percentile at 361 ng/ml) (Figure 3b) but a weaker performance in moderate COPD (AUC=0.75; cutoff 50% percentile at 158 ng/ml) (Figure 3c).

For MPO, a similar pattern was seen (Table 5). In GOLD stage I, the ROC analysis reached an AUC of 0.96 (cutoff 25% percentile at 1795 ng/ml) (Figure 3e), as opposed to 0.66 for the total group (cutoff 50% percentile at 4530 ng/ml) (Figure 3d) and 0.66 for GOLD stage II (cutoff 75% percentile at 6320 ng/ml) (Figure 3f).

DISCUSSION

This study shows that exhaled markers are significantly associated with differential cell counts and soluble sputum markers of activated neutrophils and eosinophils in mild and moderately severe COPD. These results indicate that molecular patterns in exhaled breath are related to inflammatory cell type and activation status, suggesting that breath analysis may qualify as a non-invasive marker of airway inflammation in relatively early stages of the disease after confirmation of generalisability in independent studies.

To our knowledge, this is the first study assessing the relationship and estimating the performance of molecular profiles of volatile biomarkers in exhaled air and markers of airway inflammation in sputum of patients with COPD. The present results extend findings in earlier studies, in which COPD could be distinguished from asthma using exhaled breath analysis by eNose [16] and from healthy smoking and non-smoking controls by gas chromatography and mass spectrometry [31,32]. In these studies, however, the relationships between exhaled VOC patterns and the accompanying inflammatory profiles were not examined.

In this study, we carefully selected mild and moderate COPD patients based on objective criteria including fixed airways obstruction and smoking history [1]. Because inhaled corticosteroids could have confounded the effects of inflammatory profiles, ICS treatment was withheld for 4 weeks prior to measurements. However, extended influence of ICS treatment on inflammatory profile cannot be completely excluded. This holds especially for the group of GOLD stage II COPD patients with a higher proportion of previous ICS use, which may have masked findings in this group.

Furthermore, the breathprints are critically dependent on the methods of collecting and sampling [16]. We used a validated breathing and sampling procedure with inspiratory VOC filtering to minimize any external influence on the breathprint [16,26] and corrected for ambient air and any influences from the breathing setup by background extraction. Finally, smoking status could have influenced the results as about half of the patients in this study were current smokers. Several components that were found to be associated with inflammatory markers were previously described as smoking-related, but also as (ex-smoking) COPD-related [33]. The latter is supported by observations in our previous study in which we found no difference in eNose breathprint between smoking and ex-smoking COPD patients [16].

How can we explain these findings? Earlier studies of exhaled air in inflammatory lung diseases showed a distinct signal when compared to controls [16,26,32,34]. Our present results show that the signal in COPD is most likely based on the metabolites that mediate or that are the result of the accompanying neutrophilic and eosinophilic inflammation [2,5,35]. Exhaled compounds and eNose breathprints were also associated with activation markers of eosinophils and neutrophils, namely MPO and ECP, the predominant VOCs being alkanes, methylated alkanes, aromatic compounds like benzene and toluene and alcohols. These hydrocarbons can be seen as markers of oxidative stress resulting from lipid peroxidation by the formation of reactive oxidizing and chlorinating species [33,36-38]. Indeed, one of the major biological activities of ECP [39] and MPO [40,41] is the cytotoxic effect by the formation of reactive oxygen species. Alkanes are cleared either by diffusion through the lungs, or by further oxidation to alcohols by cytochrome

p450 enzymes [42], which are also known to play a role in the pathogenesis of COPD [43]. Therefore, the complex nature of the inflammation in COPD probably makes metabolomic assessment of biomarkers a suitable approach, as a single marker that is representative of the airways inflammation in COPD has not been identified [13,44,45].

Our findings suggest that exhaled breath profiling using the quantitative method GC-MS can both identify the *type* and the *activation* of inflammation (eosinophilic vs neutrophilic), whereas multi-compound breath profiling using nano-sensor pattern recognition by eNose appeared to be more suitable for detecting *activation* of inflammatory cells, especially in COPD GOLD I. It is tempting to speculate that the inflammatory drive and activity in early stages of COPD are reflected differentially or more prominently in exhaled breath than in more advanced stages of COPD. This is in concordance with the findings by Pierrou *et al*, who observed a peak in oxidative stress gene expression in mild COPD as compared to more severe stages of the disease [17]. Therefore, the findings of the present study suggest that inflammatory activity as revealed by exhaled breath analysis depends on disease severity, being more prominent in mild COPD. This favors the hypothesis that inflammatory activity diminishes or alters with disease progression in COPD [18,19], which requires prospective follow-up studies.

The clinical implications of our findings are in the area of potential application of exhaled breath profiling in the phenotyping and monitoring of COPD. It is increasingly recognized that COPD is not a single disease entity, and that phenotypic fingerprints derived from both clinical and inflammatory markers are needed to predict disease progression and therapeutic

responses [44]. Exhaled markers are certainly appealing in this respect. Our study represents a first step towards such an application by demonstrating strong associations between differential cell counts in sputum and exhaled volatiles, and good performance of exhaled eNose breathprints for estimation of inflammatory activity in the airways. Other independent studies should be undertaken to confirm our findings by external validation [46]. In addition, it needs to be examined whether breath profiling has sufficient diagnostic accuracy for assessing early stage COPD among smokers. And finally, it should be established whether breath volatiles can adequately predict the responsiveness to inhaled steroid treatment as observed by using sputum eosinophils in mild and more severe stages of the disease [10,11,44].

In conclusion, the present data show that exhaled molecular profiling by GC-MS and by eNose is closely associated with cell counts and markers of inflammatory cell activation in induced sputum of patients with COPD. This suggests that metabolomic breath analysis can be a candidate method for the non-invasive identification of the cell type and their activation in COPD.

Acknowledgements

This study was supported by grant 3.2.06.17 of the Netherlands Asthma Foundation. The authors thank the patients that participated in the study, and Barbara Smids, Dept. of Experimental Immunology, Academic Medical Centre, Amsterdam, The Netherlands for their contribution to this study.

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Table 1 Subject characteristics.

	<i>All COPD subjects</i> <i>n=28</i>	<i>COPD GOLD I</i> <i>n=12</i>	<i>COPD GOLD II</i> <i>n=16</i>
<i>Clinical characteristics</i>			
Sex, Male / Female, %	82 / 18 %	83 / 17 %	81 / 19 %
GOLD stage I / II, %	43 / 57 %		
Age, years	58 (8)	59 (7)	58 (8)
Current / ex-smoker, %	43 / 57 %	50 / 50 %	38 / 62 %
Smoking history, pack years	40 (16)	43 (14)	39 (17)
Atopy, %	11 %	8 %	12 %
ICS before study, %	50 %	25 %*	69 %*
<i>Lung function</i>			
Postbronchodilator FEV ₁ , L	2.57 (0.63)	2.99 (0.67)	2.25 (0.39)
Postbronchodilator FEV ₁ , %pred	77 (14)	90 (7)*	67 (8)*
FEV ₁ /FVC	0.55 (0.08)	0.57 (0.07)	0.53 (0.09)
D _{L,CO} , %pred	65 (15)	64 (15)	66 (15)

Data is expressed as mean (SD). * p<0.05 when comparing GOLD stages I and II. ICS:

Inhaled corticosteroids. FEV₁: Forced expiratory volume in 1 second. FVC: Forced vital capacity. D_{L,CO}:

Diffusion capacity for carbon monoxide.

Table 2 Markers of airway inflammation

	<i>All COPD subjects</i>	<i>COPD GOLD I</i>	<i>COPD GOLD II</i>
	<i>n=28</i>	<i>n=12</i>	<i>n=16</i>
Exhaled NO, ppb	14 (9-23)	13 (9-29)	19 (8-23)
Sputum neutrophils, n (x 10 ⁴ /ml)	110 (45-243)	153 (76-326)	78 (37-229)
Sputum neutrophils, %	77.2 (70.8-86.0)	76.9 (71.3-85.2)	77.2 (67.4-86.1)
Sputum eosinophils, n (x 10 ⁴ /ml)	0.9 (0.4-3.1)	1.0 (0.5-4.1)	0.9 (0.4-6.1)
Sputum eosinophils, %	0.8 (0.4-3.1)	0.7 (0.3-2.9)	1.0 (0.3-4.2)
Sputum ECP, ng/ml	152 (90-452)	130 (57-361)	158 (100-532)
Sputum MPO, ng/ml	4530 (1187-7103)	4551 (1795-8313)	4078 (592-6318)
Sputum IL-8, pg/ml	1946 (592-6522)	1815 (579-4754)	2029 (839-8791)
Blood neutrophils, %	55.9 (9.1)	57.6 (9.2)	54.6 (9.0)
Blood eosinophils, %	2.7 (1.8-4.3)	2.9 (1.7-5.4)	2.7 (1.8-3.9)
Blood CRP, mg/L	2.1 (1.0-5.6)	2.1 (1.4-5.8)	2.1 (0.3-5.6)

Data is expressed as mean (SD) or median (IQR). NO: nitric oxide. ECP: eosinophil cationic protein. MPO: myeloperoxidase. CRP: C-reactive protein.

Table 3 Number of exhaled compounds that have a significant correlation (correlation coefficient > 0.6) with sputum inflammatory markers (p-value < 0.01)

	All COPD patients	GOLD I	GOLD II
n of compounds with sputum eosinophils	8	1	8
n of compounds with sputum neutrophils	17	2	19
n of compounds with sputum ECP	0	18	10
n of compounds with sputum MPO	2	4	3

ECP: eosinophil cationic protein. MPO: myeloperoxidase.

Table 4 Tentative exhaled compound identification for the total COPD group. *

The exact chain length of the alkyl moieties could not be determined from the mass spectra.

Retention time [min]	Tentative compound assignment	Chemical class	Sputum eosinophils %	Sputum neutrophils %	Correlation coefficient
4.82	unknown	-		X	0.56
5.01	pentane	Alkanes		X	0.55
5.38	unknown	-		X	-0.55
5.97	unknown	-	X		0.61
6.05	2-butanone	Ketones	X		0.62
7.37	unknown	-		X	-0.57
7.70	benzene	Aromatic hydrocarbons		X	-0.59
8.34	3-methylhexane	Methyl-branched alkanes		X	-0.59
8.80	trichloroethylene	Chlorinated hydrocarbons		X	-0.56
10.14	toluene	Aromatic hydrocarbons		X	-0.64
10.33	2-methylpentane	Methyl-branched alkanes		X	-0.55
10.68	alkylated cyclohexane*	Cyclic alkanes		X	-0.57
12.52	2-methylheptane	Methyl-branched alkanes	X		-0.58
12.62	styrene	Aromatic hydrocarbons		X	-0.68
12.79	alkylated cyclohexane*	Cyclic alkanes		X	-0.55
13.76	dimethyloctane	Methyl-branched alkanes		X	-0.57
15.01	branched alkane*	Alkanes		X	-0.56
15.36	alkylated benzene*	Alkyl benzenes	X	X	0.56
15.84	branched alkane*	Alkanes		X	-0.58
16.22	unknown	-	X		0.58
16.31	5-nonanol	Alcohols	X		0.56
16.50	dimethyl-3-octanol	Alcohols	X		0.61
17.38	unknown	-	X		-0.59
18.54	unknown	-		X	-0.60

Table 5 ROC analysis of breathprints predictive for airway inflammation activity.

		AUC	Optimal cutoff	Detection rate	False-positive rate
ECP	All subjects	0.860*	152 ng/ml	73	9
	GOLD I	1.000*	361 ng/ml	100	12
	GOLD II	0.750	158 ng/ml	67	17
MPO	All subjects	0.659	4530 ng/ml	50	23
	GOLD I	0.963*	1795 ng/ml	89	0
	GOLD II	0.659	6320 ng/ml	75	36

ECP: eosinophil cationic protein. MPO: myeloperoxidase. AUC: area under the curve.

*p<0.05.

Figure legends

Figure 1

Overlay of two GC-MS chromatograms. The magenta-coloured chromatogram corresponds to a GOLD stage I COPD patient and the black chromatogram to a GOLD stage II COPD patient. The two large peaks are artefacts that were not included in the analyses (Tedlar bag associated compounds being present in all samples: phenol and N,N-dimethylacetamide)

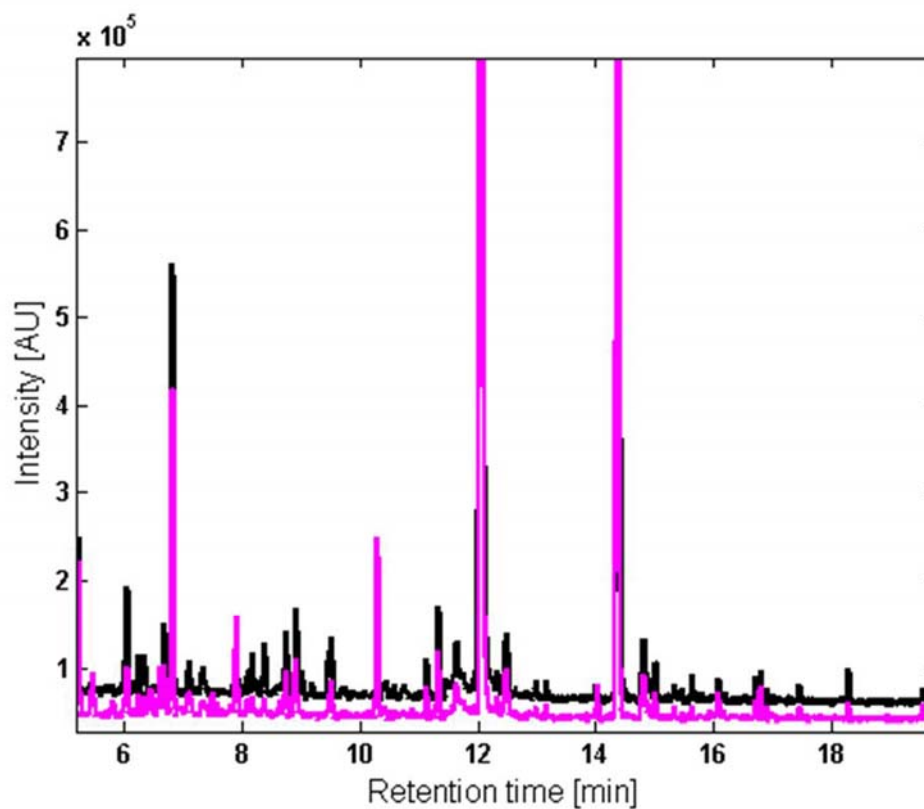


Figure 2

Correlations, linear regression lines and 95% confidence bands of eNose breathprint PC 2 in all patients, GOLD stage I and II, respectively to sputum ECP (a-c) and MPO (d-f).

▲ GOLD stage I, ● GOLD stage II.

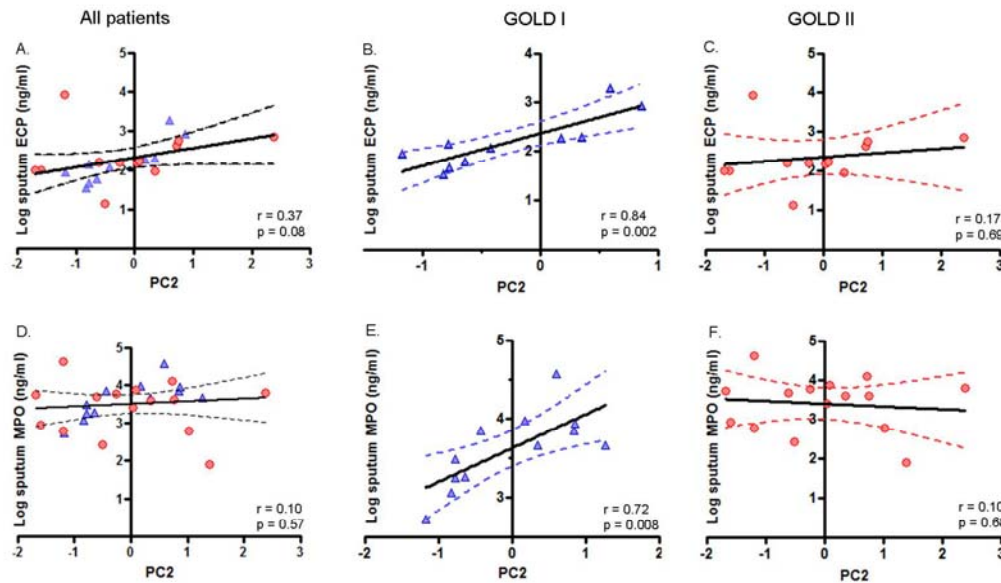
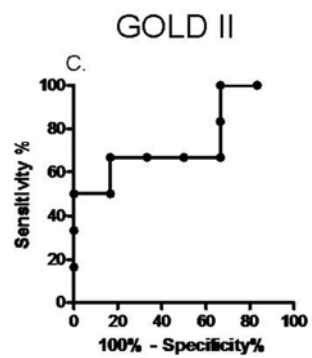
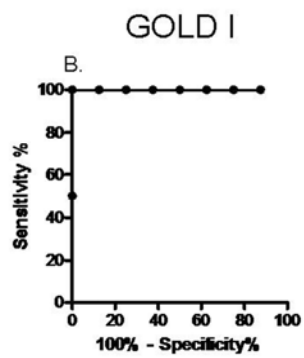
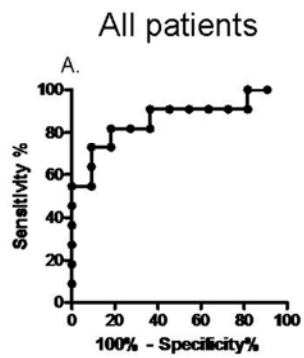


Figure 3

ROC curves with line of identity of breathprint principal component 2 predictive for sputum ECP (a-c) and MPO (d-f) for all subjects, GOLD stage I, and GOLD stage II, respectively.

ECP



MPO

