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Exhaled breath profiles in the monitoring of loss of control and clinical recovery in asthma

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Summary

Background: Asthma is a chronic inflammatory airway disease, associated with episodes of exacerbations. Therapy with inhaled corticosteroids (ICS) targets airway inflammation, which aims to maintain and restore asthma control. Clinical features are only modestly associated with airways inflammation. Therefore, we hypothesized that exhaled volatile metabolites identify longitudinal changes between clinically stable episodes and loss of asthma control.

Objectives: To determine whether exhaled volatile organic compounds (VOCs) as measured by gas-chromatography/mass-spectrometry (GC/MS) and electronic nose (eNose) technology discriminate between clinically stable and unstable episodes of asthma.

Methods: Twenty-three patients with (partly) controlled mild to moderate persistent asthma using ICS were included in this prospective steroid withdrawal study. Exhaled metabolites were measured at baseline, during loss of control and after recovery. Standardized sampling of exhaled air was performed, after which samples were analysed by GC/MS and eNose. Univariate analysis of covariance (ANCOVA), followed by multivariate principal component analysis (PCA) was used to reduce data dimensionality. Next paired *t* tests were utilized to analyse within-subject breath profile differences at the different time-points. Finally, associations between exhaled metabolites and sputum inflammation markers were examined.

Results: Breath profiles by eNose showed 95% (21/22) correct classification for baseline vs loss of control and 86% (19/22) for loss of control vs recovery. Breath profiles using GC/MS showed accuracies of 68% (14/22) and 77% (17/22) for baseline vs loss of control and loss of control vs recovery, respectively. Significant associations between exhaled metabolites captured by GC/MS and sputum eosinophils were found (Pearson $r \geq .46$, $P < .01$).

Conclusions & Clinical Relevance: Loss of asthma control can be discriminated from clinically stable episodes by longitudinal monitoring of exhaled metabolites measured by GC/MS and particularly eNose. Part of the uncovered biomarkers was associated with sputum eosinophils. These findings provide proof of principle for monitoring and identification of loss of asthma control by breathomics.

KEYWORDS
Asthma, electronic nose, exacerbation, exhaled breath analysis, gas-chromatography/mass-spectrometry, volatile organic compounds

1 | INTRODUCTION

Asthma is a chronic inflammatory disease of the airways that is associated with episodes of loss of control or exacerbations.[1] Asthma therapy with inhaled corticosteroids is targeted at the suppression of airway inflammation, which aims to maintain asthma control. Such anti-inflammatory therapy in asthma is currently guided by symptoms and lung function.[2] Because these clinical features are only modestly associated with airways inflammation,[3] there is a need for biomarkers that reflect inflammation more directly. Sputum induction is generally considered to represent a reliable, non-invasive method to assess and monitor airways inflammation in a more direct way.[4] This provides inflammatory cell differentials, from which the eosinophil counts have shown to be useful in optimizing asthma management and disease outcome.[5] Loss of asthma control or exacerbations of asthma are associated with an increase in sputum eosinophils,[6] but clinical application of sputum analysis in the monitoring asthma is somewhat limited by the requirement of laboratory facilities and the non-directly available results. Furthermore, in patients with severe and uncontrolled asthma, and especially during an exacerbation, induction of sputum can be troublesome because of saline-induced airway narrowing.[7] Therefore, there is a need for adequate surrogate markers of (changes in) airway inflammation in asthma that are easy to obtain.

Exhaled air contains volatile organic compounds (VOCs) that may be used as non-invasive biomarkers.[8] Measuring these metabolites in breath can be carried out by gas-chromatography/mass-spectrometry (GC/MS), which is required for identification of exhaled compounds and their concentrations.[9,10] Alternatively, cross-reactive sensors from electronic nose (eNose) technology allow pattern recognition of entire mixtures of VOCs.[10,11] This provides a real-time breathprint, which can be considered as a metabolomic fingerprint of exhaled air.

Previously, others and ourselves have shown that eNose breathprints and individual VOCs are related to inflammatory cell counts and markers in sputum, blood and bronchoalveolar lavage fluid in asthma and COPD patients.[12-15] Therefore, we hypothesized that exhaled breath metabolomics (breathomics) by GC/MS and eNose differs between controlled and uncontrolled episodes of the disease. For this purpose, loss of asthma control as indicated by an increase in symptoms and decrease in spirometric measures was prospectively induced by interruption of inhaled corticosteroids in patients with mild to moderate asthma.

2 | MATERIALS AND METHODS

2.1 | Subjects

Patients with a previous history of doctors diagnosed mild to moderate persistent asthma,[2] currently on ICS treatment (≥500 μg fluticasone or equivalent), were enrolled in the study. Asthma was confirmed by a positive history of recurrent wheeze, chest tightness and/or shortness of breath and the presence of airway hyperresponsiveness (PC20<8 mg/mL) or ≥12% reversibility in FEV_1 on salbutamol. Patients had either partly controlled asthma (any of the following: daytime symptoms >2x/wk; limitation of activities; nocturnal symptoms; rescue treatment >2x/wk; PEF or FEV_1<80% predicted) or controlled asthma (none of the above) based on the GINA criteria[2] and experienced at least one exacerbation or episode of loss of control during the past 2 years. A previous exacerbation or loss of control was defined as at least one of three criteria[1]: (i) start of systemic corticosteroids for at least 3 days, (ii) hospitalization or ER visit because of asthma requiring systemic corticosteroids, (iii) deterioration of symptoms, lung function or use of rescue bronchodilators >2 days leading to a GP or ER visit, requiring an increase/change in medication other than systemic corticosteroids. Patients were all current non-smokers (>12 months) with a maximum of 5 pack years, were treated with a stable dose of ICS and no systemic steroids, anti-IgE or antibiotics and experienced no respiratory infections for at least 4 weeks prior to screening.

Patients gave written informed consent. The study was approved by the Academic Medical Centre Medical Ethics Committee, registered at the Netherlands Trial Register under NTR3316 and was undertaken in accordance with the Declaration of Helsinki.

2.2 | Study design

This was a prospective intervention study.[16] Reduction in clinical asthma control and re-establishment of control was obtained by prompt and complete interruption of inhaled steroids (and LABA if applicable), followed by a course of oral steroids and restoration of inhaled steroids after loss of control. This is a model that others and ourselves have used in asthma previously.[17-19] This 14 weeks study included a screening visit and a visit for baseline measurements, followed by an open cessation phase of inhaled steroids for a maximum of 8 weeks or until loss of control, and a 4 weeks dose restoration phase. Patients were monitored daily by email, WhatsApp, phone or sms/text message contact regarding diary symptoms and daily electronic home peak flow and FEV_1. Patients paid four visits to the hospital (at screening, baseline, loss of control and recovery). The time and events schedule is shown in Figure 1.

At the screening visit, patients underwent lung function tests, methacholine challenge and skin prick tests. When fulfilling the inclusion criteria, patients returned 2-4 weeks later for the baseline visit (T0). At baseline, spirometry, sputum induction, peripheral blood sampling and exhaled NO measurements were performed. Patients were then instructed to discontinue their ICS. During the whole study, patients continued other asthma medications (except LABA) using the same dose and used their own short-acting β_2-agonist as needed. They were asked to home-monitor their morning PEF and FEV_1 values (best of three) using a portable spirometer (PiKo-1; nSpire Health GmbH; Oberthulba, Germany) and to inform the study physician of the values and their asthma symptoms (awakening during the past night due to asthma; number of rescue puffs needed in the past 24 hours) daily by email, WhatsApp, phone or sms/text message. An Asthma Control Questionnaire (ACQ)[20] was completed weekly. Exhaled breath samples were obtained during baseline, loss of control and recovery visits.

The study was suspended for a particular patient whenever loss of control occurred, or after 8 weeks if there had not been loss of control. Loss of asthma control was defined as the presence of at least two of three criteria[21]: (i) decrease in pre-bronchodilator morning PEF of ≥20% of baseline on ≥2 consecutive days, (ii) wakening due to asthma on ≥2 consecutive nights and (iii) use of ≥8 puffs short-acting β_2-agonist on ≥2 consecutive days. Measurements for the loss of control visit (T1) were performed as soon as possible. Loss of control was treated with oral prednisolone at 30-40 mg/d for 1 week and restoration of ICS. Four weeks after T1, the recovery visit was scheduled (T2) when the asthma status had returned to controlled.

2.3 | Exhaled breath collection and sampling of breath

Exhaled breath was collected as previously described[22,23] preceding sputum induction (for order of tests, see Figure 1). Patients breathed for 5 minutes at tidal volume through a two-way non-rebreathing valve and an inspiratory carbon VOC-filter (A2, North Safety, Middelburg, NL) to clean the inspired air. Next, the subject exhaled a single vital capacity volume into a 10 L Tedlar bag (SKC Inc, Eighty Four, PA, USA). Within 30 minutes after breath collection, two thermal desorption Tubes (Tenax GR SS 6 mm×7", Gerstel, DaVinci BV, Rotterdam, NL) were connected to the Tedlar bag for collection, transportation and storage of the expired VOCs. Each tube was sampled with 500 mL exhaled air at a flow of 250 mL/min using a peristaltic pump. VOCs present in exhaled breath were thereby captured onto the Tenax GR sorbent mesh in the tubes. Tubes were stored at 4°C and shipped to Philips Research (Eindhoven, the Netherlands) for GC/MS analysis and to the Academic Medical Centre, University of Amsterdam (Amsterdam, the Netherlands) for analysis by the electronic nose platform.[24] Such storage of breath VOCs has shown to preserve the eNose and GC/MS signal during 2 weeks, and therefore a 2 weeks episode was kept as the maximum storage period in this study.[25] The sampling of exhaled breath was always performed before sputum induction (Figure 1).

2.4 | Measurements in breath: electronic nose platform

After storage, the VOCs were removed from the tubes by heating (thermal desorption) in a Gerstel TDS3 desorption oven (Gerstel, Mülheim an der Ruhr, Germany) using nitrogen as carrier gas and captured in a Tedlar bag (500 mL in total). Obtained samples were used for further analysis by a composite eNose platform consisting of four eNoses from four different brands, using distinct sensor

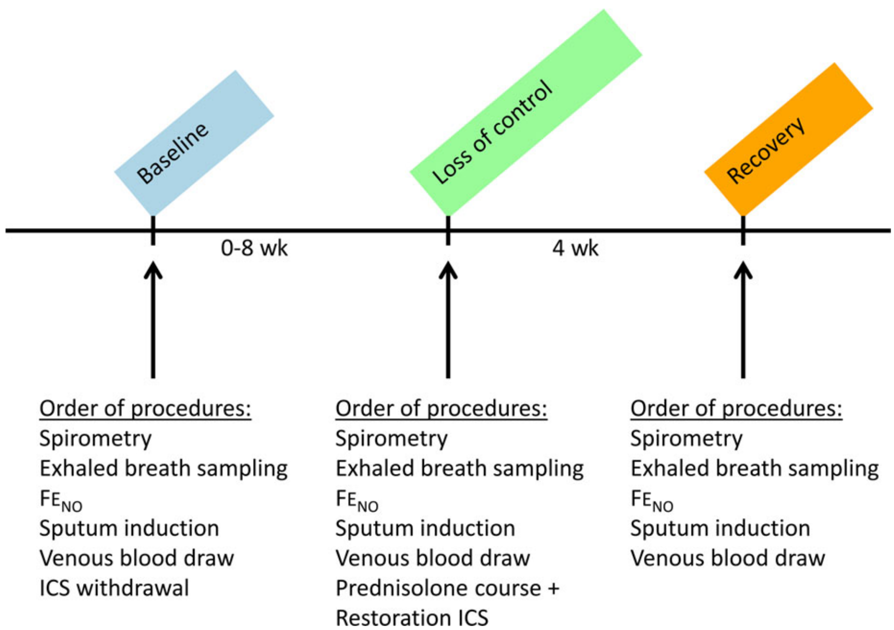

FIGURE 1 Time and events table, including order of procedures

technologies: (i) Cyranose C320 using carbon black-polymer sensors,[26] (ii) Tor Vergata eNose using quartz crystal microbalances (QMB) covered with metalloporphyrins,[27] (iii) Common Invent eNose using metal oxide semiconductor sensors[28] and (iv) Owlstone Lonestar based on field asymmetric ion mobility spectrometry.[29]

2.5 | Measurements in breath: GC/MS

GC/MS analysis was performed as described previously.[30] After transport and storage, the sorbent tubes with the VOCs were heated and thermally desorbed using a Gerstel TDS3 desorption oven (Gerstel, Mülheim an der Ruhr, Germany) with helium as carrier gas. The sample was transmitted to a packed liner, heated to 300°C for 3 minutes and transferred to a Tenax TA cold trap at −150°C, which was heated after 2 minutes to 280°C at 20°C/s and splitless injected onto the chromatographic column. The GC/MS includes separation of VOCs followed by their individual detection. To that end, the VOCs were first separated by capillary gas-chromatography with helium as a carrier gas at 1.2 mL/min (6890 N GC, Agilent, Santa Clara, CA, USA) on a VF1-MS column (30 m×0.25 mm, film thickness 1 μm, 100% dimethylpolysiloxane, Varian Chrompack, Middelburg, the Netherlands). The temperature of the gas chromatograph was adjusted in three steps: 40°C for 5 minutes, increased until 300°C with 10°C/min and held isothermal for 5 minutes. Subsequently, a quadrupole mass spectrometer (5975 MSD, Agilent, Santa Clara, CA, USA), in electron impact ionization mode at 70 eV, was used for charging the compounds and detection of resulting individual ions (ranging from 29 to 450 Da).

2.6 | Exhaled NO measurement (FE_{NO})

Exhaled NO (FE_{NO}) was measured using a portable rapid-response chemiluminescence analyzer (flow rate 50 mL/s; NIOX System, Aerocrine, Sweden) according to the guidelines of the American Thoracic Society.[31]

2.7 | Methacholine challenge

Spirometry (Masterscreen, CareFusion, Houten, the Netherlands) was performed by a trained lung function technician according to the latest ERS recommendations.[32] Airway hyperresponsiveness was assessed by methacholine challenge using MeBr (acetyl-β-methylcholine bromide) according to the standardized tidal volume method.[33]

2.8 | Allergy sensitization testing

Skin prick testing was performed using a pan-European panel of common aeroallergens. For skin testing, histamine and diluent as positive and negative controls were used.

2.9 | Sputum induction and processing

Sodium chloride aerosols 4.5% (w/v) were generated by an ultrasonic nebulizer (Ultraneb 2000; Devilbiss, Somerset, PA, USA) and administered to the patient through a 100-cm-long tube with an internal diameter of 22 mm and will be inhaled through the mouth with a 2-way valve, whilst wearing a nose clip. Prior to each induction, patients inhaled 400 μg salbutamol. Patients inhaled the saline aerosols during 3×5 minutes intervals, according to the ERS recommendations.[34] They were encouraged to cough and expectorate sputum. Sputum processing was performed using the whole sputum method.[35] Total cell counts and differential cell counts were obtained.

2.10 | Symptom score

Asthma control was assessed by the Juniper asthma control questionnaire (ACQ),[20] a validated 7-item questionnaire. The first six questions (night-time waking, symptoms on waking, activity limitation, shortness of breath, wheeze and rescue short-acting medication use) were scored by the patient, and the seventh question based on pre-bronchodilator percent predicted FEV_1 results was completed by a clinician. All items were equally weighted, and the final score was the mean outcome.

2.11 | Analysis

Pre-processing. GC/MS analysis, denoising, peak detection and alignment were performed as previously described,[28] using the XCMS package[36] (Scripps Center for Metabolomics, La Jolla, CA), and resulted in an ion fragment peak table serving as source for further analysis. As next step, all ion fragments with a mass and/or a retention time higher than n-tetradecane ($C_{14}H_{30}$, M=198 g/mol) were classified as non-volatile[37] and therefore excluded for further analysis. To make multivisit GC/MS analysis possible, fragments were reconstructed into compounds by running a principal component analysis (PCA) on all fragments within a retention time frame of 5 seconds. This time frame was set to overcome minor retention time variability during batch analysis. The total compound abundancy was calculated by adding intensities of all fragments with an absolute loading above 0.1 in Principal Component 1. A BoxCox[38] power transformation was applied to achieve optimal data distribution. Subsequently, the data were normalized by adjusting the average and standard deviation of each individual eNose sensor or GC/MS compound to, respectively, 0 and 1.

2.11.1 | Statistical analysis

In order to reduce the number of variables in comparison with the number of subjects, an initial data reduction step prior to multivariate analysis was made[39,40] by univariate analysis between exhaled markers and loss of control. To have optimal indication for level of control, ACQ scores from baseline, loss of control and recovery visits instead of the binary (yes/no) "loss of asthma control" were used. ACQ scores were associated with exhaled compound intensities, taking into account differences in ACQ between subjects at baseline by performing repeated measures analysis of covariance of multiple longitudinal data points (ANCOVA).[41] All GC/MS compounds or eNose sensors with an ANCOVA outcome of $P<.05$ and Pearson correlation ≥0.5 were determined as variable of interest. To rigorously control false discovery (FDR) and multicolinearity, we applied stringent recommendations[42] by using an FDR correction[43] of 5% and standardized QR decomposition.[44]

A principal component analysis (PCA) solely derived from baseline and loss of control visits data was performed to merge the variables of interest (molecular components for GC/MS or sensor signals for eNose) into a multivariate component. According to the Kaiser Criterion,[45] all principal components (PC's) with a eigenvalue above 1 were retained. The obtained PC's were considered as the training set. For verification purposes, the PC's of the loss of control + recovery visit and the baseline + recovery visit data sets were calculated based on the loading factors of the training set. Paired student *t* tests on the obtained PC's were performed to compare the means between the repeated measures: baseline vs loss of control, loss of control vs recovery and baseline vs recovery. *P*-values <.05 were considered significant. Boxplots, (mean) differences and 95% confidence intervals for the mean were plotted to gain overview and further insight into the results. Accuracies were determined based on the number of subjects with a similar or opposite change in signal in comparison with the group mean. Spectra of GC/MS compounds retaining after univariate analysis were provisionally identified based on NIST-library (v.2.0a) matching. Finally, the relationship between airway inflammation markers (sputum eosinophils; % and neutrophils; %) and univariate analysis persevering GC/MS compounds and PC's (GC/MS and eNose) was analysed by ANCOVA. The between-visit comparison of clinical, physiological and inflammatory variables was performed using Friedman tests. All analyses were performed in R studio (v.0.99.891) using R (v.3.1.2) as engine, combined with R packages (pwr, XCMS, MASS, HH, ggplot2, tableone).

2.11.2 | Sample size estimation

An estimated effect size (Cohen's *d*) based on the univariate and multivariate logistic regression coefficients[46] from published GC/MS analysis between controlled and uncontrolled asthma patients (ACQ<1 vs ACQ ≥1)[13] resulted in a sample size calculation of 14 patients (power 80%, significance level=0.05). We assessed that 50% of the enrolled patients would experience a loss of control following steroid withdrawal[17-19] and a dropout rate of 10%; therefore, we aimed to include 31 patients.

3 | RESULTS

3.1 | Subjects

Twenty-eight patients were tested for eligibility. Two of them did not meet inclusion criteria and three withdrew consent. From the remaining 23 asthma patients, twenty-two reached the criteria for loss of asthma control. Baseline characteristics of these subjects are described in Table 1. The median age of the participants was 25 (IQR:21-32) and 73% was female (n=16). Six patients had an inhaled corticosteroids average of 1000 μg fluticasone or equivalent, the remaining 73% had an average of 500 μg. The average post-bronchodilator FEV_1 percentage predicted at baseline was 107.5 (SD±12.09). The median time until loss of control was 22 days (interquartile range [IQR]=16.8-33.0). When comparing baseline, loss of control and recovery visit characteristics (Table 2), patients had significant differences in: ACQ scores ($P<.01$), pre- and post-bronchodilator FEV_1 ($P<.01$), FE_{NO} levels ($P<.01$) and higher eosinophil counts in sputum ($P=.01$).

TABLE 1 Demographic data and baseline characteristics of study population

Subjects n	22
Age; years [median | IQR]	25 | 21-32
Gender; female [%]	73
Body mass index (kg/m^2) [mean±SD]	25.23±4.37
Atopy; positive [%]	95
PC_{20}; mg/mL [median | IQR]	2.06 | 0.79-3.34
LABA; use [%]	77
ACQ; Juniper [median | IQR]	0.93 | 0.57-1.29
$FEV_{1\%}$ predicted [mean±SD]	101.95±11.24
$PbFEV_{1\%}$ predicted [mean±SD]	107.45±12.09
FE_{NO}; ppb [median | IQR]	19 | 10-38
Sputum eosinophils; % [median | IQR]	0.40 | 0.20-3.83
Sputum neutrophils; % [median | IQR]	31.45 | 25.60-60.55
Blood eosionophils; 10^9/L [median | IQR]	2.75 | 1.40-4.63
Blood neutrophils; 10^9/L [median | IQR]	62.45 | 52.08-64.79

Atopy, skin prick testing; PC_{20}-methacholine challenge using MeBr; LABA, regular usage of long-acting β adrenoceptor agonists; ACQ, Juniper, Asthma Control Questionnaire; FEV_1, forced expiratory volume in one second; $PbFEV_1$, post-bronchodilator forced expiratory volume in one second; FE_{NO}, fraction of exhaled nitric oxide in parts per billion.

3.2 | GC/MS

The analysis of, in total, 66 breath samples by GC/MS resulted in the detection of 729 different ion fragments. Those could be reconstructed into 144 unique volatile organic compounds. After optimization of data distribution and normalization, univariate ANCOVA between ACQ scores and VOC's identified six compounds of interest. Three compounds sustained FDR correction and QR decomposition (Methanol - CH_3OH - Mass: 32.04 g/mol, retention time: 349sec; Acetonitrile - C_2H_3N - Mass: 41.05 g/mol, retention time: 450sec; Bicyclo [2.2.2]octan-1-ol, 4-methyl - $C_9H_{16}O$ - Mass: 140.22 g/mol, retention time: 1112 seconds).

Using univariate outcomes as input for the multivariate principal component analysis and following the Kaiser criterion selection, only principal component 1 (PC1) [loadings: methanol: −0.56; acetonitrile: −0.7; bicyclo[2.2.2]octan-1-ol, 4-methyl; −0.43] retained for between-visit analysis. Paired student *t* tests with PC1 as input resulted in: baseline vs loss of control ($P=.02$), loss of control vs

TABLE 2 Visit characteristics

Visit type	Baseline	Loss of control	Recovery	*P*-value
Subjects n	22	22	22	
ACQ; Juniper [median | IQR]	0.93 | 0.57-1.29	2.86 | 2.61-3.14	0.43 | 0.29-1.07	<.01
FEV1% predicted [mean±SD]	101.95±11.24	89.59±15.50	103.14±13.29	<.01
PbFEV1% predicted [mean±SD]	107.45±12.09	102.32±12.89	108.23±13.57	<.01
FE_{NO}; ppb [median | IQR]	19 | 10-38	33 | 20-70	19 | 11-23	<.01
Sputum eosinophils; % [median | IQR]	0.40 | 0.20-3.83	3.55 | 0.40-12.78	0.60 | 0.20-1.60	.01
Sputum neutrophils; % [median | IQR]	31.45 | 25.60-60.55	52.95 | 33.53-65.98	50.00 | 24.60-65.60	.27
Blood eosinophils; 10^9/L [median | IQR]	2.75 | 1.40-4.63	4.02 | 2.13-8.16	3.13 | 1.55-6.20	.45
Blood neutrophils; 10^9/L [median | IQR]	62.45 | 52.08-64.79	56.92 | 50.89-66.11	54.86 | 49.96-60.31	.21

ACQ - Juniper, Asthma Control Questionnaire; FEV_1, Forced Expiratory Volume in one second; $PbFEV_1$, Post-bronchodilator Forced Expiratory Volume in one second; FE_{NO}, Fraction of Exhaled Nitric Oxide in parts per billion. Between visit comparisons by Friedman tests.

recovery (*P*<.01) and baseline vs recovery (*P*=.41). Accuracies based on differences, shown in Figure 2, resulted in a 68% (14/22) correct classification for baseline vs loss of control and 77% (17/22) correctness for loss of control vs recovery, respectively.

3.3 | Electronic nose platform

After pre-processing, three eNose sensors sustained ANCOVA, FDR correction and QR decomposition. This resulted in two principal components with an eigenvalue >1. There were no significant differences between the visits for PC 1 (PC1: baseline vs loss of control [*P*=.54], loss of control vs recovery [*P*=.09] and baseline vs recovery [*P*=.17]). However, there were for PC2 (PC2: baseline vs loss of control [*P*<.01], loss of control vs recovery [*P*<.01] and baseline vs recovery [*P*=.62]). Accuracies for eNose analysis resulted in 95% (21/22) and 86% (19/22) correct classification for baseline vs loss of control and correctness for loss of control vs recovery, respectively (Figure 3). The eNose that most prominently drove the discriminative signal with regard to loss of control was the ion mobility spectrometer.

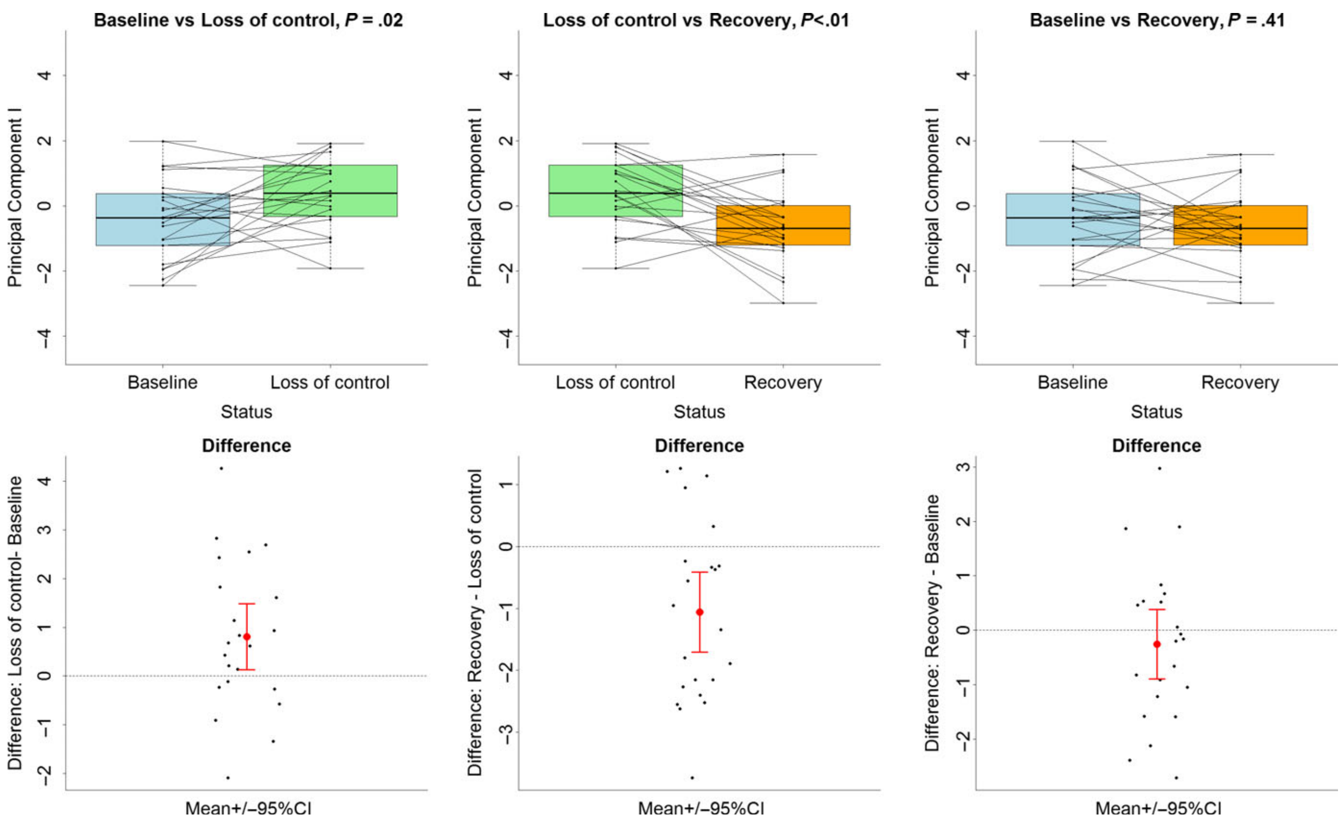

FIGURE 2 Exhaled breath profiles (principal components) obtained by GC/MS with mutual comparisons between baseline, loss of control and recovery. Upper panel: boxplots of paired *t* tests. Lower panel: difference plots, incl. means and 95% confidence intervals for the means

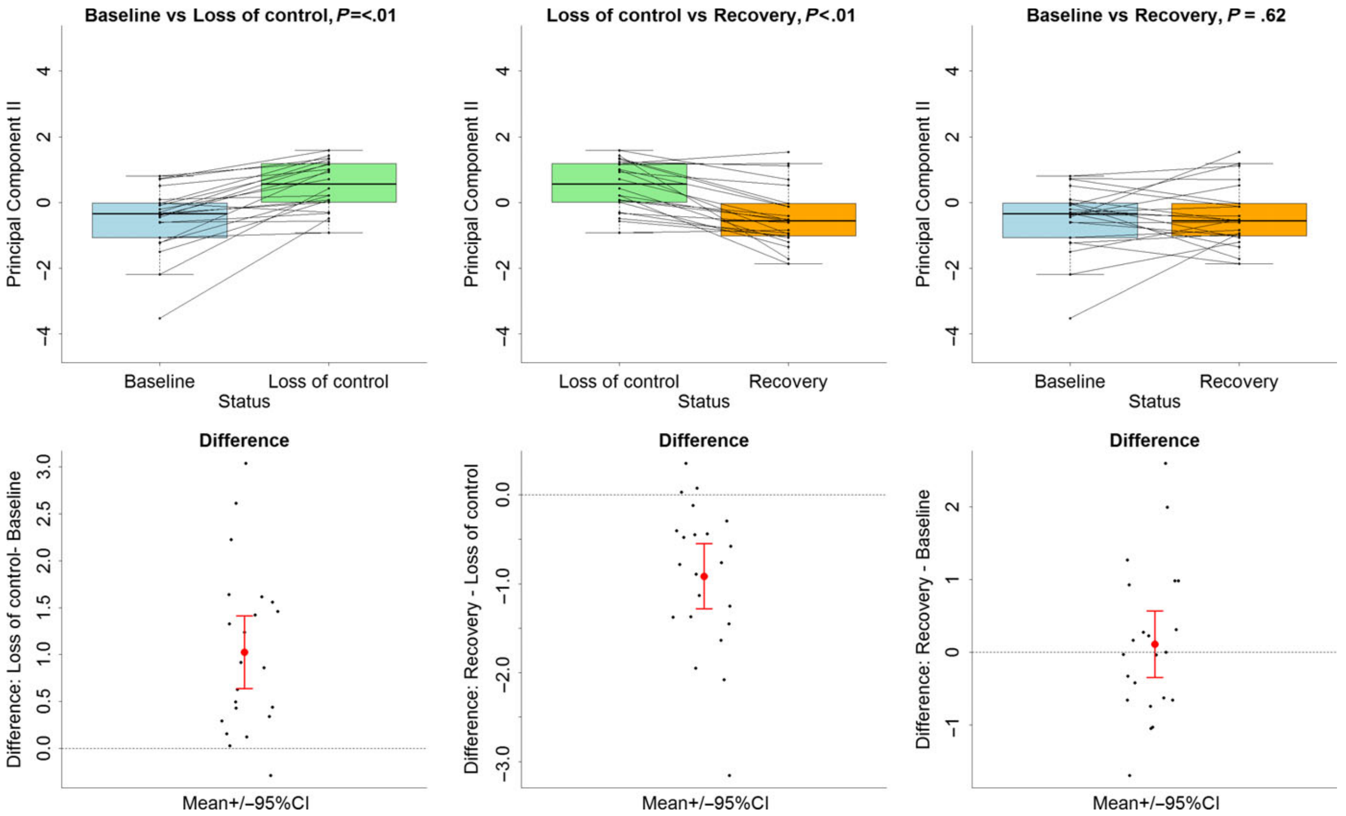

FIGURE 3 Exhaled breath profiles (principal components) as obtained by eNose with mutual comparisons between baseline, loss of control and recovery. Upper panel: boxplots of paired *t* tests. Lower panel: difference plots, incl. means and 95% confidence intervals for the means

3.4 | Association with airways inflammation and lung function

Using ANCOVA, two (acetonitrile and bicyclo[2.2.2]octan-1-ol, 4-methyl) of three remaining GC/MS compounds and GC/MS PC1 were found to be significantly correlated with sputum eosinophils, with within-patient Pearson's *r*'s of, respectively, *r*=.46, *r*=.47 and *r*=.53, all *P*<.01. No significant correlation was found with sputum neutrophils. For methanol, acentonitrile and GC/MS PC1, a significant correlation with PbFEV1% predicted was found; furthermore, 4-methyl, bicylo[2.2.2]octan-1-ol and GC/MS PC1 showed a significant association with FE_{NO}. PC's derived from eNose sensors did not show a significant relationship with sputum eosinophils nor with neutrophils, whereas there were significant associations with PbFEV1% predicted and FE_{NO} (Tables 3,4 and Figure 4).

4 | DISCUSSION

The present study prospectively examined the changes in molecular profiles of exhaled breath during loss of asthma control and subsequent clinical recovery. Two different methods of breath analysis were applied, GC/MS and eNose technology, showing similar results albeit with different strengths. Using GC/MS, the accuracies of distinguishing baseline, loss of control and recovery were relatively modest (68%-77%), whilst for eNose, the accuracies reached higher

TABLE 3 ANCOVA between univariate and multivariate outcomes vs airway inflammation markers

		Sputum eosinophils; %		Sputum neutrophils; %	
Method	Univariate outcome	*P*-value	Pearson's *r*	*P*-value	Pearson's *r*
GC/MS	Methanol	.42	−.15	.14	−.26
	Acetonitrile	<.01	−.46	.29	−.19
	bicyclo[2.2.2]octan-1-ol. 4-methyl	<.01	−.47	.40	.15
	PC1	<.01	.53	.32	.18
eNose	PC1	.12	−.27	.48	.13
	PC2	.16	.37	.30	.18

Pearson's *r*—within-patient correlation coefficient.

TABLE 4 ANCOVA between univariate and multivariate outcomes vs lung function PbFEV1% predicted and FENO

		$PbFEV_1$; % predicted		FENO; PPB	
Method	Univariate outcome	*P*-value	Pearson's *r*	*P*-value	Pearson's *r*
GC/MS	Methanol	.04	.31	.38	−.13
	Acetonitrile	<.01	.58	.13	−.23
	bicyclo[2.2.2]octan-1-ol. 4-methyl	.79	.04	<.01	−.60
	PC1	<.01	−.53	.03	.33
eNose	PC1	.12	.24	.12	−.24
	PC2	<.01	−.42	.02	.35

$PbFEV_1$, post-bronchodilator forced expiratory volume in one second; FE_{NO}, fraction of exhaled nitric oxide in parts per billion; Pearson's *r*, within-patient correlation coefficient.

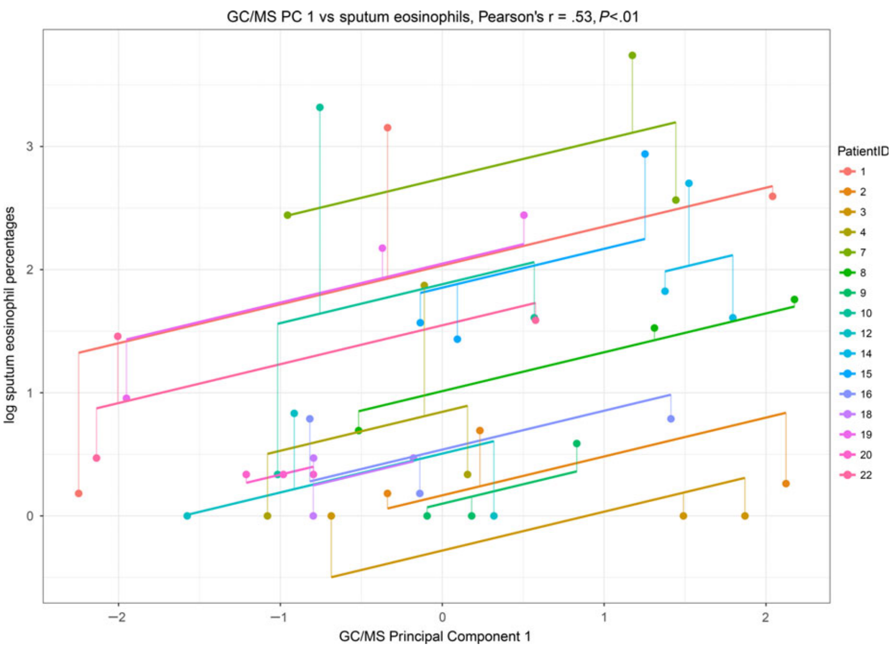

FIGURE 4 Associations between GC/MS Principal Component I and log-transformed sputum eosinophil percentages by ANCOVA analysis (p<0.01, Pearson's r=0.53) for 16 out of 22 subjects (based on three successful sputum inductions (baseline, loss of control and recovery). Bullets represent actual outcomes, parallel lines the ANCOVA modelling for each patient and vertical lines the residuals.

values (86%-95%). Our results show that exhaled breathprints can be considered as useful, composite marker for the identification of loss of control in asthma following cessation of inhaled corticosteroids. This finding needs to be extended to naturally occurring exacerbations, which merits a real-life asthma monitoring study.

The novelty of the present study is represented by the prospective follow-up of breathomics in asthma patients during the loss and recovery of clinical control. Our data therefore extend previously published cross-sectional data in adults[13] and longitudinal data in children,[47] demonstrating various accuracies in discriminating controlled and uncontrolled asthma by GC/MS analysis of exhaled breath. In addition, the present study independently confirms and extends previous data on eNose signals during loss of control by withdrawal and restoration of steroids in asthma by van der Schee et al.[48] Our data are demonstrating the longitudinal *changes* in eNose signal between baseline, loss of control and recovery, whilst relating those to the course of symptoms, lung function and inflammatory cell counts in sputum. When using FE_{NO} as singular exhaled biomarker, a recent meta-analysis showed that tailoring asthma therapy based on FE_{NO} reduces asthma exacerbations in adults, even though it does not impact day-to-day symptoms.[49] Our present data are indicating that composite molecular signatures as obtained by GC/MS, and the more so by eNose, are also capturing clinically relevant changes in asthma control.

One of the strengths of this study is the longitudinal design providing the first data on monitoring worsening as well as recovery of asthma control using exhaled breath analysis. Secondly, patients with asthma were carefully selected. They were all current non-smokers and had to have a history of at least one exacerbation in the past 2 years but stable at the commencement of the study. Finally, we used an accepted model for mimicking of asthma exacerbations by interruption of inhaled steroids.[17-19] Moreover, we applied a validated method of breath collection minimizing environmental influences.[22] Breath samples were assessed by a panel of electronic noses as well as gas-chromatography/mass-spectrometry. Both methods were analysed using stringent recommendations to avoid false discovery and led to analogous results, making it unlikely that the findings in this study came up by chance.

We realize that this study has several limitations. Firstly, the study design was uncontrolled. Our aim was to focus on the changes in exhaled breath profiles during deterioration and restoration of asthma control. A healthy control group would have allowed inference on how divergent from normal the GC/MS and eNose signals are in the asthmatics when being well controlled. Another control group of asthmatics in whom inhaled steroids were not withdrawn would have permitted more conclusive interpretation regarding the causative effects of the treatment intervention. Given the complexity of the prospective steroid withdrawal, we did not add these control groups to the design of the study. Therefore, our results should be cautiously interpreted. Second, ACQ-7 was used as gold standard for asthma control. This is largely reflecting a subjective disease marker, even though it also includes spirometry. Third, we cannot exclude that the per-protocol induced changes in (inhaled) steroid therapy have directly influenced the observed differences in breathprints between controlled and uncontrolled asthma. However, the present data are showing that the exhaled breath signal is associated with eosinophilic airways inflammation, thereby longitudinally validating previous studies by others and ourselves.[12,13,50] Another potential weakness of the study was the percentage of patients experiencing a loss of disease control. Based on studies using a similar exacerbation model as we did, loss of control percentages varied between 53 and 66%.[17–19] In this study, however, 22 of 23 patients (96%) experienced loss of control within 8 weeks after interrupting their maintenance medication. This rendered an analysis in a control group of non-exacerbators impossible. On the other hand, the sample size of those patients that did experience loss of asthma control was higher than calculated to be sufficient for determination of predictive value of exhaled breath analysis to discriminate between stable and uncontrolled asthma periods. Furthermore, it cannot be excluded that our results were affected by changes in breathing volume and expired flow during airflow limitation at loss of control. However, we believe this is unlikely, as induced bronchoconstriction by methacholine did not significantly influence the eNose signal in asthmatics.[51] It needs to be emphasized that although the breath analysis methods used in this study have been validated in earlier studies, the methods are not directly suitable for use in clinical day-to-day practice. Whereas GC/MS requires a laboratory for the handling of the samples, electronic nose technology is currently being modified for use at the doctor's office.[52] Finally, the choice of the statistics may have affected our outcomes. By applying ANCOVA for the univariate analysis, we aimed to obtain an appropriate balance between basic *t* tests and more complex linear mixed models.

The GC/MS compounds derived by univariate analysis are known from literature. Acetonitrile[53] and methanol[53,54] are both reported as common molecules in exhaled breath, for example associated with pathogenic bacteria.[55] The more complex 4-methyl-Bicyclo[2.2.2]octan-1-ol contains a characteristic bicyclic ring, which matches the compound described by Ibrahim et al. as 3,7,7-trimethyl-Bicyclo[4.1.0]hept-2-ene (known as: [+]-3-Carene), reported to be correlated with sputum eosinophils.[13] Bicyclic rings are considered as interesting moieties. Molecules with such components are known as bioactive,[56] can serve as organic core for peptides[57] and are used in drugs.[58] Two of three GC/MS compounds and the composite principal component 1 were associated with sputum eosinophil percentage, which suggests that at least part of the breath signal during loss and restoration of asthma control was derived from a flare-up and suppression of eosinophilic airways inflammation, respectively.

Notably, the cross-reactive sensor technologies of eNoses were capturing the differences between controlled and uncontrolled asthma better than the multivariate GC/MS analysis, but no significant correlations between eNose-derived PC's and sputum eosinophil or neutrophil percentages were found. This may be caused by the capacity of eNoses in potentially using many small non-significant changes in exhaled VOCs that may not be picked up by peak detection using GC/MS, in other words indicating a broader sensitivity for loss of asthma control by eNose technology. Whereas, significant compounds derived by GC/MS might reflect a more specific signal, which can be associated with more refined clinical and inflammatory characteristics such as a flare-up of local eosinophilic inflammation. These findings are underlining the methodological strengths of both methods of breath analysis: GC/MS as technology for the assessment of pathophysiological background against eNose as tool that is primarily suitable for producing diagnostic probabilities and possibly monitoring.

What are the clinical implications of our data? The present composite eNose platform was not designed for clinical usage nor for benchmarking various eNose brands for clinical application. This proof of principle study shows that exhaled breath analysis techniques, such as eNose technology, are capable of monitoring asthma control that is associated with a flair-up of airways inflammation. This may qualify in fulfilling the long outstanding clinical need for novel (composite) biomarkers that warrant simple and accurate management of asthma patients. Tailoring asthma therapy in adults by inflammatory biomarkers such as sputum eosinophils[5] and more recently by FE_{NO}[49] has been shown to reduce exacerbations. Considering the accuracy of eNose to identify loss of control together with the association between specific VOCs and sputum eosinophilia as shown in this present study and by previous investigators,[12,13] the application of metabolomic fingerprints derived from exhaled breath should be developed into a quick and non-invasive approach for asthma monitoring and management. Real-life loss of control and exacerbations are mostly driven by other factors than reduction in inhaled steroids, including respiratory virus infections, allergens and other environmental exposures.[59] Therefore, the present experimental study should be followed by a real-life monitoring study of asthma control and exacerbations using GC/MS and eNose.

In conclusion, metabolomics of exhaled breath enables discrimination between stable periods and periods of loss of control during longitudinal follow-up of patients with asthma, which is partly associated with sputum eosinophils. The present proof of principle supports bringing eNose technology to point of care[52] for broad clinical validation in the monitoring and management of asthma.

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CONFLICTS OF INTEREST

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