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Predicting steroid responsiveness in patients with asthma using exhaled breath profiling

Running Title: Predicting steroid responsiveness in patients with asthma using exhaled breath profiling

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KEYWORDS

Asthma, Exhaled Breath, Steroid Responsiveness, VOCs, Volatile Organic Compounds, eNose, Electronic Nose, Oral steroids.

ABSTRACT

Background Exhaled breath contains disease-dependent volatile organic compounds (VOCs), which may serve as biomarkers distinguishing clinical phenotypes in asthma. Their measurement may be particularly beneficial in relation to treatment response.

Objective Our aim was to compare the performance of electronic nose (eNose) breath analysis with previously investigated techniques (sputum eosinophils, exhaled nitric oxide (F_{ENO}) and airway hyper-responsiveness) to discriminate asthma from controls and identify steroid responsiveness in steroid free patients. Trial registration ACTRN12613000038796.

Methods Twenty five patients with mild/moderate asthma had their inhaled steroid treatment discontinued until loss of control or 28 days. They were subsequently treated with oral prednisone 30mg/day for 14 days. Steroid responsiveness was defined as an increase of either $>12\%$ FEV_1 or >2 doubling doses $PC_{20}AMP$. Steroid free assessment of sputum eosinophils, F_{ENO} and exhaled breath VOCs were used to construct algorithms predicting steroid responsiveness. Performance characteristics were compared by ROC analysis.

Results The eNose discriminated between asthma and controls (area under the curve = 0.766 ± 0.14 ; $p=0.002$) with similar accuracy to F_{ENO} (0.862 ± 0.12 ; $p<0.001$) and sputum eosinophils (0.814 ± 0.15 ; $p<0.001$). Steroid responsiveness was predicted with greater accuracy by VOC-analysis (AUC = 0.883 ± 0.16 ; $p=0.008$) than F_{ENO} (0.545 ± 0.28 ; $p=0.751$) or sputum eosinophils (0.610 ± 0.29 ; $p=0.441$).

Conclusions and Clinical Relevance Breath analysis by eNose can identify asthmatic patients and may be used to predict their response to steroids with a greater accuracy than sputum eosinophils or F_{ENO} . This implies a potential role for breath analysis in the tailoring of treatment for asthma patients.

INTRODUCTION

Asthma is a pathologically heterogeneous disease, and may be characterised with reference to the underlying airway inflammatory cell type e.g. eosinophils , neutrophils . This is helpful in that the cell type is related to the clinical and treatment-response phenotypes.[1,2] Phenotyping patients has the potential to enable therapy to be targeted towards those most likely to benefit, thus avoiding wasteful treatment or potentially harmful side-effects. This is true for existing as well as novel therapies, notably inhaled corticosteroids (ICS).

Induced sputum cell counts and fractional exhaled nitric oxide measurements ($F_{E}NO$) are markers of airway inflammation which have also been validated as predictors of steroid-responsiveness.[3,4] However, for induced sputum, the need for technical expertise limits the provision of a widely available quality service, and $F_{E}NO$, although easily available, is associated with high negative but only modest positive predictive values for steroid responsiveness.[5] There is still a need for alternative, simple, non-invasive, validated techniques for assessing clinical asthma phenotypes.

Over the last 5 years, interest in analyzing the constituents of exhaled breath as a means of characterizing airway pathology has expanded rapidly.[6,7] Exhaled breath contains a vast range of volatile organic compounds (VOCs) and these alter in relation to both airways as well as systemic disease.[8] The candidate methodologies for VOC analysis include chemical analytical techniques (e.g. NMR, GC-MS, PT-RMS, SIFT-MS) and methods based on pattern recognition, a so-called “electronic nose” (eNose).[9] The last of these closely resembles mammalian olfaction.[10] eNoses employ an array of cross-reactive sensors that exhibit reversible interactions with the total VOC mixture[11]. Combined sensor responses can be integrated using pattern recognition algorithms to derive a “breathprint” for an

underlying disease state or pathological phenotype.[12,13] eNose technology has been successfully used to diagnose respiratory tract cancer,[14–16] and in airways disease, can discriminate patients with asthma[17,18] from healthy controls or COPD.[19,20] More recently, two studies have independently reported an association between exhaled breath VOCs and markers of airway inflammation.[21,22] Given that responsiveness to steroids is closely related to the presence of an eosinophilic cell type,[23] this suggests that VOC analysis may be able to identify those subjects responsive to treatment with steroids.

In this study we hypothesised that exhaled VOC profiles would differ significantly between patients with asthma and controls, and also between patients who were responsive to steroid treatment compared to those who were unresponsive. Our aims were twofold; Firstly, we wished to compare the diagnostic accuracy of an eNose against existing measurements of airway inflammation (sputum eosinophils and $F_{E}NO$) in discriminating patients with asthma from healthy controls. Secondly, we wished to assess the eNose as a predictor of steroid responsiveness in patients with asthma, and compare its performance with other pre-treatment predictors: airway hyper-responsiveness (AHR to hypertonic saline), sputum eosinophils and $F_{E}NO$.

METHODS

Subjects

Patients with mild to moderate asthma[24] and healthy controls participated. Patients were aged 18 to 75 and were recruited from primary care centres in 2010 and 2011. Each had doctor-diagnosed asthma, and the majority were taking regular inhaled corticosteroid treatment for more than 6 months. They were excluded if they had experienced any respiratory tract infection or had used oral prednisone during the previous 4 weeks. The healthy controls were non-atopic and non-asthmatic, and had negative skin prick allergen

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tests, $F_{E}NO < 25$ ppb, no evidence of airways hyper-responsiveness to hypertonic saline, and no reversibility to bronchodilator. Smoking subjects had smoked < 10 pack years and had ceased smoking > 10 years ago. The study was reviewed by the Lower South Island Ethics Committee (approval number:LRS/09/10/043) and all participants provided written informed consent. The study was registered in the Australia New Zealand Trial Registry under ACTRN12613000038796.

Design & Procedures

For patients with asthma, there were three phases in the study: run-in (phase 1), steroid withdrawal if appropriate (phase 2), and open label treatment with oral steroid (phase 3) (see Figure 1). The study was designed as an open label study because the placebo effect on the VOC-profile and parameters used to assess steroid responsiveness can be assumed to be very limited.

Initial measurements consisted of $F_{E}NO$, spirometry and skin prick testing. During phase 1, patients were provided with a 14-day diary to generate data to determine prospective individualized loss of control (LOC) criteria. Criteria for LOC were: $> 20\%$ decrease in morning/evening peak expiratory flow (PEF) for 2 days; $> 40\%$ decrease in morning/evening PEF on any one day; $> 10\%$ decrease in mean morning PEF over a week; reliever beta-agonist use > 4 doses in any one day more than the average daily use during run-in; waking due to asthma > 2 nights per week above run-in weekly mean.[25]

During phase 2, ICS and other maintenance asthma treatment was withdrawn. Patients were contacted thrice weekly, and instructed to contact the investigators if/when LOC criteria were reached. The next study visit was planned within 24 hours of LOC occurring, or after 28 days, whichever occurred sooner. If LOC was due to likely respiratory infection, based on

clinical judgment, patients were excluded from the study. Data obtained at the end of phase 2 were used for comparisons with healthy controls.

Patients entered phase 3 only if they had AHR ($PD_{15}HS < 12\text{mL}$ (provocative dose of hypertonic saline causing a 15% fall in forced expiratory volume in one second (FEV_1)) and/or a $>12\%$ improvement in post-bronchodilator FEV_1 at LOC. This was done to prevent a ceiling effect in which patients would be unable to respond to the initiated steroid treatment because they were clinically stable. Other measurements at LOC/28 days included, in sequential order: Asthma Control Questionnaire (ACQ),^[26] $F_{E}NO$, exhaled breath analysis by eNose, spirometry, hypertonic saline challenge and sputum induction. AHR to adenosine monophosphate (AMP) was assessed on the subsequent day, but omitted for safety reasons if FEV_1 was $<50\%$ predicted or $<1.2\text{L}$.

Patients then commenced a 14-day course of oral prednisone 30mg/day, at the end of which interval all study procedures except for hypertonic saline challenge and sputum induction, were repeated in the same order. Data obtained at the end of phase 3 were used to identify steroid responsive and steroid unresponsive patients. Steroid responsiveness was defined as one or both of the following: increase in FEV_1 of $\geq 12\%$; increase in provocative concentration of AMP causing a 20% fall in FEV_1 ($PC_{20}AMP$) of ≥ 2 doubling doses. If the AMP challenge was omitted for safety reasons and the patient was not steroid responsive on basis of an increase in FEV_1 , they were possible false negatives and therefore excluded from analysis of steroid responsiveness.

Induced sputum was collected and processed according to a standard protocol by a blinded qualified researcher.^[27] $F_{E}NO$ was measured using the NiOX MINO (Aerocrine, Solna, Sweden).^[28] AMP challenge tests were conducted according to ATS and ERS guidelines.^[29,30] Exhaled breath collection using the eNose was performed according to previously validated and published methodology showing adequate repeatability and

reproducibility.[17,19,31,32] In short subjects inhaled VOC-filtered air during a 5-minute wash out period, and thereafter breath was collected during a vital capacity manoeuvre into a inert bag. Breath was subsequently analyzed within 10 minutes by a Cyranose 320® eNose.

Sample size estimation

Based on data from a previous study[17], it was known that 10 subjects per group were sufficient to distinguish asthma from healthy controls using the eNose. There were no prior data available to estimate the study numbers required to compare steroid responsive versus unresponsive patients. Therefore a *post hoc* analysis of the power of this study based on the established effect size was done to assess whether subject numbers in phase 3 were sufficient. To minimize the risk of overfitting data was internally validated by bootstrapping procedure.

Data-analysis

Principal component analysis (PCA) was used to capture the variance of the eNose sensor data into a set of orthogonal principal components. Discriminating components were selected by unpaired t-test. Selected principal components, $F_{E}NO$ values and sputum eosinophils (% of total cell count) were individually used in a canonical discriminant analysis (CDA) to classify subjects according to each of these predictors. The canonical functions were used to construct receiver operator characteristic curves. These were internally cross-validated by bootstrapping procedure. Optimum (upper left corner) single spot sensitivity, specificity, positive and negative likelihood ratios were calculated. Pearson correlation coefficients were used to assess associations between breath profile and the other predictors.

RESULTS

Thirty two patients with diagnosed asthma were enrolled into the study, of whom 25 fulfilled entry criteria for the steroid trial and completed the study. There were 20 healthy controls. The baseline characteristics of the study subjects are shown in Table 1. After steroid withdrawal (phase 2), 13/25 patients with asthma (52%) fulfilled loss of control (LOC) criteria within 28 days. PC₂₀AMP, FEV₁ and FVC values after steroid withdrawal did not differ significantly between steroid responsive and unresponsive patients. Seven of these subjects could not undergo an AMP challenge at LOC for safety reasons (FEV₁ <50% predicted), and were also steroid unresponsive based on post-bronchodilator changes in FEV₁. To prevent a false negative outcome data for these 7 patients had to be excluded from the analyses which focused on predicting steroid responsiveness.

Following the course of oral prednisone (phase 3), 11/18 (61%) were steroid responsive as defined by *a priori* criteria. Six of the 11 steroid responsive patients had experienced LOC compared to 1 steroid unresponsive subject (Fisher's exact, $p=0.15$). As expected, following the course of prednisone, there were significant changes in all of the measured end-points: FEV₁ ($p=0.001$), FVC ($p=0.003$), airways hyper-responsiveness to AMP ($p=0.001$), ACQ ($p=0.001$) and F_ENO ($p<0.001$). The magnitude of these changes was consistently greater in the responsive group (Table 2).

Discrimination between patients with asthma and controls

The eNose significantly discriminated patients with asthma from healthy controls (AUC $\pm$ 95% C.I. = 0.766 ± 0.14 ; $p=0.002$). A scatter plot depicting the separation of the two groups by principal components is shown in Figure 2A. Sputum eosinophils (%) and F_ENO (ppb) identified patients with similar accuracies (AUCs 0.814 ± 0.15 ; $p<0.001$ for eosinophils, and 0.862 ± 0.12 ; $p<0.001$ for F_ENO respectively). Importantly, the discrimination between asthma patients and healthy controls using the eNose was maintained even after the former

group had received oral prednisone ($AUC = 0.842 \pm 0.12$; $p < 0.001$). In contrast, the diagnostic value for F_{ENO} was somewhat weakened ($AUC = 0.738 \pm 0.15$; $p = 0.007$). Receiver operator characteristic curves for each of the diagnostic tests are shown in Figure 2B.

Discrimination between patients who did / did not lose control after steroid withdrawal

Although not used specifically to detect LOC after withdrawal of ICS treatment, the ability of each of the diagnostic tests (eNose, sputum eosinophils and F_{ENO}) to discriminate between patients who did and did not lose control after steroid withdrawal was also tested. Both the eNose and sputum eosinophils were able to distinguish LOC from no-LOC (AUCs 0.814 ± 0.17 ; $p = 0.008$, and 0.868 ± 0.17 ; $p < 0.002$ respectively. F_{ENO} was not able to make this distinction ($AUC = 0.673 \pm 0.22$; $p = 0.142$).

Discrimination between patients who were / were not steroid responsive

The eNose VOC-profile obtained at LOC successfully predicted responsiveness to subsequent treatment with oral prednisone. The discrimination between steroid-responsive and steroid-unresponsive patients was significant ($AUC = 0.883 \pm 0.16$; $p = 0.008$). A scatter plot depicting the separation of the two groups by principal components is shown in Figure 3A. Somewhat surprisingly, sputum eosinophils (%) and F_{ENO} values at LOC were unable to predict response to steroids $AUC = 0.610 \pm 0.29$; $p = 0.441$ and $AUC = 0.545 \pm 0.28$; $p = 0.751$, respectively. Likewise airway hyper-responsiveness to hypertonic saline ($PD_{15} < 12\text{mL}$) did not predict steroid response ($AUC = 0.597 \pm 0.29$; $p = 0.497$). Figure 3B shows the receiver operator characteristic curves for the prediction of steroid responsiveness using these four methods. Detailed performance characteristics for all models are reported in Table 3.

Correlations between breath profile and other predictors of steroid responsiveness

eNose breathprints showed a strong correlation ($r = 0.601$, $p = 0.002$) with the percentage of sputum eosinophils. $F_{E}NO$ was not associated with the VOC-profile ($r = 0.141$, $p = 0.502$).

PD_{15} was mildly correlated with the breath profile ($r = 0.483$, $p = 0.020$).

DISCUSSION

This study shows that, as well as discriminating patients with asthma from controls, exhaled breath analysis by electronic nose technology is capable of predicting steroid responsiveness in steroid-free patients with established asthma. Patients experiencing loss of control after steroid withdrawal could also be identified. These findings imply that exhaled breath analysis may be a valuable tool in assessing asthma with respect to treatment response phenotype and future risk. Although pathological (e.g. eosinophilic versus non-eosinophilic) and clinical (e.g. chronic persistent versus brittle) phenotypes are important, in practice the ease with which treatment-response phenotypes can be identified holds the most potential for improving asthma management.

Our study is the first to provide a direct comparison of the eNose, $F_{E}NO$, sputum eosinophils and airway hyper-responsiveness for diagnosing asthma and assessing steroid responsiveness. Despite statistically significant differences in their performance characteristics, the study is too small to conclude that any one test ranks more highly than another. Our results are in keeping with those reported by Ibrahim *et al*[22] who demonstrated a strong association between sputum eosinophils and exhaled VOCs. Our study extends data from previous studies of VOCs as biomarkers in asthma by showing the potential for breath analysis to be used as a predictor of response to steroid therapy. Given that the present study was designed as a mechanistic study to confirm the principle of predicting steroid responsiveness by VOC analysis, it therefore requires external validation

in an intention-to-treat population of symptomatic steroid-naïve subjects using both inhaled and oral corticosteroids.

In the present study the sensitivity and specificity for the diagnosis of asthma by eNose (80% and 65%, respectively) are less robust than those reported previously by Dragonieri *et al.*[17] (90-100% overall accuracy). Paradoxically, this may be due to the effects of inhaled drug treatment on the eNose signal. In the study by Dragonieri *et al.*, patients did not discontinue their treatment. Importantly in the present study, the diagnostic accuracy for asthma was maintained even when patients were on treatment.

Our subjects were well characterized in terms of their symptoms, lung function and underlying disease activity. Control subjects were significantly younger compared to asthmatic subjects. However, in a previous study by Dragonieri *et al.*, there was no significant effect of age on the VOC-profiles of controls.[17] All asthmatic subjects but none of the control subjects were atopic, similar to previous studies [17,18]. It is possible that this may have confounded the discrimination of asthma from healthy controls, but this would not apply to the assessment of LOC or steroid responsiveness. Studies comparing asthmatic and COPD subject have shown significant discrimination irrespective of the atopic status of the subjects [19,20]. The exact effect of atopy on exhaled breath profiles is the subject of current studies.

All subjects fulfilled the diagnostic criteria for asthma at study entry even though nearly half (12/25) of patients with asthma did not fulfil loss of control criteria during the 28-day period after steroid withdrawal. This can be explained by the fact that, for safety reasons, only a limited deterioration in asthma control was permitted, and the time frame was limited to 28 days. The LOC outcomes are comparable to a previous study in our department showing that 19% of asthmatic patients did not reach loss of control over a period of 2 months when taken off inhaled corticosteroids.[33]

We found that those subjects reaching loss of control within 28 days were very likely to be steroid responsive. It might be argued that subjects not reaching loss of control were not steroid responsive because they no longer or had never in fact required steroid treatment at all. We aimed to prevent this ceiling effect by ensuring that all patients entering the steroid trial had proven airway hyper responsiveness and/or bronchodilator reversibility. This ensured that all subjects had the potential to improve in relation to the parameters we used to define steroid responsiveness. The success of this approach is underlined by the fact that 45% of patients not reaching loss of control were responsive to steroid treatment.

Unfortunately, for safety reasons, an unexpectedly high number of subjects were excluded from the analysis of steroid responsiveness due their inability to undergo an AMP challenge (n=7). This may have reduced the statistical power of the study, potentially explaining the apparently limited performance of sputum eosinophils and F_ENO as predictors of steroid response. Nonetheless % sputum eosinophils reached a higher specificity for the prediction of steroid-responsiveness than eNose (85,7% vs 71.4%). *A priori* power calculations for the eNose as a predictor of steroid response were not possible due to the complete lack of previous data. A *post hoc* power calculation based on the currently established effect size indicated a statistical power of 87%, sufficient to reliably establish the potential of the eNose to predict steroid response with the current sample size.

The mechanisms determining response to corticosteroids in asthma are strongly linked to the nature of the underlying inflammatory cell type.[34] In turn, as shown by both Fens and Ibrahim[21,22] the inflammatory cell phenotype is associated with specific VOCs exhaled by the subject. The present study shows a strong correlation between the breath profile parameters and the % of sputum eosinophils. Pattern recognition provided by the eNose, which detects a spectrum of VOCs related to the underlying pathophysiological changes in

the airways, includes far more biomarkers than a single marker such as sputum eosinophils or F_ENO. This may also explain why somewhat to our surprise, both F_ENO and % sputum eosinophils performed worse than the eNose in predicting steroid response.

Our study is the first breath analysis study to provide insights into the potential strengths of this methodology in clinical practice. Firstly we showed that the performance characteristics of the eNose for diagnosing asthma were not influenced by administering steroids even though we cannot exclude an effect of steroids on the VOC-profile itself. In practice this would be helpful given that so many patients receive empiric inhaled corticosteroid treatment before there is an opportunity for diagnostic tests to be carried out. More importantly our study clearly demonstrates the potential of non-invasive, rapid, on-site analysis of exhaled breath to provide guidance in clinical decision making regarding steroid treatment. This may complement currently available techniques in the monitoring of asthma and help to target treatment to patients who are most likely to benefit. Additionally the detection of loss of control in our subjects suggests that in the future, breath analysis could be used as a tool for monitoring brittle asthma by both clinicians and patients or aid in identifying patients whom no longer require ICS therapy.

In summary, the principal finding of this study is that exhaled breath analysis by eNose is useful not only to diagnose asthma but also in predicting steroid responsiveness in steroid-free patients. Further prospective studies focussing on analysis of VOCs are required in an intention to treat population to externally validate our findings. Given the perplexing heterogeneity of the asthma syndrome in clinical practice, the concept of defining phenotypes using simple non-invasive techniques, such as breath analysis, represents an advance in asthma research with the potential to facilitate more personalized management.

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COMPETING INTERESTS

All authors declare that they have no competing interests

AUTHORS' CONTRIBUTIONS

MS, research fellow, analyzed the data and wrote the manuscript.

RP, research technician, conducted the study procedures.

JC, research technician, conducted the study procedures.

DRT, principal investigator, designed and supervised the study, and wrote the manuscript.

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LEGENDS TO FIGURES

Figure 1. Study design

Steroid responsiveness was defined as one or both of the following: increase in FEV₁ of 12% or greater; increase in PC₂₀AMP of 2 doubling doses or greater.

Abbreviations: ACQ, asthma control questionnaire; F_ENO, fraction of nitric oxide in exhaled air; LOC, loss of control; PD₁₅HS, provocation dose of hypertonic saline causing a 15% fall in FEV₁; PEF, peak expiratory flow; PC₂₀AMP, concentration of adenosine monophosphate resulting in a 20% fall in FEV₁; SABA, short acting beta-agonist.

Figure 2a. Discrimination of steroid free patients with asthma from healthy controls using the eNose.

Discrimination of steroid free patients with asthma (full circles) from healthy controls (empty triangles) by electronic nose: sensitivity = 80.0%; specificity = 65.0%; positive likelihood ratio = 2.3; negative likelihood ratio = 0.3. Axes depict two orthogonal linear recombinations of the original 32 sensor data by means of principal component analysis.

Figure 2b. Receiver operator characteristic curves for the diagnosis of asthma by eNose, sputum eosinophils and F_ENO.

Receiver operator characteristics curve for the diagnosis of asthma by electronic nose (black solid line), sputum eosinophils (grey dashed line) and F_ENO (light grey dotted line). The areas under the curve ± 95% confidence interval (AUC ± 95%CI) with associated p-value were: eNose: 0.766 ± 0.14; <0.002; percentage sputum eosinophils: 0.814 ± 0.15 ; <0.001; F_ENO: 0.862 ± 0.12; <0.001.

Figure 3a. Prediction of steroid responsiveness in steroid free patients with asthma using the eNose.

Prediction of steroid responsiveness (responsive = full circles ; unresponsive = empty triangles) to oral prednisone, 30mg daily for 14 days in steroid free patients with asthma by electronic nose. Sensitivity = 90.9%; specificity = 71.4%; positive likelihood ratio = 3.2; negative likelihood ratio = 0.1. Steroid responsiveness was defined as one or both of the following: increase in FEV₁ of 12% or greater; increase in PC₂₀AMP of 2 doubling doses or greater. Axes depict two orthogonal linear recombinations of the original 32 sensor data by means of principal component analysis.

Figure 3b. Receiver operator characteristic curves for the prediction of steroid responsiveness in steroid free patients with asthma by eNose, sputum eosinophils, F_ENO and airway hyper-responsiveness to hypertonic saline.

Receiver operator characteristics curve for the prediction of steroid responsiveness to oral prednisone, 30mg daily for 14 days in steroid free patients with asthma by electronic nose (black solid line), sputum eosinophils (black dashed line), F_ENO (grey dashed line) and PD₁₅HS (light grey dotted line). The areas under the curve $\pm$ 95% confidence interval (AUC $\pm$ 95%CI) with associated p-values were: electronic nose: 0.883 $\pm$ 0.16 ; 0.008; percentage sputum eosinophils: 0.610 $\pm$ 0.29; 0.441; F_ENO: 0.545 $\pm$ 0.28; 0.751; PD₁₅HS: 0.597 $\pm$ 0.29; 0.497. Steroid responsiveness was defined as one or both of the following: increase in FEV₁ of 12% or greater; increase in PC₂₀AMP of 2 doubling doses or greater.

TABLES

Table 1. Baseline Subject Characteristics

	Healthy controls	Patients with Asthma		
	(n=20)	All patients (n=25)	Steroid responsive (n=11)	Steroid unresponsive (n=7)
Age, yr (Median[range])	22.5 [18-62]*	46 [27-75]	42 [27-63]	53 [38-75]
Sex, % ♀	55	56	55	43
Ex-Smoker, %	5	20	15	37
Atopy, %	0 [‡]	100	100	100
ICS use, %	-	88	82	86
ICS dose, µg/day (med [range])	-	500 [0-1000]	500 [0-1000]	500 [0-1000]
FEV ₁ , L	3.77 $\pm$ 0.88*	2.50 $\pm$ 0.87	2.84 $\pm$ 0.57 [‡]	2.25 $\pm$ 0.49
FEV ₁ , % predicted	100.9 $\pm$ 11.9*	79.8 $\pm$ 19.2	90.1 $\pm$ 17.3 [†]	67.0 $\pm$ 12.5
FEV ₁ / FVC, %	83.3 $\pm$ 7.0*	62.8 $\pm$ 9.6	68.2 $\pm$ 4.6 [†]	55.0 $\pm$ 11.8
PD ₁₅ HS, mL (gm $\pm$ sd) [§]	NA	3.7 $\pm$ 2.6	4.6 $\pm$ 2.5	3.3 $\pm$ 1.8
F _E NO, ppb	17 $\pm$ 5 [‡]	40 $\pm$ 36	56 $\pm$ 48	24 $\pm$ 8
Sputum successful, %	95	96	100	100
Sputum eosinophils, % [§]	0.3 $\pm$ 0.6*	17.2 $\pm$ 17.1	19.3 $\pm$ 16.2	7.5 $\pm$ 11.1
PC ₂₀ AMP [§]	NA	6.7 $\pm$ 4.3	5 $\pm$ 4.0 [‡]	11 $\pm$ 5.3
Reaching LOC, n	NA	13	7	1
Decrease of PEF, %[∞]	NA	70	57	100
Increase of β2-rescue, %[∞]	NA	39	29	0
Increase nighttime	NA	39	43	0
Days to loss of control	NA	10.6 $\pm$ 5.3	11.8 $\pm$ 3.8	16

All values are reported as mean $\pm$ SD unless stated otherwise. Values were obtained at inclusion, prior to steroid withdrawal unless stated otherwise. Full assessment of steroid responsiveness using AMP challenge was only possible in 18/25 of patients who underwent the trial of oral steroid for safety reasons.

[§] Value for PD₁₅HS, sputum eosinophils and PC₂₀AMP in asthma were obtained after steroid withdrawal.

[∞]Percentage of subjects meeting criteria for LOC are presented as percentage of total subjects reaching LOC. Total may exceed 100% if subjects met multiple criteria.

[‡] p<0.01 for comparison with all patients with asthma.

* p<0.001 for comparison with all patients with asthma.

[‡] p<0.05 for comparison with steroid unresponsive patients.

[†] p<0.01 for comparison with steroid unresponsive patients.

Abbreviations: F_ENO, fraction of nitric oxide in exhaled air; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; ICS, inhaled corticosteroids; **LOC, Loss of control**; **NA**, not applicable; PD₁₅HS, provocation dose of hypertonic saline causing a 15% fall in FEV₁.

Table 2. Changes in FEV₁, FVC, PC₂₀AMP, ACQ and F_ENO after treatment with oral prednisone.

	All patients with asthma (n=18 [†] , LOC=13)				Steroid unresponsive patients (n=7, LOC=1)				Steroid responsive patients (n=11, LOC=6)			
	Pre	Post	$\Delta \pm$ SE M	p	Pre	Post	$\Delta \pm$ SE M	p	Pre	Post	$\Delta \pm$ SE M	p
FEV ₁ , L	2.2 $\pm$ 0.6	2.5 $\pm$ 0.8	0.31 $\pm$ 0.08	0.0 01	2.1 $\pm$ 0.5	2.1 $\pm$ 0.4	0.01 $\pm$ 0.09	0.9 52	2.4 $\pm$ 0.6	2.9 $\pm$ 0.8	0.46 $\pm$ 0.12	0.0 03
FVC, L	3.7 $\pm$ 1.1	4.0 $\pm$ 1.1	0.30 $\pm$ 0.09	0.0 03	3.9 $\pm$ 0.6	3.9 $\pm$ 0.3	0.01 $\pm$ 0.11	0.4 75	4.0 $\pm$ 1.2	4.3 $\pm$ 1.3	0.30 $\pm$ 0.11	0.0 23
PC ₂₀ AMP, % $\pm$ sd	6.7 $\pm$ 4.3	50 $\pm$ 6 .4	2.9 $\pm$ 2 .9*	0.0 01	11 $\pm$ 5.3	23 $\pm$ 3.4	1.1 $\pm$ 0 .3*	0.0 20	5 $\pm$ 4. 0	83 $\pm$ 7 .7	4.0 $\pm$ 1 .0*	0.0 02
ACQ, units	1.5 $\pm$ 1.1	0.7 $\pm$ 0.6	0.7 $\pm$ 0 .2	0.0 01	0.7 $\pm$ 0.5	0.5 $\pm$ 0.7	0.1 $\pm$ 0 .2	0.5 61	1.7 $\pm$ 1.4	0.6 $\pm$ 0.5	1.1 $\pm$ 0 .4	0.0 12
F _E NO, ppb	52 $\pm$ 22	30 $\pm$ 1 5	23 $\pm$ 5	<0. 001	37 $\pm$ 16	23 $\pm$ 6	15 $\pm$ 5	0.0 29	68 $\pm$ 49	34 $\pm$ 1 6	34 $\pm$ 1 0	0.0 08

All values are reported as mean $\pm$ SD unless stated otherwise. Steroid responsiveness was defined as one or both of the following: increase in FEV₁ of 12% or greater; increase in PC₂₀AMP of 2 doubling doses or greater.

* Difference between pre and post prednisone PC₂₀AMP values is reported as doubling doses.

[†] Not all subjects underwent an AMP challenge for safety reasons.

Abbreviations: $\Delta \pm$ SEM, mean change in endpoint with standard error of the mean; ACQ, asthma control questionnaire; F_ENO, fraction of nitric oxide in exhaled air; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; gm $\pm$ sd, geometric mean $\pm$ standard deviation; LOC, subjects reaching loss of control criteria after inhaled steroid withdrawal, PC₂₀AMP = concentration of adenosine monophosphate resulting in a 20% fall in FEV₁; SEM, standard error for the mean.

Table 3. Performance characteristics of eNose, sputum eosinophils, F_ENO and AHR predicting asthma, LOC and steroid responsiveness.

	AUC $\pm$ 95% CI	p-value	Sensitivity	Specificity	+ LR	- LR
<u>Distinguishing asthma from healthy controls (n=45)</u>						
eNose pre steroid	0.766 $\pm$ 0.14	0.002	80.0	65.0	2.3	0.3
eNose post steroid	0.842 $\pm$ 0.12	<0.001	84.0	80.0	4.2	0.2
F _E NO pre steroid	0.862 $\pm$ 0.12	<0.001	80.0	90.0	8.0	0.2
F _E NO post steroid	0.738 $\pm$ 0.15	0.007	84.0	65.0	2.4	0.3
Sputum eosinophils % [†]	0.814 $\pm$ 0.15	<0.001	83.3	84.2	5.3	0.2
<u>Distinguishing loss of control(LOC) after steroid withdrawal (n=25)</u>						
eNose	0.814 $\pm$ 0.17	0.008	69.2	91.7	8.3	0.3
Sputum eosinophils %	0.868 $\pm$ 0.17	0.002	83.3	83.3	5.0	0.2
F _E NO	0.673 $\pm$ 0.22	0.142	75.0	62.5	2.0	0.4
<u>Predicting steroid responsiveness in steroid-free asthmatics (n=18)</u>						
eNose	0.883 $\pm$ 0.16	0.008	90.9	71.4	3.2	0.1
Sputum eosinophils %	0.610 $\pm$ 0.29	0.441	63.6	85.7	4.5	0.4
F _E NO	0.545 $\pm$ 0.28	0.751	72.7	42.9	1.2	0.6
PD ₁₅ HS	0.597 $\pm$ 0.29	0.497	72.7	57.1	1.7	0.5

Sensitivities, specificities, positive and negative likelihood ratios are reported for the respective optimum cut-points. Steroid responsiveness was defined as one or both of the following: increase in FEV₁ of 12% or greater; increase in

PC₂₀AMP of 2 doubling doses or greater.

[†] Sputum cell counts were not obtained after treatment.

Abbreviations: AUC ± 95% CI, Area Under the Curve with 95% Confidence Interval; F_ENO, fraction of nitric oxide in exhaled air; + LR, positive likelihood ratio ; - LR, negative likelihood ratio; LOC, subjects reaching loss of control criteria after inhaled steroid withdrawal; PD₁₅HS, provocation dose of hypertonic saline causing a 15% fall in FEV₁.

LIST OF ABBREVIATIONS

ACQ	Asthma control questionnaire
AHR	Airway Hyperresponsiveness
AMP	Adenosine monophosphate
AUC	Area under the curve
CDA	Canonical Discriminant Analysis
COPD	Chronic obstructive pulmonary disease
eNose	Electronic Nose
FEV ₁	Forced expiratory volume in 1 second
FeNO	Fraction of nitric oxide in exhaled air
FVC	Forced vital capacity
GC-MS	Gas-chromatography Mass-spectrometry
ICS	Inhaled corticosteroids
LOC	Loss of control
PC ₂₀ AMP	Concentration of adenosine monophosphate resulting in a 20% fall in FEV ₁
PCA	Principle component analysis
PD ₁₅ HS	Provocation dose of hypertonic saline causing a 15% fall in FEV ₁
PEF	Peak expiratory flow
ROC	Receiver operator characteristic
SEM	Standard error for the mean
VOCs	Volatile Organic Compounds





