Phenotyping Asthma using an Electronic Nose

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Abstract

Rationale

Asthma is a chronic respiratory condition affecting many millions of people worldwide. The recommended long-term preventative treatment for asthma is corticosteroid medication. Improvement in asthma symptoms in response to corticosteroids is not universal, and prescription to non-responsive patients is costly and potentially dangerous. The assessment of whether patients will respond to corticosteroid treatment is an important clinical problem.

This study investigated the utility of a gas sensor array, the ‘Electronic nose’, in predicting response to steroid among asthmatics and in differentiating asthmatics from healthy controls using exhaled breath. The anti-inflammatory actions of steroids and the biochemical basis of steroid response are complex and may be better quantified by the electronic nose than by single biomarkers.

In parallel with the assessment of steroid response a study was conducted on the ability of the electronic nose to predict sputum eosinophil counts among asthmatics. Eosinophil count is a predictor of steroid response and can be used as a guide to asthma treatment.

Statement of problems

Can steroid-responsive and non-steroid responsive asthmatics be distinguished using electronic nose analysis of exhaled breath?

Is sputum eosinophil count able to be predicted by electronic nose analysis of exhaled breath?

Methods

47 patients (27 asthmatics, 20 healthy controls) participated in the study.

Asthmatic patients completed a two-week trial of oral prednisone. Asthmatics were classified as steroid responsive if FEV$_1$ improved by 15%, PC$_{20}$AMP improved by $>300\%$, or ACQ
Asthma control questionnaire improved by >0.5 points over the steroid course. 16 asthmatics were steroid responsive and 11 asthmatics were steroid unresponsive.

Breath samples were taken before and after the steroid course. A sputum sample was taken prior to the steroid course. Asthmatics were defined as eosinophilic if their sputum cell count contained more than 3% eosinophils. Healthy controls provided a single breath sample and sputum sample.

**Main results**

Steroid responsive and non-steroid responsive asthmatics were unable to be distinguished either before or after the steroid course (no significant principal component differences, M-distances < 2, all p > 0.2). Healthy controls were significantly differentiated from pre-steroid asthmatics (PC2, PC4, PC6, p = 0.0090, 0.000060, 0.0090 respectively, M-distance = 4.66, p = 0.031) and less differentiated from post-steroid asthmatics (PC6, p = 0.0016, M-distance = 3.80, p = 0.16). A multilayer perceptron based predictive model for the comparison was associated with a cross-validation value (CVV) of 83% between controls and pre-steroid samples and 70% between controls and post-steroid samples. Improvement in ACQ (PC6, p = 0.0041) and improvement in FEV1 (PC6, p = 0.045) could be detected based on differences in samples before and after the steroid course.

A principal component from pre-steroid asthmatics was strongly correlated with sputum eosinophil counts (coefficient = 0.615, p = 0.0082). Breathprints from eosinophilic and non-eosinophilic asthmatics were significantly differentiated (PC4, p = 0.0033, M-distance = 2.00, p = 0.0020). A predictive model for the comparison was associated with a CVV of 77%.

**Conclusions**

Steroid responsive and non-steroid responsive asthmatics cannot be differentiated on the basis of exhaled breath analysis by electronic nose. Healthy controls and asthmatics can be significantly differentiated on this basis. Electronic nose readings are correlated with sputum eosinophil counts and eosinophilic and non-eosinophilic asthma can be significantly differentiated.
Preface

The study described in this thesis established that the steroid-responsive and steroid-unresponsive patient groups were not able to be differentiated on the basis of electronic nose breathprints. As expected, asthmatics and non-asthmatics were able be differentiated on this basis. I stand unreservedly by all the results obtained.

I would like to thank my family and friends who have been of great help in addressing adversities during and after this project. Thanks should also go to the staff and students at University College, 2010. This study and subsequent events gave me considerable insight into the integrity of medical research and data analysis which may come in useful in future.
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List of abbreviations

AATD – Alpha 1 AntiTrypsin Deficiency
ACQ – Asthma Control Questionnaire
ACT – Asthma Control Test
AMP – Adenosine MonoPhosphate
cAMP – cyclic Adenosine MonoPhosphate
ANN – Artificial Neural Network
ANOVA – ANalysis Of VAriance
AUC – Area Under Curve
CBD – Chronic Beryllium Disease
CC – Correlation Coefficient
COPD – Chronic Obstructive Pulmonary Disease
COX – Cyclo-Oxygenase
CPAP – Continuous Positive Airway Pressure
CVV – Cross Validation Value
ECV – External (distinct training/test set) Cross Validation
EA – Eosinophilic Asthma
ECP – Eosinophil Cationic Protein
EN – Electronic Nose
F\textsubscript{e}NO – Fractional Exhaled Nitric Oxide
FEV\textsubscript{1} – Forced Expiratory Volume in 1 second
FVC – Forced Vital Capacity
GC – GlucoCorticoid
GC-MS – Gas Chromatography – Mass Spectrometry
GINA – Global INitiative for Asthma
GM-CSF – Granulocyte-Macrophage Colony Stimulating Factor
GR – Glucocorticoid Receptor
HC – Healthy Controls
ICS – Inhaled Corticosteroid
IgE – ImmunoGlobin E
ICV – Internal (leave-one-out/bootstrap) Cross Validation
IL – InterLeukin
ILD – Interstitial Lung Disease
KNN- K-Nearest Neighbour
LABA – Long-Acting Beta Agonist
LDA – Linear canonical Discriminant Analysis
LT – LeukoTriene
MD – Mahalanobis Distance
MHC- Major Histocompatibility Complex
MOS – Metal Oxide Semiconductor
MOSFET – Metal Oxide Semiconductor Field Effect Transistor
NEA – Non-Eosinophilic Asthma
NF-kB – Nuclear Factor kappa B
NO- Nitric Oxide
NSCLC – Non-Small Cell Lung Carcinoma
PCA – Principal Component Analysis
PD_{15}HS – Provocative Dose of Hypertonic Saline causing a 15% drop in FEV_{1}
PC_{20}AMP – Provocative Concentration of AMP causing a 20% drop in FEV_{1}
PEFR – Peak Expiratory Flow Rate
PERFUME – Prediction of Eosinophilia and Responsiveness to steroid From Unspecified Molecules in Exhaled breath.
PLSR – Partial Least Squares Regression (projection to latent structure)
PTR-MS – Proton Transfer Reaction – Mass Spectrometry
RMS – Root Mean Square
ROC – Receiver Operator Characteristic curve
RV – Residual Volume
SABA – Short Acting Beta Agonist
SR – glucocorticoid (Steroid) Resistant
SS – glucocorticoid (Steroid) Sensitive
SVM – Support Vector Machine
TLC – Total Lung Capacity
TNF-alpha – Tissue Necrosis Factor alpha
VOC – Volatile Organic Compound
Summary of introduction section

This introduction provides a background to the study described in this thesis. It does not exhaustively cover the theory and history of all topics of relevance, but serves as a contraction of the extensive range of literature and scientific theory relating to the experiment at hand.

The first section of this introduction reviews the use of clinical biomarkers in exhaled breath, and describes the motivation for development of such biomarkers based on electronic nose-based analysis. The section includes a discussion on where molecules in exhaled breath arise, and detail on ‘direct’ and ‘indirect’ methods for detecting them. Some detail is given on requirements for electronic nose componentry and the common types of sensors currently used in electronic nose systems. Specific focus is made on the Cyranose 320 electronic nose, which was used in this study. The purpose of this section is to introduce some of the breath tests currently and historically used in medicine as the background to developing a breath test based on the electronic nose, and to overview the mechanism by which electronic noses function.

The second section is a short systematic review of the current literature on electronic nose-based analysis of exhaled breath. The methods and criteria of the review are covered, and a summary of the studies is made. Particular attention is given to the type of nose used, the groups being discriminated, the study sizes, and the analysis techniques employed.

The third section contains an overview of the steroid response in asthma with particular reference to the biology of eosinophils and the mechanisms of eosinophilic asthma. A considerable research effort in the past several decades has focused on the biochemical effects of corticosteroids, and the molecular mechanisms which underlie the response. This study attempts to quantify differences in VOC patterns related to steroid response, and this section of the introduction summarises some such molecular differences which may be present. The steroid response is linked to the presence and proliferation of eosinophils, and a second part of the introduction focuses on the biology of eosinophils in relation to the action of steroids. A secondary aim of this study was the quantification of eosinophilia using electronic nose techniques, and this section provides a background to the biochemical basis of eosinophilia.
The field of electronic olfaction is draws from a wide range of scientific topics. In particular, these include neurobiology, organic and inorganic chemistry, physics and dispersion theory, electronics, statistics, combinatorics, artificial intelligence and computer science. In the interests of brevity a full explanation of electronic nose engineering and data analysis is not given here. A more extensive exploration is available in Gardner and Bartlett (1).

1. Exhaled breath analysis

1.0 Overview

Exhaled breath analysis is an area of medicine with considerable potential to be further developed (2-3). Breath-based diagnosis has been used extensively throughout history (2), mostly as subjective smell-based tests, such as the detection of ketones. Recent technology is allowing similar analyses to be conducted objectively. The electronic nose is a method for objectifying breath analysis.

The analysis of exhaled breath can broadly be divided into ‘direct’ and ‘indirect’ methods, corresponding to whether or not specific compounds are identified. Direct analysis of exhaled breath analysis can either be holistic, analysing all molecules on the breath, or can involve the measurement of individual molecules (2). Because many techniques for indirect breath analysis, in particular the design of gas sensors, were developed in parallel with those for direct breath analysis (4), techniques of both holistic and individual molecular identification are covered in some detail here.

 Constituents of exhaled breath can arise from several different sources, dependent on the chemical properties of molecules. Molecules such as carbon dioxide diffuse from the bloodstream (2) whereas molecules such as nitric oxide arise almost exclusively from the airways (5). Volatile compounds of interest as biomarkers generally make up only a very small proportion of exhaled breath (2). It is generally these compounds which are of interest in diagnosis (2) and consequently of greatest interest in this study.

Traditional breath analysis has focused on the measurement of individual compounds in breath. Nitric oxide is one such compound which has been used both for the diagnosis of asthma and the prediction of steroid response (6). Analysis of exhaled breath by electronic
nose has been found by one study to be more sensitive and specific for the presence of asthma than $F_{E}NO$ (7), and the current allows a comparison of the potentials of each to predict steroid response. Exhaled nitric oxide measurements were conducted on all patients in this study.

There are several ways which levels of exhaled nitric oxide may be tested for on the breath (8) and these are covered in regard to the methods used in this study. Other molecules such as alcohol (9) and acetaldehyde (10) have also been successfully measured on exhaled breath.

Techniques for holistic direct breath analysis are complex. The two main such techniques are gas chromatography mass spectrometry (GC-MS) and proton transfer reaction mass spectrometry (PTR-MS), both of which have been used in conjunction with electronic nose measurements in earlier studies. Both of these involve a technique to separate a gas into constituent molecules followed by identification of the individual constituents (11-12). While GC-MS is highly sensitive, it is impractical for real-time breath analysis (11).

The operation of instruments for indirect breath analysis differs somewhat to that of direct breath analysis. Like the human nose, electronic nose designs work on the principle of gaining sensitivity through an array of non-specific sensors, in contrast to the measurement of molecules such as nitric oxide by specific sensors. The ability to distinguish breath samples is based on subsequent integrative processing of sensor responses (13).

Several types of sensor have been used for electronic noses. Sensors attempt to discriminate gases based on their adsorptive, chemisorptive, optical, or reactive properties, with the assumption that different molecules will exhibit different profiles (14). In this sense, the aim of sensor design is similar to that of GC-MS – the discrimination of different compounds based on chemical properties. Each type of sensor used in electronic noses has advantages and disadvantages. The electrical response to a sensor must be processed according to several assumptions in order to gain a meaningful sensor signal (15). An ideal sensor assigns a reproducible and distinct signal to each gas it is exposed to.

This study uses techniques of indirect breath analysis to analyse exhaled air. The use of indirect rather than direct breath analysis is indicated due to the complexity of direct breath analysis and the number of molecules present on exhaled air (16). The electronic nose used in
this study is the Cyranose 320, which is a current global standard for breath analysis. The Cyranose uses 32 polymer-based sensors (17).

1.1 Basis of exhaled breath analysis

1.1.1 Biomarkers on exhaled breath

1.1.1.1 Generation of VOCs in breath

In the same way as oxygen and carbon dioxide, small molecules may freely diffuse across the alveolar wall between the blood and the air in the lungs, at a rate proportional to their concentration differences across the alveolar-capillary junction. The only requirement for this diffusion to occur is that the diffusing substance be able to exhibit a significant partial vapour pressure; that is, it must be volatile (2). Compounds such as ethanol enter the breath in this way.

The other significant source of exhaled VOCs is from cells adjacent to air spaces (2). It is generally this component of exhaled VOCs which are of interest when breath analysis is used to assess airway pathology. Sources of exhaled VOCs include cells of the mouth, nose, sinuses, airways, and GI tract (2).

1.1.1.2 General makeup of exhaled breath

The main gaseous constituents of exhaled breath are similar to those of ambient air. Nitrogen, oxygen, carbon dioxide, water vapour and inert gases make up almost all of the volume, with other compounds generally accounting for less than 100 parts per million of total breath (2).

Concentrations of exhaled breath constituents vary due to a range of biological phenomena. Many detectable compounds on the exhaled breath, including ethane, ethylene, pentane and longer-chain hydrocarbons, are associated with lipid peroxidation. Bacteria of the digestive tract produce compounds including methane, hydrogen, carbonyl sulphide and carbon disulphide. Other metabolic processes produce ammonia, methanethiol, and methylamine. Acetone, methane, hydrogen, and carbon monoxide are found in exhaled breath in the parts-per-million range. Concentration of other compounds are generally in the parts-per-billion range (2).
1.1.2 Medical use of exhaled breath biomarkers

1.1.2.1 History of smell-based analysis

The use of smell as a tool for medical diagnosis and assessment has a long history. In general, the smell of exhaled breath provides a non-invasive and simple, if quantitative, method for diagnosis and assessment of respiratory and other conditions. Hippocrates identified the odour of 'Fetor Hepaticus' (literally ‘smell of the liver’) as a clinical marker, a characteristic now suspected to be associated with portal hypertension and liver failure (3). Other odours historically considered as markers of 'bad humour' are now known to be associated with conditions such as diabetes, bacterial infection, and dental disease (2).

Lavoisier identified that carbon dioxide is present in exhaled breath in 1784, in what was essentially the first quantification of a gaseous compound in a human. As modern analytical chemistry developed in the later part of the 20th century, analysis of exhaled breath for compounds such as ethanol and acetone became possible (2).

Within the last century, the field of odour analysis has expanded considerably, and has become more objective as better hardware becomes available. Although most research on volatile organic compounds in medicine has focused on exhaled breath, studies on the 'headspace' surrounding other tissue samples have also been used in several studies. Urine vapour (18), blood (19), and faeces (20) have all been utilised as headspace generators.

1.1.2.2 Early breath analysis

A study by Pauling et al. (18) was one of the first 20th century investigations of the VOC components in human tissue headspaces. Around 250 quantifiable substances were found in a breath sample and around 280 in a sample of urine vapour. This study pioneered a technique involving gas-liquid chromatography to perform the analysis.

The study was motivated by the concept of ‘orthomolecular medicine’ – essentially that while in good health the concentrations of all molecules in a tissue sample (biomarkers or otherwise) are consistent to within a certain range. This was based on the finding that, when diet was controlled, the concentrations of molecules in exhaled breath remained relatively constant (standard deviation 10%) day-to-day. The electronic nose is in a way a way to
quantify this consistent state; a variation in concentrations of organic constituents of breath (without knowledge of exactly what the constituents are or what they do) may be a predictor of pathology.

The study by Pauling et al. was significant in that it identified such a use of ‘indirect’ breath analysis as a biomarker. Although the study identified a large number of molecules found in breath, it acknowledged the actual identification of the molecules was not necessary for diagnostic use. Quantifications such as the area under peaks on the chromatograph could be used without knowing what the peaks represented. This is analogous to several analysis techniques for electronic nose data.

Other studies of exhaled breath have focused on measurement of individual known compounds and the development of methods for testing for the presence of individual gases. Dannecker (10) developed a method for assessing acetaldehyde levels in breath using a highly sensitive technique involving gas chromatography. Perhaps the earliest test of this type was the quantification of exhaled ethanol. This can be accurately measured with a small portable device called a breathalyser (21). The presence of water vapour in exhaled breath has also been used as a qualitative biomarker for general mortality (2).

This study, and others using electronic nose technology, are extensions of Pauling’s use of indirect analysis. Electronic nose technology is a more efficient way of gaining such indirect measurements; superfluous information such as the exact composition of the exhaled breath is not obtained.

1.1.2.3 Problems with exhaled breath analysis

Breath collection is one of the least invasive sampling techniques available to a physician, and representative samples can be gathered in a comparatively short time (2). However, the use of breath analysis as a clinical tool is difficult for several reasons. One problem is that exhaled air samples are difficult to handle, transport and store, and instruments for analysis tend to be large and unwieldy, making on-site analysis difficult. The collection of exhaled breath is difficult to standardise; factors such as composition of inhaled air, expiratory flow rate, collected exhaled breath fraction, mouth pressure, mouth carbon dioxide concentration, and nasal contamination can all affect the concentrations of constituent molecules and must be
closely monitored (2).

In order to devise exhaled breath-based tests which can be used conveniently in a clinical context, these problems must be addressed. In this study, the use of an electronic nose for breath analysis is proposed as an alternative to larger and more expensive instruments. The electronic nose used in the study was small (~10x5x25 cm), light (~1.5 kg), and portable, with a low power consumption. Such a device could conceivably be used in a clinical setting. Analysis can also be performed on-site and in real time, reducing the need for storage and transport of breath samples.

To obtain reproducible measurements of exhaled breath samples, a well-prescribed procedure for breath collection must be followed and the conditions of the ambient air must be controlled (2). In this study, the composition of inhaled air was controlled by a five-minute tidal breathing period through a filter, and an identical physical location at which each sample was taken. Nasal contamination was limited through the use of a nose-peg, and an entire vital capacity was collected from all patients, rather than a prescribed volume of air.

1.1.3 Example of VOC biomarker – Nitric Oxide
1.1.3.1 Exhaled nitric oxide as a clinical test

Nitric oxide is a volatile organic compound found in the exhaled breath which is known to vary in concentration according to presence of airway pathology. It is a volatile organic compound and is one example of a substance probably detectable by the electronic nose.

Nitric oxide has been shown to be an effective predictor of asthma (5) and indicator of asthma control (22) as well as predicting steroid response (6). Montuschi et al. (7) found that electronic nose data could be used to predict asthma with greater accuracy than F\textsubscript{E}NO. One of the aims of this study was to asses the utility of the electronic nose in indicting asthma control and predicting steroid response.

Because F\textsubscript{E}NO is able to detect the clinical phenotype of steroid responsiveness based on a purely biochemical measurement, it appears that the phenotype is associated with a reasonably consistent pathophysiological state. The assumption that the electronic nose (also a
purely biochemical measurement) could predict steroid response was based on the fact that this state was measurable by $F_E NO$.

### 1.1.3.2 Biochemical activity

Nitric oxide acts in homeostatic feedback pathways and as an inflammatory mediator. It acts both as a paracrine and intracrine signalling molecule. The main effect of nitric oxide is to induce relaxation of smooth muscle via the secondary action of cGMP. While the small and lipophilic nature of NO allows it to easily diffuse between adjacent cells, the very short half-life (about 5 seconds in biological tissues) means that NO only acts locally (23).

### 1.1.3.3 Synthesis and biokinetics

Nitric oxide principally enters exhaled breath by diffusion across the membrane of airway epithelial cells. It is produced in these cells from the amino acid L-arginine in a reaction catalysed by one of three nitric oxide synthase enzymes. Two of these – endothelial and neuronal nitric oxide synthase – are constantly active in endothelial cells and are responsible for the basal level of exhaled NO. The third, inducible nitric oxide synthase, has a variable level of activity which and may be upregulated by cytokines and similar inflammatory mediators. Differences in levels of nitric oxide produced between individuals are mostly due to variance in levels of iNOS activity (24).

Nitric oxide synthases are found in both the upper and lower airways. However, the relative contribution to total exhaled nitric oxide from the upper and lower airways varies between individuals. In healthy patients, the majority of exhaled nitric oxide comes from the nose and upper airways, while in patients with a raised $F_E NO$ a greater proportion often comes from the lower airways. This suggests that the nose and upper airways are the primary sources of basal exhaled nitric oxide, with raised $F_E NO$ being caused by an increase in NO synthesis and release in the lower airways (5).
1.2 The electronic nose
1.2.1 Emulation of the sense of smell
1.2.1.1 The human olfactory system

Human technology is often directed toward the emulation of the human special senses. Beginning with the development of the phonograph in the 1870s, the recording of sound has long since exceeded the capabilities of human hearing. Likewise, the camera, in the manner of imitating sight, now has developed well past the capabilities of the eye. Technologies to mimic the sense of touch, and associated dermal sensations, have also been produced. However, the last two special senses - taste and smell - have been comparatively unexplored (1).

The sense of smell arises from specialised neurons in the olfactory epithelium. Olfactory receptors on the dendrites of neurons bind to volatile compounds adsorbed in the mucus covering the epithelium, and cause depolarisation of the neuron if the stimulus is sufficiently strong. Movement of an odorant molecule across the mucus may be by diffusion or by transport aided by 'odorant binding proteins'. The range of odorant binding proteins and olfactory receptors expressed determines the range of odours detectable by an organism. Surface areas for interaction between odours and olfactory receptors is maximised by the highly ciliated morphology of olfactory neurons (25).

Olfactory neurons differ from most human sensory neurons in that they regenerate throughout the life of an organism, with life spans ranging from a few days to several weeks (1) with an average lifespan of around 22 days (13). The implication of this is that the sense of smell is malleable and varies in relation to age and well-being (1). Although the sensitivity of any particular neuron is generally in the parts-per-million range, and specificity is poor, sensitivity is increased by several orders of magnitude by subsequent neural processing (13). Outputs from olfactory neurons are also combined with outputs from neurons involved in gustation (taste) and sensory information from the trigeminal nerve (1) before being processed by the brain, allowing sensation from these alternative sources to contribute to the sense of smell.

The olfactory receptor genome contains around 900 genes in humans. The production of a large variety of odorant receptors is accomplished in a similar way to the production of antibodies. Like antibodies, odorant receptors are generally nonspecific, and a single receptor...
will respond to a variety of compounds with varying response amplitudes. Each odorant receptor is expressed on a subset of neurons on the epithelium, and in general each neuron will contain only one type of odorant receptor (25). There is no known spatial organisation of receptors for specific odours; neurons expressing a particular receptor are spread with consistent density across the epithelium (1).

1.2.1.2 **Quantification of odour**

The degree to which a molecule is detectable by smell is described by the ‘olfactory threshold’ and ‘intensity curve’. The olfactory threshold of a molecule is the minimum concentration of the molecule in air that is detectable by the human nose. The intensity curve of a molecule is the plot of perceived odour intensity against concentration in air. Intensity curves are generally non-linear and unpredictable (1), although they are to some degree described by the Weber-Fechner law

\[
I \propto \log \left( \frac{C}{C_0} \right)
\]

where \( I \) is odour intensity, \( C \) is the concentration of a molecule in gas, and \( C_0 \) is the olfactory threshold of the molecule (26).

In general, the sense of smell is applied to a ‘headspace’ – the gas directly surrounding a sample. The concentration of a given molecule in the headspace of the sample is dependent on the volatility of the molecule. Consequently, the concentrations of molecules in the headspace are not proportional to concentrations of the same molecules in the sample (1). It is important to distinguish the headspace concentration and the sample concentration of a molecule.

The constituents of a liquid or solid sample which have a high volatility will generally have higher relative concentrations in the headspace of the sample than constituents with low volatility. Although the olfactory threshold of molecules varies widely, molecules with low volatility are less likely to be detected in a sample because a much higher sample concentration of such molecules is required to reach their threshold headspace concentrations. For this reason most molecules which are detectable in a sample are highly volatile.
Detectable molecules in a sample are usually small (30-300 g mol$^{-1}$), as larger molecules generally have low volatility. Non-polar molecules tend to have weaker intermolecular bonds than polar molecules and consequently greater volatility, meaning that detectable molecules tend to have one or at most two polar functional groups (1).

A sample may be ‘simple’, containing only one molecular compound, or ‘complex’. Natural smells and flavours are generally complex, arising from large numbers of odorant molecules. Molecules with a low olfactory threshold tend to dominate the odour of a sample, and are termed ‘Key Impact Odours’. When such molecules are added to a gas sample, they may cause the sample to be misclassified according to odour (27). This effect may be strong enough to cause misclassification of samples in breath analysis (28).

In biological olfaction, even identical samples can smell different between individuals due to the subsequent neural processing of the response of the olfactory neurons (1). Electronic noses are generally more reproducible in this respect as the processing techniques are prescribed.

### 1.2.1.3 Electronic olfaction

An electronic nose can be defined as “an instrument which comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern recognition system, capable of recognising simple or complex odours” (13). The earliest such device was developed by Moncrieff in 1961, and worked on mechanical rather than electrical principles. This was followed by Wilkens and Hatman in 1964, who developed an odour sensor which worked by oxidation-reduction reactions at various electrodes. The first electronic nose in the modern sense, consisting of an intelligent array of gas sensors, was developed independently by Persaud and Dodd in 1982 and by Ikegami et al. in 1985-87 (14).

The general approach to produce an electronic nose-like device is the construction of an array of chemical sensors. Output from the sensors can then be analysed by pattern recognition. Ideally, when a sample is presented to the sensors, an array of outputs characterising the sample is produced, and pattern recognition techniques can be used to classify the sample (14). The use of neural-network based pattern recognition algorithms is indicated by the phenomena of biological olfaction.
1.2.2 Gas sensors
1.2.2.1 Requirements for gas sensors

All gas sensors comprise a chemically sensitive material interfaced to a transducer. The interaction of odorant molecules with the chemically sensitive material generates a physical change which is detected by the transducer and converted into an output signal (1). In order to be useful in electronic nose applications, gas sensors have to satisfy several requirements, in some ways reminiscent of the properties of biological olfactory receptors. Firstly, 'reversibility' - the return of the sensor to a consistent state after a measurement- is necessary in order that the sensor can be re-used. Reliability is also important; the sensor must give a consistent response when presented with identical samples. The sensor must be reasonably sensitive and selective in order to be useful, and robustness is needed if the sensor is to be used long-term (14).

These simultaneous requirements often mean that trade-offs must be used in sensor design so that no one requirement is compromised. In the same way as in biological noses, sensitivity and specificity are gained through the use of multiple sensors of different types (14).

Compounds may bind to sensors in several different ways. Binding mechanisms include adsorption (the attraction of molecules in a liquid or gas to an adjacent solid surface), absorption (the incorporation of molecules in a gas or liquid phase into a liquid or solid phase), chemisorption (adsorption with an additional reaction at the adsorptive surface), and co-ordination bonding (electromotive attraction between a molecule and an inert surface). Often the interaction between a compound and sensor will be a combinations of these bind types (14).

Chemisorption is a binding technique which allows high selectivity, due to the requirement of a chemical interaction between the sensor and the substance. However, because binding in this way involves a chemical reaction rather than only intermolecular attraction, the reversibility of binding is often compromised. Purely adsorptive binding, by contrast, is easily reversible, though selectivity is poor (14).
1.2.2.2 Carbon black conducting polymer sensors

The electronic nose which was used in this study contained 32 carbon black conducting polymer sensors. Each sensor consisted of a volume of a non-conducting polymer interspersed with a carbon black ‘conducting filler’. A different polymer was used in each sensor (17).

Conducting polymer sensors of this type characterise a gas by a change in resistance through the sensor. Fillers are homogenously spread throughout the polymer, and allow the sensor to conduct electricity. When the sensor size is at a minimum, the density of the filler in the sensor is high, and many conducting pathways exist in the form of 'tracks' of filler through the polymer. As the polymer sensor absorbs, adsorbs or chemisorbs a gas sample, the density of filler decreases, and less conducting pathways are present (fig.1) causing an increase in resistance through the sensor (14). The change in resistance is analogous to the faster spread of a forest fire through a dense forest than a sparse one, or the faster spread of disease in a dense population (17). See figure 1.

![Figure 1. Carbon black conducting polymer sensors.](image)

The upper diagram shows a carbon black conducting polymer sensor prior to exposure to a gas sample. The sensor is at minimum size, with a high density of conducting ‘filler’ and consequently many conducting paths with low resistance.

The lower diagram shows a carbon black conducting polymer sensor after exposure to a gas sample. The sensor has increased in size after adsorbing the sample, and has a lower density of conducting filler, with less conducting paths through the sensor and consequently higher resistance.

Figure adapted from (17).
1.2.2.3 Other sensor types

Sensors used in electronic noses vary according to the desired function of the nose. Broadly, sensors can be divided into four categories: electrochemical, optical, piezoelectric, and thermal. Sensors vary according to operating temperature, power consumption, long-term accuracy, and detection range. In consideration of the operating environment and expected use of an electronic nose, the choice of sensor type is important (1). Generally sensors consist of a volume of a gas-sensitive material which changes in character (volume, absorbance spectrum, or temperature) when exposed to a gas sample. The change is then measured and recorded as the sensor response (14).

Absorbent polymers are widely used as gas-sensitive materials in electronic nose applications. Polymers respond to a wide variety of gases and can operate at ambient temperatures, although they can be sensitive to humidity and their response can drift over time. The polymers used in such sensors absorb VOCs, causing swelling of the sensors. The degree to which a gas causes this swelling is dependent on the molecular properties of the gas (14).

Other gas sensitive materials include porphyrins and other organometallic compounds, and metal oxides (14).

Conductivity sensors generally are generally formed of either metal oxides or polymers. Metal oxide semiconductors (MOSs) generally utilise oxides of strontium, tungsten, zinc or titanium together with a palladium or platinum catalyst. Resistance changes in response to oxidation-reduction reactions involving VOCs on the oxide surface (1). Metal Oxide Semiconductor Field Effect Transistors (MOSFETs) are metal oxide based devices used as semiconductors in conventional electronics which can be adapted as gas sensors. MOSs generally operate at temperatures of 300-500 °C, and MOSFETs (when used as sensors) at around 150 °C (14). MOSs generally have a relatively high power consumption when compared to other sensor types (1). Conductivity sensors formed of polymers without any conducting filler can also be used but have lower sensitivity and specificity than those with fillers (14).
Optical sensors are widely used in chemical testing although they have not been used extensively in electronic nose technology. One type of sensor that has been used successfully (29) characterises samples by the change in colour of a sensor exposed to the sample. Sensors based on differentiation by colorimetry in this way often utilise transition-metal bound complexes such as porphyrins to chemisorb a gas (14).

Another type of optical sensor differentiates gases by fluorescence, in a technique analogous to the chemiluminescent measurement of exhaled nitric oxide. Fluorescent sensors are formed by coating one end of an optical fibre with a fluorescent polymer, which reacts with gases non-specifically in the same way as conducting polymers do. The resulting fluorescence can be measured from the other end of the optical fibre.

Optical sensors can also be used to characterise a gas absorbance spectrum, refractive index, or reflection. Because of the wide range of characteristics of a sensor which can be measured simultaneously with optical sensing, it is highly versatile. (14).

Piezoelectric sensors operate by measuring the change in fundamental resonant frequency of a crystal due to chemisorption of a gas sample by an adsorbent layer (usually a polymer) on the crystal surface. Crystals can be induced to undergo mechanical strain in response to an applied charge (the piezoelectric effect) and can thus be caused to vibrate by application of an alternating current. The fundamental frequency of vibration of a crystal can be found by the frequency of alternating current at which circuit reactance is minimised. When a compound binds to the polymer layer on the surface of the crystal, the total volume of the crystal and polymer layer is increased. The consequent change in resonant frequency of the crystal can be used to characterise the gas (1).

A type of thermal sensor, the Pellistor, has been used in some electronic nose applications. Pellistor sensors discriminate gases based on the heat released when they are catalytically oxidised. Although these types of sensors are typically non-sensitive to humidity (14) and give a rapid response, they require a high operating temperature (65) and offer a comparatively small range of detection (1).
1.2.2.4 Sensor reaction to compounds

The numerical reading across a gas sensor exposed a compound changes over time. The numerical reading $V$ of a sensor $s$ exposed to a sample $i$ for time $t$ can be considered a function $V(i,s,t)$, assuming the sensor is fully reversible and reproducible. As long as a sensor is exposed to an odour in a consistent way (with regard to flow rate and sample concentration) $V$ will tend toward a fixed $V_0$ and $\partial V/\partial t$ will tend to 0 as $t$ increases. When $V$ is close to $V_0$ the sensor is essentially in equilibrium or a ‘steady state’ with the compound. The behaviour of $V$ in the time before the steady state is reached is dependent on the delivery system to the sensor, reaction kinetics and diffusion rates of the odour, the nature of the sensor and supporting substrate, and ambient conditions. Generally the sensor response is considered to be the value of the sensor signal at the steady state. There has been little analysis done of the discriminatory potential of the function $V(i,s,t)$ prior to the steady state (13).

Several methods are used to characterise the steady state and give a numerical value $X(i,s)$ representative of the response of sensor $s$ to sample $i$. Because $V$ is generally monotonically increasing or decreasing with time $t$, $X$ can be defined in terms of a maximum value of $V$, $V_{\text{max}}$, taken during the time the sample is exposed to the sensor and a baseline value of $V$, $V_{\text{min}}$, taken prior to sample exposure (71). The definition of $X$ in terms of $V_{\text{max}}$ and $V_{\text{min}}$ is decided based on the nature of the sensor (15). In this study, the fractional difference $X = (V_{\text{max}} - V_{\text{max}})/V_{\text{min}}$ is used, as recommended by the manufacturer (17).

1.2.2.5 Percolation and conducting polymer sensor response

In the measurement used in this study, it was assumed that sensors would reach a satisfactory equilibrium with a baseline air sample after 30 seconds of exposure, and with a breath sample after 60 seconds of exposure.

The sensors used in the study exhibit a non-linear relationship between the swelling of the sensor and the corresponding increase in resistance. If the sensor swells enough, all conducting pathways through it will be broken, and a ‘percolation threshold’ will be reached as the last pathway breaks. At this critical point, a small increase in volume will cause a large increase in resistance (17). It is assumed that no sensor will swell enough to cause this threshold to be reached. See figure 2.
17

Figure 2. Relation of sensor resistance to sensor volume near percolation threshold.

Three percolation curves show the different volume changes required to reach the percolation threshold for three carbon black/polymer composite materials containing 34%, 35%, and 40% carbon black. Caption and figure from (17).

1.3 Direct analysis of exhaled breath
1.3.1 Direct analysis of entire breath makeup
1.3.1.1 Utility of entire breath makeup analysis

Establishing the exact composition of a gas sample requires complex techniques from analytical chemistry. Most early studies in the area of breath analysis (3, 18-20) used the method of gas-chromatography - mass-spectrometry (GC-MS) although more recently the method of proton-transfer reaction– mass-spectrometry (PTR-MS) has been used in several studies (30). Although GC-MS is generally preferred as a method of direct analysis, PTR-MS is faster and can be performed in closer to real time (12).

GC-MS or PTR-MS are commonly used as secondary analysis techniques in studies using electronic noses (28, 31). The concurrent use of direct breath analysis in this way can allow
the molecules responsible for differences in electronic nose readings to be identified. However, there is only limited advantage in such identification; clinically, indirect breath profiles are as adequate as biomarkers, and knowledge of the molecules causing the difference is not necessary (2). Recent studies (32-35) have used electronic nose measurements to analyse breath samples without any use of direct analysis. In this study, no direct breath analysis was used, as no information about actual breath composition was sought.

1.3.1.2 Methods of holistic direct breath analysis

Direct breath analysis differs from indirect breath analysis mainly in that constituents of a gas are separated prior to detection. In indirect breath analysis, there is no need to include a separation technique, as individual constituents are not of interest.

In GC-MS, the gas in question is first separated into constituent molecules by chromatography, a method of separation of molecules based on volatility. A gas sample is combined with an inert gas, heated, and injected at one end of a 'column', consisting of a long tube coated with a 'stationary phase' composed of a single organic compound. The stationary phase will adsorb the various components of the gas at different rates, meaning that the components of the gas interact with the walls of the column at different rates. The rate at which molecules pass through the column is proportional to the degree to which they interact with the wall, meaning that the various components of the gas emerge from the end of the column at different times. Generally, the less volatile a compound is, the more it will interact with the stationary phase, and the later it will emerge from the column (11).

Once a gas has been separated in this way, mass spectrometry can be used to identify the compounds that have eluted. This involves the ionisation of molecules in a gaseous compound and subsequent acceleration of the ions by an electric field. The path of the ions through the electric field can be used to determine their mass-to-charge ratios and hence identify them (11). GC-MS is capable of achieving sensitivities as low as 0.1 parts per billion (12). By contrast, the human nose is capable of detecting some compounds at less than ten parts per billion (36).

PTR-MS is a similar technique in that it involves the separation of a gas sample into constituent parts followed by spectrometry of the constituents. In PTR-MS, a quantity of near-
pure $\text{H}_3\text{O}^+$ ions are formed, and mixed with the sample at one end of a drift tube, which is subject to an electric field. In general, VOCs in the sample will be protonated by the $\text{H}_3\text{O}^+$ and will thus become ionised. The electric field of the drift tube then accelerates the ions. Collisions between ions and other molecules in the tube provide a braking force on the ions in proportion to their velocity, meaning that each type of ion in the sample eventually reaches a terminal velocity inversely proportional to its gas number density. The variance in terminal velocities of components of the gas sample means that constituents reach the end of the drift tube at different times, where they can be analysed by mass spectrometry as for GC-MS (12).

1.3.2 Measurement of individual substances in exhaled breath

1.3.2.1 Methods for analysing exhaled nitric oxide

The main two methods for analysing nitric oxide concentrations in exhaled breath are chemiluminescence and electrochemical sensing. Chemiluminescence is a technique in which NO is quantified according to the intensity of light emitted when NO is reacted with ozone. This is a preferred technique for clinical NO measurement (8).

In electrochemical gas sensing a gas is oxidised or reduced at an electrode and the resultant current is measured to establish the gas concentration. Devices which work by this property have the advantage of being much smaller and more portable than chemiluminescence analysers, although they are not as accurate (8).

1.3.2.2 Measurement of breath ethanol

One of the most easily-measured compounds on exhaled breath is ethanol. Ethanol concentration on the inhaled breath is a good predictor of concentration in the bloodstream, meaning that exhaled breath measurement of ethanol provides a useful clinical measure of ethanol intoxication. The earliest attempts to measure alcohol level in breath were made in 1927, where a football was used as a device for standardising breath collection and quantification of alcohol was measured by titration against potassium dichromate (9). Later techniques identified the need to use end-exhalation air for the sample, and modern ‘breathalysers’ operating on the same principles were developed in the 1950s. The 'Intoxilyser', which has been in use since 1992, uses infrared spectroscopy to determine concentration somewhat more accurately. Other techniques for breath alcohol analysis use
semiconductors and fuel cells (21). One study successfully measured breath alcohol concentrations using an electronic nose, in a rare example of the use of indirect breath analysis techniques for a direct measurement (31).

Because ethanol is known to be present in relatively high concentrations on the breath following ethanol ingestion, and is a highly chemically reactive compound, it is likely that ethanol presence due to ingestion would be a major confounding variable in electronic nose measurements. One study (31) found that electronic nose measurements were influenced in this way. For this reason, it was necessary to control the effect of ethanol consumption. This was achieved by the requirement that all participants did not consume ethanol in the 24 hours before a breath sample was taken.

2 Literature review
2.0 Overview

A summary of the literature review is shown in Table 1.

A literature search was conducted to find papers which described an electronic nose-based analysis of exhaled breath.

The Ovid database was used to search all OVID Medline records from 1950 to present, and the EMBASE records from 1947 to present, for papers which had ‘electronic nose’ and ‘breath’ as keywords.

Only papers reporting an original experiment were included. All studies used an electronic nose (defined as an array of non-specific gas sensors) as a primary or secondary study endpoint. The search was conducted on 2 October 2010.

One additional study (37) was found subsequently and was included in the review.

2.1 Summaries
2.1.1 Available and appropriate studies

Five hundred and eighty nine papers were found with ‘Electronic Nose’ as a keyword, and 63,
Twenty one papers were found which had both keywords. Fifty eight papers were found which had both keywords. Twenty one papers were found to be repeated within these results. Of the 37 papers remaining, 10 were reviews or opinion pieces, 2 were available only in Chinese, 3 used only direct gas analysis, 2 analysed VOCs from sources other than exhaled breath, and 2 could not be accessed. After this filtering, 18 papers were found that described a clinical study involving exhaled breath analysis by electronic nose. One study was added later, for a total of 19 papers.

All but three studies attempted to use the electronic nose to discriminate groups of participants. Two of the remaining studies analysed levels of a characteristic of a single condition (37-38) and the third was a small crossover trial (31). Of the studies which sought to discriminate groups, all but three compared one or several conditions to a healthy control group. The other three attempted to distinguish a subject group (such as lung cancer) from a control group with non-specific lung pathologies (33, 38-39).

A range of electronic nose instruments and breath sampling techniques were used. Most studies used a similar set of analyses for EN data, although many also employed more complex techniques in addition to this.

### 2.1.2 Sensor types and breath sampling techniques

The most common EN used in the reviewed papers was the Cyranose 320, used in 9 studies (32-35, 37-38, 40-43). Other noses used included a 10-MOSFET array (31), a 6-MOS array (44), a 36-colorimetric sensor array (29), an array of unknown size based on gold nanoparticles (45), and a 7 or 8-quartz microbalance (metalloporphyrin-based piezoelectric) sensor array (7, 27-28, 39, 46). Other than the Cyranose, the only device used in more than one study was an 8-quartz microbalance sensor array. There was no observed relation between the type of EN used and the type of experiment being performed.

Breath sampling techniques varied somewhat between papers. The most common technique was the collection of a vital capacity following five minutes of tidal breathing through a VOC filter (32, 35, 37, 40-41, 43). All papers which used this technique of breath collection also used the Cyranose for analysis. Three earlier studies used a vital capacity manoeuvre without the five-minute tidal breathing period (28, 31, 34).
Several other techniques were used which were customised to the experiment being conducted. Three studies whose groups consisted of mechanically ventilated patients used samples taken from expiratory ports of the patients’ ventilators (33, 38, 42). Two studies on rhinosinusitis sampled only expired air from the nasal passages; one by means of a CPAP mask (29) and the other by a bifurcated nasal tube insertion following nasal mucus induction (46). A study on halitosis sampled only the upper part of exhaled breath by an unreported technique (27), and a study on lung cancer sampled only end-exhalation breath (39).

Montuschi et al. (7) performed a comparison between two sampling techniques, as part of a wider study comparing diagnostic performance of several tests. The paper found that samples consisting of full vital capacities were less well-separated between groups than samples containing a vital capacity first 150ml of exhaled air discarded.

### 2.1.3 Study sizes and groups

All studies were conducted on fewer than 150 participants. The smallest included only four individuals in a crossover design (31). The largest used separate ‘training’ and ‘discovery’ groups and had a total of 133 patients (34). Otherwise, total study size ranged from 10 to 115. The minimum size for a group varied; while one study accurately discriminated chronic beryllium disease with a group of only 6 patients (34), and another discriminated prostate cancer and head/neck cancer with only 5 (45), another, with two groups of five participants each, was unable to obtain a significant separation (46) although group data appeared visually separated. The shortcomings of such a small size were noted in the paper.

### 2.1.4 Analysis techniques

Most studies used principal component analysis (PCA; see methods section 2.2.3.1) as an initial unsupervised method to reduce sensor data (13 out of 18 studies where analysis techniques were reported). This was often followed by a linear discriminant analysis (LDA; also called ‘Fisher’s linear discriminant’, see methods sections 2.3.2.1, 2.3.2.2, and 2.3.3.1) on the extracted components, with error estimated by leave-one-out cross validation (7 out of 13 studies). All but one (38) of the papers which used the Cyranose 320 performed a principal component extraction as a first stage of data reduction.
Many studies also used more complex analysis techniques, although usually as a secondary analysis following LDA. Three papers used an artificial neural network in the architecture of a multilayer perceptron (MLP; see methods sections 2.3.2.1 and 2.3.3.2) as a classifier (7, 31, 46). Three papers used support vector machine analysis with internal (33, 42) or external (34, 42) cross-validation (CVV; see methods section 2.3.2.1). Four studies used partial least squares (projection to latent structure; see Friedman et al. (47)) discriminant analysis (28, 31, 39). Papers which attempted to use the nose to predict an ordinal or ratio variable rather than a category used linear and nonlinear partial least squares regression (38), multiple linear regression (37, 44), or Spearman’s rank correlation (37).

2.2 Results

2.2.1 General

A range of diseases and conditions were successfully distinguished by electronic nose and many studies noted the advantage of the non-invasive sampling technique. To date, however, there have been no studies of a sufficient size to justify the introduction of the electronic nose as a clinical tool, and no attempts to assimilate raw electronic nose data from multiple studies were found.

2.2.2 Asthma

The earliest use of the electronic nose to diagnose asthma was by Dragonieri et al. in 2007 (32). This study attempted to discriminate asthma and controls after stratifying by age. A total of 40 patients were recruited, encompassing 10 asthmatics and 10 controls under 35, and 10 asthmatics and 10 controls over 35. The younger asthmatics had less severe asthma than the older asthmatics. Breath samples were collected by a vital capacity manoeuvre following 5 minutes of tidal breathing, and were analysed using a Cyranose 320. Data from the nose were reduced by PCA and analysed by linear discriminant analysis, with leave-one-out CVV as the marker of success. A CVV of 100% was found between young controls and young asthmatics, and a 90% CVV between old controls and old asthmatics. No significant difference was found between the young and old controls, or between the young and old asthmatics.

Fens et al. in 2009 (35) conducted a study comparing the breathprints of patients with asthma
and patients with COPD. The study groups consisted of 30 patients with COPD, 20 patients with asthma, 20 control patients who were current smokers and 20 controls who were non-smokers. Breath samples were collected and analysed in the same manner as Dragonieri. The study found that asthma and COPD could be successfully distinguished with 96% CVV, asthma and non-smoking controls could be separated with 95% CVV, and asthma and smoking controls could be separated with 92.5% CVV. COPD and smoking controls were less well separated with 66% CVV.

Montuschi et al. in 2010 (7) compared the ability of the electronic nose, FeNO and spirometry in diagnosing asthma. The study groups consisted of 27 asthmatics and 24 healthy controls. Breath was collected through a single vital capacity manoeuvre. No tidal breathing of filtered air was undertaken prior to collection. Exhaled breath analysis was done firstly on the entire exhaled volume and secondly on the exhaled volume after the first 150ml of exhaled air had been removed. An array of eight quartz microbalance (piezoelectric) sensors was used. The electronic nose method correctly diagnosed 87.5% of asthmatics compared with 79.2% for FeNO and 70.8 by spirometry. Better discrimination was observed when the breath sample with the first part removed was analysed than when the whole breath was analysed (87.5% vs 75%).

2.2.3 COPD

Two studies used the electronic nose to analyse exhaled breath from COPD patients. Fens et al. (37) compared electronic nose samples of COPD patients to their differential sputum eosinophil counts (obtained using a method similar to that in this study; see methods section 1.1.5.1) by rank correlation. The study included 19 patients. A significant correlation (-0.50, p=0.03) was found between a principal component of electronic nose samples and sputum eosinophil counts. The study also searched for a correlation between principal components and neutrophil counts, but found no significant correlation.

Dragonieri et al. (43) found an 85% CVV when discriminating non-small-cell lung carcinoma (NSCLC) from COPD and a 90% CVV when distinguishing NSCLC from controls.

2.2.4 Cancer
Several studies focused on identifying lung cancer from biomarkers in breath. Generally the cross-validation value was the principal finding recorded. Di Natale et al. (28) found a 91.6% CVV when discriminating lung cancer pre- and post-treatment from controls, and a 100% CVV when discriminating cancer from non-cancer. Machado et al. (34) found a 71.6% internal CVV (leave-one-out) when discriminating lung cancer, alpha-1-antitrypsin deficiency, chronic beryllium disease and controls, and an 85% CVV on external cross-validation (separate testing set). Abdah-Bortnyak et al. (45) compared lung, breast, colon, prostate and head/neck cancer with controls and reported a 92% sensitivity when distinguishing all of them. D’Amico et al. (39) found a sensitivity of 85%, specificity of 100%, NPV of 90% and PPV of 100% when comparing lung cancer with other non-specific lung disease and controls. Collectively, there appears to be good evidence that breath analysis by electronic nose can distinguish lung cancer from controls, other lung diseases, and other cancers.

2.2.5 Pneumonia

Three studies attempted to diagnose pneumonia among mechanically ventilated patients. Hockstein et al. in 2004 (42) found an 80% external cross-validation value when comparing pneumonia with non-pneumonia as diagnosed with a CT scan. Hockstein et al. in 2005 (33) and Hanson and Thaler (38) used a ‘pneumonia score’ based on several pathological variables to quantify the presence and severity of pneumonia. Hockstein et al. distinguished a ‘high’ pneumonia score from a ‘low’ pneumonia score with 77% CVV. Hanson and Thaler found that a correlation of 0.81 between an electronic nose-based score and a clinical pneumonia score. All of these studies took breath samples directly from the expiratory ports of mechanical ventilators. The ease of sampling and the successful results obtained suggest that EN analysis of mechanically exhaled air could be a good biomarker for the presence and severity of pneumonia.

2.2.6 Halitosis

Two studies explored the ability of the electronic nose to detect halitosis. Neither study attempted to define halitosis, instead seeking to identify specific compounds on the breath which corresponded to them, albeit using an electronic nose to do so. Nonaka et al. (44) found that a score derived from electronic nose readings could predict the presence of certain
compounds on the breath as well as GC-MS. Pennazza et al. (27) found that the breath of individuals with and without halitosis could be discriminated. Although both these studies reported some success in discrimination, the principal aim behind each was simply to test the abilities of the nose rather than to develop a clinical tool.

2.2.7 Other

Other studies used the nose to quantify breath alcohol, diagnose sinusitis, detect smoking status, and diagnose interstitial lung disease. Paulsson and Winquist (31) found that an electronic nose could measure breath ethanol in a small group of subjects. Mohamed et al. (46) found a 60% CVV when comparing patients with chronic rhinosinusitis to controls, and Thaler et al. (29) found a 90% CVV when comparing acute or chronic sinusitis to controls. Cheng et al. (41) found a 95% CVV when comparing current smokers with non-smoking controls. Dent and Bowman (40) found a 76.3% CVV when comparing patients with interstitial lung disease to controls. The range of applications of electronic nose technology in analysing exhaled breath is evident, and there appears to be potential for several conditions to be tested for concurrently.
### Table 1. A summary of available literature describing exhaled breath profiling by an Electronic Nose

<table>
<thead>
<tr>
<th>Paper*</th>
<th>Study groups or dependent variable</th>
<th>Total participants†.</th>
<th>Type of electronic nose</th>
<th>Sampling technique</th>
<th>Analysis techniques‡.</th>
<th>Main results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paulsson (1999) (31)</td>
<td>Alcohol in breath (quantification)</td>
<td>4 (crossover)</td>
<td>10 MOSFETs and CO2 sensor</td>
<td>Patient breathed in, held for 20 seconds, then exhaled into bag</td>
<td>PCA, ANN, PLSR (RMS error of ICV, ECV)</td>
<td>Using PLSR, RMSE of 0.065 mg/L. Using ANN, RMSE of 0.037 mg/L.</td>
<td>Unusual instance of the direct measurement of a compound using an electronic nose.</td>
</tr>
<tr>
<td>Mohamed (2003) (46)</td>
<td>Chronic rhinosinusitis vs controls</td>
<td>10 (5 patients with chronic rhinosinusitis, 5 HC)</td>
<td>8 quartz microbalance sensors with metalloporphyrin coating</td>
<td>Collection of nasal outbreath following stimulation of nasal secretion</td>
<td>PCA, ANN (ICV).</td>
<td>80% CVV.</td>
<td></td>
</tr>
<tr>
<td>Di Natale (2003) (28)</td>
<td>Lung cancer pre treatment vs lung cancer post treatment vs control</td>
<td>60 (35 lung cancer pre-treatment, 9 lung cancer post-treatment, 2 lung cancer pre and post-treatment, 18 HC)</td>
<td>8 quartz microbalance sensors with metalloporphyrin coating</td>
<td>Patients with identical diet and mouth hygiene took samples in morning before eating. Patients breathed directly into a tedlar bag.</td>
<td>PLSR (ICV)</td>
<td>100% CVV between cancer and non-cancer; 91% CVV (3-way) between all groups.</td>
<td></td>
</tr>
<tr>
<td>Hockstein (2004) (42)</td>
<td>Pneumonia vs non-pneumonia. All patients mechanically ventilated.</td>
<td>25 (13 pneumonia, 12 non-pneumonia)</td>
<td>Cyranose 320</td>
<td>Breath sample from exhalation port of mechanical ventilator.</td>
<td>Exploratory PCA, SVM analysis (ICV,EVC)</td>
<td>100% CVV using SVM, 80% external cross validation value</td>
<td></td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Subject Description</td>
<td>Sample Size</td>
<td>Methodology</td>
<td>Classification</td>
<td>Prediction Model</td>
<td>Validation</td>
<td>Results</td>
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<tr>
<td>Nonaka (2005) (44)</td>
<td>Patients with oral malodour vs patients without oral malodour</td>
<td>66 (46 oral malodour, 20 no oral malodour)</td>
<td>Six metal oxide semiconductors</td>
<td>Unknown.</td>
<td>MLR between an EN score and organoleptic score, a measure of oral malodour intensity. ROC curve for ability of EN score to predict oral malodour presence (AUC, CC).</td>
<td></td>
<td>Electronic nose correlated with organoleptic score (CC 0.81), AUC of 0.975. EN performed better than measurement of volatile sulphur compounds on breath in predicting oral malodour (coef. 0.73). Extraction method for EN score was prescribed before the study.</td>
</tr>
<tr>
<td>Hockstein (2005) (33)</td>
<td>High pneumonia severity score vs low pneumonia severity score. All patients mechanically ventilated.</td>
<td>44 (15 high pneumonia score, 29 low pneumonia score)</td>
<td>Cyranose 320</td>
<td>Breath sample from exhalation port of mechanical ventilator.</td>
<td>Fishers discriminant analysis, KNN, SVM (ICV)</td>
<td>70% CVV using KNN</td>
<td>66% CVV using FDA.</td>
</tr>
<tr>
<td>Machado (2005) (34)</td>
<td>Training stage - Healthy controls vs lung cancer vs chronic beryllium disease vs AAT deficiency. Discovery phase - cancer vs non-cancer</td>
<td>133 (Training stage (59 total) - 14 lung cancer, 19 AATD, 6 CBD, 20 HC. Testing stage (74 total) - 14 untreated lung cancer, 12 COPD, 11 Asthma, 7 pulmonary hypertension, 30 HC)</td>
<td>Cyranose 320</td>
<td>Collected one FVC following exhalation to residual volume and inhalation through a filter.</td>
<td>PCA, LDA, SVM (ICV, ECV, MD).</td>
<td>71.6% CVV (training set). SVM algorithm correctly identified 85% of discovery set</td>
<td>71.4% sensitivity and 91.9% specificity reported for SVM model</td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Disease/Condition</td>
<td>Sample Size</td>
<td>Methodology</td>
<td>Classification</td>
<td>Model</td>
<td>Results</td>
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<tr>
<td>Hanson (2005) (38)</td>
<td>Pneumonia score prediction. All patients mechanically ventilated.</td>
<td>38 (19 low pneumonia score, 19 high pneumonia score, 38 total.)</td>
<td>Cyranose 320 Breath sample from exhalation port of mechanical ventilator.</td>
<td>Linear and nonlinear PLSR (CC)</td>
<td>CC of 0.81 for PLSR</td>
<td></td>
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</tr>
<tr>
<td>Dragonieri (2007) (32)</td>
<td>Young patients with mild asthma vs young controls vs old patients with severe asthma vs old controls</td>
<td>40 (10 mild asthma, 10 young control, 10 severe asthma, 10 old control)</td>
<td>Cyranose 320 Collected one FVC following 5 minutes tidal breathing VOC-filtered air</td>
<td>PCA, LDA (ICV)</td>
<td>100% CVV comparing ‘mild asthma’ and ‘young control’ groups, 90% CVV comparing ‘severe asthma’ and ‘old control’ groups. 65% CVV comparing mild and severe asthma, 50% CVV (insignificant) comparing young and old controls</td>
<td></td>
<td></td>
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<tr>
<td>Thaler (2008) (29)</td>
<td>Sinusitis vs control</td>
<td>20 (11 sinusitis, 9 control)</td>
<td>Colorimetric sensor array using metalloporphyrin dyes. 36 sensor dots on a paper cartridge photographed at 2 minute intervals. Collected exhaled gas from nasal passages using CPAP mask.</td>
<td>PCA, LDA (ICV)</td>
<td>90% CVV</td>
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<tr>
<td>Pennazza (2008) (27)</td>
<td>Individuals without halitosis vs individuals with halitosis vs individuals without halitosis with VOC compounds added to breath</td>
<td>Not reported</td>
<td>Seven quartz microbalance (piezoelectric) sensors with metalloporphyrin coating. ‘Upper part’ of breath sampled (exact method unknown).</td>
<td>PCA (CC)</td>
<td>Apparent separation – no reported CVV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Comparison</td>
<td>Participants</td>
<td>Equipment</td>
<td>Methodology</td>
<td>CVV</td>
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<tr>
<td>Cheng (2009) (41)</td>
<td>Non-smokers vs smokers</td>
<td>39 (15 smokers, 24 non-smokers)</td>
<td>Cyranose 320</td>
<td>Collected one FVC following 5 minutes tidal breathing VOC-filtered air</td>
<td>95%</td>
<td></td>
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<tr>
<td>Fens (2009) (35)</td>
<td>Asthma vs COPD vs healthy control (non-smoking) vs control (smoking)</td>
<td>90 (30 COPD, 20 asthma, 20 smoking control, 20 non-smoking control)</td>
<td>Cyranose 320</td>
<td>Collected one FVC following 5 minutes tidal breathing VOC-filtered air</td>
<td>96%</td>
<td></td>
<td></td>
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<tr>
<td>Dragonieri (2009) (43)</td>
<td>Non-small cell lung carcinoma vs COPD vs healthy controls (non-smoking)</td>
<td>30 (10 NSCLC, 10 COPD, 10 HC)</td>
<td>Cyranose 320</td>
<td>Collected one FVC following 5 minutes tidal breathing VOC-filtered air</td>
<td>85%</td>
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</tr>
<tr>
<td>Abdah-Bortnyak (2009) (45)</td>
<td>Healthy controls vs lung cancer vs breast cancer vs colon cancer vs prostate cancer vs head and neck cancer</td>
<td>115 (30 lung cancer, 15 breast cancer, 20 colon cancer, 5 prostate cancer, 5 head and neck cancer, 40 HC)</td>
<td>Molecularly modified gold nanoparticles</td>
<td>Not reported</td>
<td>92%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First author (year) (reference number)</td>
<td>Total number (number in each group)</td>
<td>Training techniques (evaluation of success)</td>
<td>Diagnostic Test</td>
<td>Diagnosing asthma (CVV)</td>
<td>EN performed better than spirometry and F$_E$NO in diagnosing asthma (CVV 87.5 vs 79.2, 70.1%). A better EN measurement was obtained from alveolar air only than from an FVC (CVV 87.5 vs 75%).</td>
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<tr>
<td>Montuschi (2010) (7)</td>
<td>Asthma vs control</td>
<td>51 (27 Asthma, 24 HC). 27 patients randomly assigned to ‘training group’ and 24 to ‘testing group’ for each diagnostic test.</td>
<td>Eight quartz microbalance sensors with metallocorphyrin coating</td>
<td>Total exhaled breath (1) and total exhaled breath discounting first 150ml (2) following inhalation of ambient air to TLC.</td>
<td>PCA, ANN(ECV) 87.5% CVV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D'Amico (2010) (39)</td>
<td>Lung cancer apart from non-specific other pulmonary condition</td>
<td>82 (28 lung cancer, 28 diverse lung disease, 36 HC)</td>
<td>Eight quartz microbalance sensors with metallocorphyrin coating</td>
<td>End-exhalation breath collection (exact method unknown)</td>
<td>PLSR (ICV) Sensitivity 85%, specificity 100%, NPV 90%, PPV 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dent (2010) (40)</td>
<td>Interstitial lung disease vs healthy controls</td>
<td>37 (17 ILD, 20 HC)</td>
<td>Cyranose 320</td>
<td>Collected one FVC following 5 minutes tidal breathing VOC-filtered air</td>
<td>PCA, LDA (ICV) 76.3% CVV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fens (2010)</td>
<td>COPD by eosinophil count</td>
<td>19</td>
<td>Cyranose 320</td>
<td>Collected one FVC following 5 minutes tidal breathing VOC-filtered air</td>
<td>PCA, Spearman’s correlation Correlation of -0.50, p=0.03 No correlation between PCs and neutrophil count.</td>
<td></td>
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</tbody>
</table>
Asthma is a chronic inflammatory disease of the airways affecting more than 300 million people worldwide (48). Diagnosis of adult asthma is currently based on a characteristic pattern of symptoms, such as chronic cough, wheeze, and impaired airway calibre, without the presence of other explanations (49). Asthma is also suggested by presence of atopic disease or widespread eosinophilia (49), although non-atopic and non-eosinophilic asthma exist (50).

Asthma has a wide range of pathophysiological phenotypes, which may be identified using biomarkers such as sputum eosinophilia and atopy to common allergens (50).

Inhaled glucocorticosteroids (ICS) are the preferred medications for long-term control of asthma and prevention of symptoms. In addition, oral glucocorticosteroids (OCS), particularly prednisone and prednisolone, are recommended in short courses following acute exacerbations and for long-term control in severe asthma (48).

Response to OCS and ICS varies widely (51) and a small proportion of patients do not respond at all. In patients for whom the response to OCS is low, conventional approaches to asthma management may be insufficient to achieve control of symptoms. Studies of dose-response relations for ICS indicate that above a certain threshold, additional steroid use produces diminishing improvements in asthma symptoms (52) and may be harmful (6), contraindicating the management of such patients by increased steroid dose.

Other treatments including theophyllines, leukotriene antagonists and immunosuppressant drugs may be indicated in severely steroid-unresponsive asthma (52-53). Because of the differences in management of steroid-unresponsive asthmatics, and in the interest of avoiding potentially harmful and ineffective steroid treatment of such patients, the prediction of steroid responsiveness is an important clinical problem (6).

Steroid responsiveness has been shown to be associated with changes in sputum cytology (54), exhaled nitric oxide (6), and smoking (55). This suggests that steroid response is related to asthma pathophysiology, and knowledge of pathophysiological phenotype may be of use in predicting steroid response. Indeed, several biomarkers based on these associations show
good promise for prediction; sputum eosinophilia (56), exhaled nitric oxide (6), and blood eosinophilia (54) have been tested.

Glucocorticoid actions are pleiotropic and vary considerably between individuals (57). The principal action of glucocorticoids is regulatory, and many regulatory effects have been identified (54, 57-59). Glucocorticoids also interact directly with cytosolic proteins (57). In general, glucocorticoids have an anti-inflammatory effect, achieved by the upregulation and increased activation of a wide range of secondary messengers.

The effect of glucocorticoids is difficult to measure directly. Although several potential biomarkers have been found to quantify the effect (54, 60), no single biomarker has been found to give an accurate representation of the degree of response (61). Assessment of steroid response by quantification of specific compounds is therefore difficult, and specificity and sensitivity are low. It is possible that an approach based on 'patterns' of increased levels of non-specific biomarkers could better assess steroid response.

Although no study to date has established the capability of the electronic nose in differentiating pathophysiological phenotypes of asthma, the utility of the nose in diagnosing and differentiating other lung pathologies, such as cancer, COPD, and asthma (32, 43) is clear. The use of $F_{E\text{NO}}$ as a predictor of steroid response (6) suggested that the exhaled breath may be effectively used as a sample. Because the electronic nose operates under principles of indirect breath analysis it is suited to the detection of patterns of changes in molecules, rather than changes in individual molecular concentrations (1). The steroid response is associated with a large number of subtle chemical changes in orthomolecular makeup, rather than a small number of marked changes in key molecules (59, 61-63). These characteristics of the steroid response suggested the investigation of prediction and detection of steroid response by electronic nose.

The steroid response is closely related to the biology of eosinophils (50, 64-65) and one possible means of assessing steroid response in asthma is to quantify changes in sputum or blood eosinophil levels. Eosinophil levels can give a good indicator of steroid responsiveness (56). Although many asthmatics are 'eosinophilic' (exhibit a proportionally large number of eosinophils in the airways) when not on steroid medication, although several non-eosinophilic phenotypes exist (64).
Eosinophilic and non-eosinophilic subtypes have been shown to have different clinical characteristics (50). Non-eosinophilic asthma can be further broken down into neutrophilic asthma, paucigranulocytic asthma, and other subgroups (64). The mechanisms by which eosinophilic inflammation leads to asthma differs from the mechanisms giving rise to non-eosinophilic forms of asthma (50). The knowledge of whether asthma cases are eosinophilic or non-eosinophilic is useful clinically, and the recent results by Fens et al. in prediction of eosinophilia in COPD (37) suggested that such determination in asthma may be achievable using the electronic nose.

3.1 Current status of asthma definition and preventative asthma control

3.1.1 Definitions of asthma and assessment of asthma control

3.1.1.1. Definition of asthma

Asthma is a chronic obstructive disease of the airways defined by a characteristic set of symptoms. Such symptoms include wheezing, breathlessness, chest tightness and cough. The diagnosis of asthma generally requires that at least two symptoms are present in the absence of another known cause (49).

According to current guidelines, the diagnosis of asthma is made on a clinical basis. There are no standardised cutoffs of type, severity or frequency of symptoms which characterise asthma from non-asthma (49), although several attempts have been made to produce rigorous definitions (66). The recommended initial clinical test for asthma in adults is spirometry. Further tests include airway hyper-responsiveness tests and spirometry response following the administration of a short acting beta agonist (reversibility) (49).

3.1.1.2 FEV$_1$, PEFR, and questionnaires

Forced expiratory volume in one second (FEV$_1$) and peak expiratory flow rate (PEFR) are commonly used objective measures of airway calibre in asthmatics (67). Both are easily and accurately measured, and can be safely tested even in patients with severe airway pathology. It is often useful to measure airway calibre throughout a study rather than only at certain points (67) and PEFR is the easiest way to do this. FEV$_1$ is the standard measure used for lung-function based testing of asthma severity by ATS/ERS guidelines (67).

FEV$_1$ is related to PEFR in that PEFR is the maximum gradient and FEV$_1$ is the average
gradient of the lung volume/time curve over the time interval [0,1s]. The curve is non-linear; average PEFR for a 35 year old healthy 175 cm male is about 635 L/min, or 10.6 L/s (68) while the average FEV$_1$ for the same demographic is about 4.3 L (69).

Although as a rough measure FEV$_1$ and peak flow are helpful as continuous measurement techniques to observe short-term changes in airway calibre, PEFR shows too much day-to-day variability to be used realistically as a study endpoint (67) and FEV$_1$ may vary considerably day-to-day and is affected by factors outside lung pathology.

As a differential marker of asthma control, patient questionnaires can be a useful indicator of perceived symptom level. Standardised validated questionnaires such as the ACQ (70) and ACT (71) have been developed to meet this requirement. The ACQ was used as an endpoint in this study.

3.1.1.3 Bronchial challenge testing

Airway hyper-responsiveness tests are more stable indicators of asthma and asthma control than spirometric measurements (67). Measurements of airway hyper-responsiveness can be obtained from bronchial challenge testing, which, according to the type of test, can be highly sensitive or highly specific (49).

Bronchial challenge tests establish the minimum concentration or dose of an inhaled bronchoconstrictor required to lower FEV$_1$ by a predetermined percentage (PD%/PC%). The provocative amount is evaluated using a series of inhalations of increasing amounts of bronchoconstrictor, with FEV$_1$ measured between each inhalation. The provocative dose or concentration is extracted by interpolation (72). See methods sections 1.2.2.2-1.2.2.3.

A range of substances have been used as bronchoconstrictors for bronchial challenge tests, including histamine, hypertonic saline, methacholine, mannitol, and adenosine monophosphate (AMP). Airway challenge tests are either ‘direct’ or ‘indirect’ depending on which substance is used. In direct airway challenge tests, the most commonly used of which are histamine and methacholine challenges (73), bronchoconstriction is caused by interaction of the inhaled substance with a specific receptor on airway smooth muscle cells (74). In ‘indirect’ bronchial challenge tests, the most common of which are hypertonic saline, mannitol, and AMP challenges (73), bronchoconstriction is caused by the release of
endogenous substances from inflammatory cells or nerves secondary to the introduction of a bronchial irritant (74).

Direct airway challenges generally have a high sensitivity, whereas indirect challenges have a high specificity (73). A positive indirect challenge is therefore a strong indicator of the presence of asthma. Bronchial challenge tests are also a very strong indicator of asthma control (67).

Indirect challenges are essentially dose-limited in that the inhaled dose or concentration cannot be raised past a physiological or practical limit (73). If patients are not hyper-responsive to the inhaled substance, the dose or concentration limit may be achieved with minimal or insignificant change in FEV$_1$. This limits the usefulness of indirect challenge tests to individuals sensitive to the bronchoconstrictor.

In contrast, among severe asthmatics, even a small dose of an indirect irritant can cause a marked drop in FEV$_1$. This means that bronchial challenge tests can only be safely performed when the patient’s baseline FEV$_1$ is at a satisfactory level (72), usually above 50% of the predicted value and above 1.2 L.

Although indirect bronchial challenge testing is a highly useful tool in the diagnosis of asthma and estimation of asthma control, negative tests may occur even among severe asthmatics, and the test may not be appropriate if asthma control is poor. Regarding these shortcomings, PC/PD value alone cannot be used as the sole marker for steroid responsiveness.

3.1.1.4 Measurements of asthma control used in this study

All asthmatic participants in this study had previously been diagnosed with asthma by a medical professional. In addition to a previous diagnosis asthmatic patients were required to demonstrate hyper-responsiveness to hypertonic saline (a highly specific indirect challenge test) or a significant reversal of airway obstruction following administration of a short acting beta agonist.

During a period of steroid withdrawal asthma control was monitored using serial PEFR measurements. Asthma control before and after a course of oral corticosteroids was measured by spirometry, an ACQ questionnaire, and an AMP challenge (indirect).
See methods section for further details on the tests used in this study.

3.1.2 The use of corticosteroids as asthma treatment

3.1.2.1 Inhaled corticosteroids

Inhaled corticosteroids (ICS) are currently the first-line treatment for asthma (49). Although intermittent asthma may be treatable by short-acting beta agonists (SABAs), the use of preventers is strongly recommended in most cases. ICS have been found to be the most effective preventer drug for adult asthma. Current recommendations indicate introduction of ICS when asthma has exacerbated in the previous two years, when SABAs are used three or more times a week, when asthma symptoms are present three or more times a week, or when asthma is causing night wakening one or more nights per week (49).

Typically corticosteroids are taken by inhaler although other delivery systems are occasionally indicated. Most ICS are prescribed for twice-daily usage. ICS vary in potency, with identical doses of different steroids often having marked differences in magnitude of effect. ICS dose is measured on the basis of the equivalent dose of Beclamethasone Dipropionate (BDP equivalent)(49).

3.1.2.2 Oral corticosteroids

Long-term use of oral corticosteroids (OCS) is considered for asthma treatment when asthma is poorly controlled even on concurrent high-dose ICS, SABAs, and other drugs (such as long-acting beta agonists). More frequently, OCS are used in short term courses (from five days upward) to treat exacerbations of asthma. Short term OCS use has been found to reduce mortality, relapses, and subsequent admissions to hospitals (49).

OCS have been found to be as effective as injected steroids in controlling asthma. When prescribed for exacerbations, OCS can be stopped abruptly, as long as ICS are continued. Generally 40-50 mg/day is prescribed for exacerbations, and long-term OCS are prescribed with the minimal effective dose. The most widely used OCS is prednisone (49).
3.1.2.3 Problems with steroid therapy and the utility of steroid response assessment

The use of steroids in asthma treatment has several drawbacks, generally due to side effects of steroid medication. For this reason, current protocol recommends that the dose of ICS and OCS used in asthma treatment is progressively down-titrated until a minimum level of treatment to achieve asthma control is reached (49).

ICS may cause side effects of oral candidiasis (thrush) and dysphonia. There is a possibility that high doses of ICS may lead to decreased bone mineralisation, although doses up to 1000 µg BDP have shown no such effect (49). Long-term OCS use may lead to Cushing’s syndrome, which is characterised by a range of systemic effects. These include decreased bone mineralisation, weakness of collagen fibres leading to striae and purpura, increased central adiposity, increased blood pressure, reduced glucose tolerance, muscle wasting, and increased fluid retention (75).

The high rate of prescription of ICS and OCS means that many unresponsive patients are placed on these therapies. This is both costly and harmful (6), and can be highly dangerous in ‘brittle’ asthma where steroids are ineffective in controlling acute exacerbations (52). Because of this, rapid estimation of level of steroid response holds considerable clinical application (6).

This study explored a means to predict individual steroid response prior to a treatment course. The electronic nose is a portable and easy-to-use device, which evaluates breath samples in real time, and could have been adapted for use in situations where rapid and non-invasive patient evaluation was required.

3.2 The steroid response

3.2.1 Anti-inflammatory mechanisms of steroid action

3.2.1.1 Pharmacokinetics

Chemically, glucocorticoids are 21-carbon steroid hormones with four rings (61). They are plasma bound to specific globulins (as for cortisol) or bound to albumin (57). Free glucocorticoids will readily cross plasma membranes and bind to receptors in the cell (61). Glucocorticoid receptors (GRs) are cytosolic proteins expressed in almost all cells of the
body, which are bound to chaperones (often heat shock proteins) prior to activation (57, 62). See figure 3 for a summary of glucocorticoid actions in the cell.

On binding glucocorticoids, GRs are activated, and may either translocate to the nucleus and up- or down-regulate specific genes, or may interact with other regulatory proteins in the cytosol to repress their action. Isoforms of GRs have diverse cytoplasm-to-nucleus trafficking patterns and transcriptional activity. An estimated 10 to 100 genes per cell are directly regulated by glucocorticoids with many others regulated indirectly (61).

### 3.2.1.2 Regulatory effects

Glucocorticoids have been found to have downregulatory effects on several genes implicated in asthma. Direct regulation of a gene by GRs requires the presence of glucocorticoid receptor binding sites (called glucocorticoid response elements, or GREs) in the promoter region for the gene (62). Indirect regulation involves the activation or inhibition of a secondary molecule involved in transcription. Glucocorticoid receptors also induce changes in histone protein acetylation, which affects chromatin structure and DNA packing, causing further regulatory effects (57). Evidence also suggests that GRs may be able to alter mRNA stability, altering translation of pro-inflammatory molecules without effects in the nucleus (61).

One important secondary messenger is the NF-κB molecule, a powerful pro-inflammatory mediator. GRs have been found to inhibit NF-κB with consequent anti-inflammatory effects. NF-κB is activated by several extracellular stimuli including cytokines and immune stimuli, and can enhance activity of other inflammatory transcription factors as well as directly regulating transcription itself. Data suggest that GRs can bind directly to NF-κB as well as repressing it by indirect mechanisms including induction of other inhibitors (61). See figure 3.

One of the best-documented effects of corticosteroids is downregulation of the pro-inflammatory cytokines, a group of molecules which are involved in immune system activation and control of vascular permeability (61). Because of the lack of known GR-binding sites in the promoter regions of the genes which code for these molecules, the downregulation is apparently due to a downstream effect (62). Glucocorticoids have been found to downregulate IL-1β, IL-3, IL-4, IL-5, IL-6, TNF-alpha and GM-CSF in this manner (62). See figure 3.
The downregulation of the pro-inflammatory cytokines causes several downstream regulatory effects. One notable action of many cytokines is the upregulation of inducible nitric oxide synthase (iNOS) causing increased nitric oxide synthesis. Nitric oxide (NO) is a potent paracrine vasodilator which causes observable increases in plasma exudation and bronchial blood flow (62) and is also implicated in immune reactions both as an activator and an effector. Nitric oxide synthases are also found in the airway epithelium and are upregulated in atopic asthma. It is likely that the regulation of iNOS is also controlled partially by NF-κB (62). GRs inhibit many of the cytokines responsible for iNOS regulation, as well as NF-κB, reducing these effects. Exhaled nitric oxide is used as a marker of OCS activity in this study (see introduction section 1.1.3, methods section 1.1.3.1).

Glucocorticoids inhibit the inducible form of the cyclo-oxygenase enzyme (COX2) in monocytes, airway smooth muscle cells, and endothelial cells (62). Cyclo-oxygenases are necessary for the production of prostaglandins, leukotrienes, and thromboxanes, which promote inflammation and platelet aggregation. The downregulation of COX2 is also partially accomplished by repression of NF-κB (57). See figure 3.

Glucocorticoids also affect the production of adhesion molecules such as ICAM-1 and E-selectin. These molecules are expressed on the surface of endothelial cells and are essential to the trafficking of inflammatory cells. The regulation of adhesion molecules is thought to be partially via a direct mechanism and partially through downregulation of cytokines (62). See figure 3.
Figure 3. Anti-inflammatory actions of glucocorticoids.
*Caption over page*
Anti-inflammatory actions of steroids include the downregulation of pro-inflammatory cytokines (A), the inhibition of NF-kB (B), the inhibition of the COX2 enzyme in some cell types (C), the alteration of histone acetylation patterns (D) and the downregulation of endothelial adhesion molecules (E). Free glucocorticoids (GCs) in the plasma readily cross the cell membrane and bind to glucocorticoid receptors (GCRs) in the cytoplasm. Prior to GC binding, GCRs are bound to chaperone proteins, usually of the heat shock protein class. The inhibition of NF-kB is achieved both by direct interaction of the GC-GCR complex with the NF-kB molecule and via the downregulation of NF-kB activators. NF-kB both upregulates and enhances the activation of the pro-inflammatory cytokines, and activates the COX2 enzyme. Cytokines upregulate ICAM-1 and E-selectin.

Caption and diagram adapted from (57), (59).

3.2.1.3 Cytological and morphological effects

The effects of glucocorticoids in the airways bring about clear changes in inflammatory cell presence and tissue morphology, some of which can be used as biomarkers of steroid response (54, 60). The most prominent effects are increased apoptosis among inflammatory cells, morphological changes in airway walls, and decreased mucus secretion. Systemically, long-term use of steroids (particularly when taken orally) cause the morphological effects of increased adiposity, decreased bone mineralisation, and thrombocytopenia (52).

The most prominent increase in apoptosis occurs in eosinophils. Eosinophil level is used as a biomarker for the presence of atopic asthma and a reduction in blood or sputum eosinophil levels is a strong indicator of steroid response (54). Eosinophil survival requires the presence of the cytokines IL-5 and GM-CSF, both of which are downregulated by corticosteroids (54). As well as an increase in apoptosis, there is evidence of decreased eosinophil production in the bone marrow in response to steroids (62). Eosinophil degranulation is also somewhat affected by steroids, in that release of inflammatory mediators inhibited. However, release of reactive oxygen species and eosinophil basic protein is unaffected by steroid response (62).

Other inflammatory cell types also exhibit changes when exposed to glucocorticoids. The activation of T lymphocytes, a prominent step in the humoral immune response, is significantly muted by glucocorticoid effects. Neutrophils are relatively steroid insensitive (61).

Glucocorticoids may also to some extent reverse airway remodelling features in asthma such as increased basement membrane thickness (59). They also have a direct inhibitory effect on plasma exudation from post-capillary venules at inflammatory sites (62). Airway mucus secretion is inhibited by steroid use, possibly due to direct action of steroids on submucosal
gland cells (62). Inhibition of plasma exudation and decreased mucus secretion are both partially due to downstream effects following COX and cytokine inhibition (62).

3.2.2 Heterogeneity of the steroid response

3.2.2.1 Steroid resistant and steroid unresponsive asthma

There is a wide variation in the degree to which asthmatic patients respond to corticosteroid treatment (76). Although the response to steroid varies from ‘totally unresponsive’ to ‘strongly responsive’, asthmatics are generally stratified into ‘steroid responsive’ and ‘steroid unresponsive’ groups rather assigned a ‘level’ of steroid response. This approach has been followed in many studies, and is generally accepted as standard (77).

The first documented cases of steroid resistance were recorded by Schwarz in 1968 (77), and later by Carmichael in 1981 (78). The term ‘steroid resistant asthma’ was first used at this time. Later publications recommended that since this definition covered asthmatics with a reduced response to prednisone, rather than an unmeasurably small response, the collective term for such patients should be ‘steroid insensitive’ rather than ‘steroid resistant’ (79).

There is some malleability in steroid response in individual patients, in that tachyphylaxis is known to occur (80) and response varies with lifestyle factors such as cigarette smoking (59). For this reason, level of steroid response was re-assessed at the beginning of this study in all asthmatics, although many had already had response assessed in previous studies.

3.2.2.2 Definition of steroid response

Several different thresholds for resistance to oral glucocorticoid have been used for research and for clinical protocol. A task force from the European Respiratory Society (52) described steroid resistance qualitatively as one of three forms of difficult asthma, in which patients with ‘a persistent pattern of airway obstruction… requiring oral corticosteroid treatment, to which they show an incomplete response’. The threshold suggested for ‘difficult’ asthma is on the basis of the minimum dose of inhaled corticosteroid in order to maintain asthma control, and is set at beclamethasone 2000 µg or fluticasone 1000 µg per day.

From a clinical perspective, steroid responsiveness can be broadly considered to be the need for high doses of corticosteroids in order to control asthma (77). This can be objectified as a
failure of measures of asthma control to significantly improve following a course of high-dose oral corticosteroids.

Woolcock (77) found that many studies defined steroid resistant asthma on the basis of failure of peak expiratory flow rate to improve following 10-14 days of high-dose oral corticosteroid. Exact cutoffs to specify resistance vary between studies. In one of the first major studies on steroid resistance, Carmichael et al. (78) used a definition of less than a 15% improvement in FEV$_1$ following 7 days of 20 mg/day oral prednisone. Alvarez et al. (81) defined resistance as a failure of FEV$_1$ to improve beyond 60% of predicted value during a 2 week course of >40mg/day oral prednisone. A review by Nimmagadda et al. (53) defined steroid resistance as failure of FEV$_1$ to respond by 15% to a 7 to 14 day course of oral prednisone.

### 3.2.2.3 Management of steroid-unresponsive asthma

The management of steroid-unresponsive asthma is difficult. Steroid-unresponsive asthma accounts for around 50% of the total healthcare costs for asthma, although steroid-unresponsive asthmatics represent far less than this proportion of all asthmatics (61).

First-line treatment for partially steroid-resistant asthma remains increased doses of inhaled corticosteroid, although the dose-response curves for ICS suggest that increasing dosage beyond certain thresholds has little additional effect and may cause systemic side effects (15). Other anti-inflammatory drugs such as methotrexate and cyclosporine can be used in severe steroid-insensitive asthma. The use of such ‘steroid-sparing agents’ during a reduction of glucocorticoid dose may partially reverse tachyphylaxis-related glucocorticoid insensitivity, leading to an increase in steroid responsiveness. However, the toxicity of such drugs means they are ideally avoided (61).

### 3.3 Associated phenotypes and prediction of steroid responsiveness

#### 3.3.1 Clinical phenotypes associated with steroid responsiveness

#### 3.3.1.1 Relationship of asthma severity to steroid responsiveness

The relationship between steroid response and asthma severity is complex. According to current definition (67), the severity of asthma is defined by the level of treatment necessary to achieve control of symptoms. Although a steroid-responsive asthmatic and a steroid-unresponsive asthmatic may have similar symptoms when untreated, the steroid-responsive
asthmatic will automatically be classified as less severe by this rationale.

An implication of this is that steroid insensitivity is a contributor to asthma severity. There have been cases of steroid-unresponsive asthma which are relatively well-controlled when untreated, indicating that steroid non-response is not limited to steroid-dependent asthma (82).

3.3.1.2 Clinical characteristics of steroid-responsive asthma

Patients with steroid-insensitive asthma differ in several ways on clinical presentation from those with steroid-sensitive asthma. In a study comparing 58 steroid-responsive and 58 steroid-resistant asthmatics, Carmichael found that steroid-resistant patients tended to have had asthma for longer than steroid-sensitive patients, and a family history of asthma was more common. Regular nocturnal or morning wheeze was more common among steroid-resistant patients, and PD20 Methacholine was significantly lower (78).

Steroid-insensitive asthma has been associated with poorer clinical outcomes than steroid-sensitive asthma. In one study of asthmatic children both patients with steroid insensitive asthma and their caregivers reported a lower quality of life following treatment than their steroid-sensitive counterparts (83).

3.3.1.3 Demographics associated with steroid responsiveness

Steroid insensitivity appears to vary somewhat in incidence between demographics. One study (79) found a higher proportion of African Americans among steroid insensitive asthmatics. The same study reported earlier ‘first use of glucocorticoids’ among the steroid insensitive group. Steroid insensitive asthmatics often had a ‘chaotic’ pattern of FEV1 values, with large day-to-day fluctuation, both on and off oral steroids. It was acknowledged that several confounding factors may have been present.

Asthmatic patients who smoke have an impaired response to both oral and inhaled corticosteroids. However, some GC sensitivity can be restored following cessation of smoking (59).
This study was not large enough to establish any demographic differences between the steroid-responsive and the steroid-unresponsive group. All patients had ceased smoking at least three months before the first visit.

3.3.2 **Pathophysiological basis of steroid responsiveness**

3.3.2.1 **Mechanisms of response impairment**

Steroid resistance in asthma has been shown to occur independently of malabsorption or altered pharmacokinetics and cell extracts from steroid-insensitive asthmatics have been found to show reduced response to corticosteroids in vitro (82). This indicates that resistance may arise in deficiencies in the anti-inflammatory actions of glucocorticoids. Identified deficiencies included abnormal glucocorticoid-GR interactions, abnormal GR function, and metabolic abnormalities affecting secondary glucocorticoid effects (61).

Cellular insensitivity to steroids is apparent in regions of cytokine production (inflammation) but normal glucocorticoid action is generally observed at non-inflamed sites (58). This is consistent with observations of a normal feedback mechanism regulating adrenal cortisol release among steroid-insensitive asthmatics (61).

3.3.2.1 **Genetic bases**

A possible cause for steroid resistance is a mutation in the glucocorticoid receptor gene. Glucocorticoid action is essential to life and individuals with mutations causing severe loss of function often cannot survive (62). However, mutations resulting in reduced but present GR function are well-documented.

Familial glucocorticoid resistance is a condition in which mutations of the GR receptor gene inhibit systemic response to glucocorticoids, causing a homeostatic increase in basal cortisol level without development of Cushingoid features. Such mutations include deletions in the ligand binding domain of the protein, causing decreased ligand affinity, and insertions at splice sites, causing loss of an allele. Possible genetic causes of glucocorticoid resistant asthma are an area of current research interest (61).

Most asthmatics with corticosteroid resistance, however, have normal cortisol levels. This suggests that asthmatics with steroid resistance do not generally have familial glucocorticoid
resistance (61). Several studies have shown (82) that steroid resistant asthmatics have no obvious differences in GR structure. This suggests that most steroid resistance in asthma arises from abnormalities elsewhere in the glucocorticoid response pathways.

### 3.3.2.2 Biochemical basis of resistance

An increase in tissue concentrations of pro-inflammatory factors, especially cytokines, has been reported to occur in common with glucocorticoid resistance. Although steroids suppress cytokine production (61), high cytokine levels, notably IL-2, IL-3, and IL-4, also reduce the affinity of GR binding to many inflammatory cells, especially T lymphocytes (62). Due to this mechanism, pro-inflammatory cytokines and steroids both repress the action of the other to an extent (61), implying that steroid resistance may arise secondary to conditions which cause high levels of pro-inflammatory cytokines.

Cytokines can also lead to inhibition of other areas in the steroid response pathway. Inadequate translocation of the GC-GR complex to the nucleus has been identified as a common cause of steroid resistance (61) and can arise both due to mutations in the GC-binding region of the GR and due to secondary effects. Interleukins 2 and 4 have been found to be over-expressed in steroid-resistant asthma and incubation with IL-2 has been shown to cause glucocorticoid resistance in cell lines due to inhibition of nuclear translocation (82). It is proposed that the mechanism of reduction of nuclear translocation is via phosphorylation of the GR by MAP kinase, which has been found to have a greater degree of activation in asthmatics with a poor response to glucocorticoids (82).

Post-translational modification of phosphorylation patterns on the GR can lead to secondary glucocorticoid resistance. Phosphorylation patterns on the GR are known to affect ligand binding, nuclear translocation, and DNA binding (59). GC resistance can also be due to more obscure causes such as GR downregulation, impaired cytoplasmic GR-protein interactions, reduced ability of GRs to bind to DNA (59), and increased steroid efflux (82).

### 3.3.2.3 Tachyphylaxis

Adequate response to glucocorticoids requires the presence of functional GRs in the cytoplasm. Studies on patients with haematological malignancies have demonstrated that low GR levels are associated with poor response to glucocorticoids. Tachyphylactic effects from
prior glucocorticoid exposure are known to influence cytoplasmic GR concentration (58, 80).

The downregulation of GRs in response to glucocorticoid therapy is due to several mechanisms. Regulation of the GR may occur at multiple points in its production, including transcription, post-transcription, and post-translation. GR degradation is also increased in response to glucocorticoids. Mechanisms are complex, involving secondary messengers such as cAMP and histamine. There is evidence to show that in addition to reversible downregulation of GRs, cells may also become steroid-resistant due to other non-reversible mechanisms resulting from prolonged glucocorticoid exposure (80). One study (54) found a decrease in the steroid sensitivity of eosinophils in steroid-dependent asthmatics (those requiring >20mg/day oral prednisone for asthma control) compared to cells from steroid-sensitive asthmatics.

3.3.3 Prediction of steroid responsiveness
3.3.3.1 Histological and biochemical characteristics of steroid responsiveness

Several studies have found evidence of structural and biochemical differences between steroid-responsive and steroid-unresponsive asthmatics. Steroid-unresponsive asthmatics have thicker airway epithelium and basement membranes and altered expression of markers for epithelial proliferation than steroid-sensitive asthmatics, despite similar levels of epithelial shedding. The difference in airway structure was associated with altered expression of biomarkers linked to epithelial proliferation, particularly regulators of apoptosis (59).

Cytokine analysis in bronchial-alveolar lavage fluid has also been explored as a means of genomically analysing steroid response. Cytokines such as IL-2 and IL-4 were associated with low GC response (59).

3.3.3.2 Exhaled nitric oxide as a predictor of steroid response

A study from the Otago Respiratory Research Unit by Smith et al. (6) established the utility of fractional exhaled nitric oxide (FENO) as a marker of steroid response. The study also compared the prediction accuracy of FENO with that of spirometry, peak flows, bronchodilator response and airway hyper-responsiveness at baseline. Steroid responsiveness was measured by changes in symptoms, peak flow, spirometry or airway hyper-
responsiveness before and after a course of ICS. Response was found to be significantly higher in asthmatics with \( \text{FeNO} > 47 \) ppb for each endpoint. \( \text{FeNO} \) was found to be a more accurate predictor than each of the other measurements.

In another study from the department, \( \text{FeNO} \) was also found to be a strong predictor of steroid response in non-eosinophilic asthmatics (64).

### 3.3.3.3 Eosinophilia as a predictor of steroid response

Eosinophils are strongly indicated in asthma pathophysiology, and a raised level of eosinophils in the sputum is a strong indicator of the presence of asthma (49). There is some potential for eosinophils to predict steroid response but generally the prediction efficacy is poor. In general, eosinophilic asthma appears to be responsive to steroid, but response in non-eosinophilic asthma is variable. In order for sputum eosinophilia to be useful as a predictor of steroid response in this way, ICS use must be withdrawn for a period of time (64).

A study from the Otago Respiratory Research Unit identified the effect of inhaled fluticasone on asthmatic patients after stratification by inflammatory cell subtype. Patients underwent a steroid withdrawal period after which proportions of inflammatory cells in induced sputum were measured. All patients then underwent a 28+ day trial of inhaled fluticasone, before and after which asthma control was measured. Steroid responsiveness was found to be significantly higher in eosinophilic asthma than in non-eosinophilic asthma.

A study by Meijer et al. (56) compared the blood and sputum eosinophil counts of steroid-responsive and steroid-unresponsive asthmatics. Participants were treated with prednisone 30 mg/day, fluticasone 1000 µg/day or fluticasone 250 µg/day for two weeks. Quality of life, \( \text{FEV}_1 \) and \( \text{PC}_{20} \) (methacholine) were measured before and after the course of steroid. For each endpoint, an attempt was made to use blood and sputum eosinophil counts to predict a change. Prediction of response was poor with only 55-66% of patients correctly identified.

The use of blood eosinophil counts to predict steroid responsiveness was considered in a study by Bosse et al. (54). Patients in the study were given a 14-day course of 40 mg/day oral prednisone, and were defined as ‘steroid sensitive’ if their \( \text{FEV}_1 \) improved by more than 25%. Patients with less than 15% improvement in \( \text{FEV}_1 \) were defined as ‘steroid resistant’. Both steroid sensitive and steroid resistant patients had reduced blood eosinophil counts following
the steroid course.

3.4 **Eosinophilic and non-eosinophilic asthma and relation to steroid response**

3.4.2 **Clinical relevance of eosinophilic and non-eosinophilic asthma**

3.4.2.1 **Histological phenotyping in asthma**

In the attempt to classify asthma by its underlying pathology, several studies have analysed the presence of inflammatory cells in the tissues of asthmatic patients (54, 60, 64). Biomarkers based on relative proportions of inflammatory cells in tissues have been shown to be useful in distinguishing visible asthma phenotypes (84), predicting steroid responsiveness (54, 64) and assessing asthma severity (60).

The main cell type used as a basis for biomarkers in assessment of asthma is the eosinophil. In particular, high eosinophil counts in blood and sputum are known to correspond to a particular phenotype in asthma and frequent asthma exacerbations (84).

Eosinophilic asthma causes a rise in eosinophil levels in several tissues as well as in sputum. In each tissue type, the level of eosinophilia defining eosinophilic asthma varies. Several recent studies have compared the diagnostic use of eosinophil counts from different tissues. Sources of tissue for assessing the level include bronchial biopsy specimens (84), induced sputum (64, 84-85) and blood (60, 85). A large amount of research has also focused on the use of eosinophilic cationic protein (ECP) as measured from a multitude of tissues (86).

3.4.2.2 **Eosinophilia as a guide to asthma treatment**

Eosinophil count can be used in asthma diagnosis. A sputum differential cell count with more than 1% eosinophils in an untreated individual has a sensitivity of greater than 80% and specificity of 95% in predicting current asthma (87). The lower sensitivity arises due to the well-described phenomenon of non-eosinophilic asthma.

Within asthma, the primary use of sputum eosinophil level is in the prediction and assessment of steroid response (see section 3.3.3.3). In this application, eosinophilia may be considered as an interval variable; degree of steroid response can to some extent be predicted by level of eosinophilia (56). Airway eosinophil level may also be used as a biomarker for steroid
response by measurement before and after a steroid course. Eosinophil counts in eosinophilic asthma usually decrease in response to steroid treatment (54, 56).

Although eosinophilia can be used both to diagnose (87) and to stratify (88) asthma, specificity is reduced by the presence of eosinophilic non-asthmatic conditions. Asthma also exists for which airway eosinophilia is present but is not the main cause. Eosinophilia is also observed in the sputum of patients without asthma (50).

This study attempted to relate sputum eosinophil counts to breathprints from the electronic nose. In theory a predictive model may be able to be developed allowing eosinophilic asthma to be predicted by analysis of exhaled breath.

Collection and processing of sputum is currently difficult and requires trained experts. A rapid and simple estimation of sputum eosinophil level, as could be performed by electronic nose, could potentially be a useful tool in asthma assessment.

3.4.3 Molecular basis of eosinophilic and non-eosinophilic asthma

3.4.3.1 Eosinophilic inflammation

Eosinophils are inflammatory cells which develop from granulocyte precursor cells in the bone marrow. Under non-inflammatory homeostatic conditions, most migrate to the GI tract after formation, with a smaller proportion trafficked to the thymus, mammary glands, and uterus. Trafficking to other sites is controlled by cytokines and other inflammatory mediators (65).

Proliferation of eosinophils requires the presence of GM-CSF (granulocyte-macrophage colony stimulating factor) (54) and interleukins (65). Of the cytokines involved in leukocyte recruitment (essentially the inflammatory cytokines), only IL-5 and eotaxins regulate eosinophil trafficking selectively. IL-5 has been shown to regulate growth, differentiation, activation, and survival of eosinophils (54, 65) and is an essential factor for trafficking eosinophils from the bone marrow to the lung following antigen exposure (65). Antigen induced eosinophilia can occur in other tissues without the presence of IL-5. The eotaxins are also important in trafficking eosinophils to the lung (65).
Eosinophils express a variety of membrane receptors common to other inflammatory cell types. These include cytokine and chemokine receptors, complement proteins, and major histocompatibility complex (MHC) class I and II molecules. This allows them to be highly sensitive and responsive to local inflammation. Prostaglandin, histamine and leukotriene receptors are also prominent, which are partially responsible for the reaction of eosinophils to allergen-induced mast cell degranulation. Toll-like receptors have also been found, although in lower levels than in other granulocytes (65).

Eosinophils contain large numbers of basic granules in their cytoplasm, which are released into the extracellular fluid when the eosinophil is ‘activated’ or ‘degranulated’. Granules contain histamines and pro-inflammatory cytokines as well as four major cationic proteins, which promote inflammation and cell damage when released (65).

Eosinophil cationic protein (ECP) is one of the four main cationic proteins found in eosinophil granules. It is released upon eosinophilic degranulation (86) and acts as a cytotoxic pore-forming protein. ECP is a better measure of eosinophil ‘activation status’ than direct eosinophil number, which is raised in subtly different circumstances (85). ECP is not specific to asthma, which is one reason for its relatively poor specificity as a diagnostic tool (86). It also exhibits circadian and diurnal variations and is raised in smokers (86).

Eosinophil activation is also highly linked to mast cell regulation and activation. Activation of eosinophils by chymase (a mast-cell protease) leads to production of eosinophil-derived stem cell factor, a critical factor for mast cell growth. In this way, eosinophil and mast cell activation promote each other in a positive feedback loop (65).

Eosinophils possess the ability to perform and mediate a variety of immune functions and rapidly enhance inflammatory responses due to their ability to release a large range of relatively pre-formed cytokines and lipid mediators. MHC molecules on eosinophils have been shown to present a variety of microbial and viral antigens, as well as superantigens (65).

3.4.3.2 Eosinophilic asthma

The eosinophil is hypothesised to be the central effecter cell responsible for long-term airway inflammation. Airway remodelling features characteristic of asthma (including goblet cell
proliferation and increased basement membrane thickness) are partially a consequence of eosinophil activation (65).

Eosinophil granule proteins have the potential to cause airway damage through several mechanisms. Granule-associated basic proteins damage epithelial cells and nerve endings, and lipid mediators cause bronchoconstriction and mucus hypersecretion. Eosinophil granules also release several reactive oxygen species, which injure mucosal cells. Eosinophils in asthmatic patients tend to be hyperadhesive both to other cells and to collagens (65).

The generally accepted mechanism for atopic asthma is a result of IgE mediated reaction to an allergen, which causes a Th2 lymphocyte response and subsequent IL-5 mediated eosinophilic inflammation (50). In this pathway, IgE immunoglobins are released from activated plasma cells and bind to mast cells, which along with eosinophils co-operatively degranulate to release inflammatory mediators and cause asthmatic symptoms (89).

There is however sparse evidence that eosinophils or their products are directly causative of airway hyper-responsiveness. While hyper-responsiveness is associated with eosinophilia, there is little correlation between eosinophilia and degree of hyper-responsiveness (65).

3.4.3.3 Non-eosinophilic asthma

Around 20% of asthmatics do not show raised eosinophil counts in the sputum (64) although this figure is highly variable (50). Such asthma is collectively called ‘non-eosinophilic asthma’ (88) although asthmatics without raised eosinophil counts can display a range of histological phenotypes (64). Subtypes of non-eosinophilic asthma include neutrophilic and paucigranulocytic asthma (64).

One identified non-eosinophilic asthma subtype is characterised by neutrophilic inflammation. This may be as a result of exposure to several non-allergenic irritants including air pollution, bacterial endotoxins, and viral infection (50). Symptoms resemble those of atopic asthma but can develop without any sensitisation or latency period.

Several studies have noted an asthma phenotype in which tissues show no inflammatory cell infiltrate. This type of asthma is termed paucigranulocytic asthma, and the mechanism by which it develops is unclear. It has been suggested that mast cells, epithelial cells, or smooth
muscle cells may act as effector cells in the absence of granulocytes. Airway smooth muscle cells may also undergo hypertrophy independent of inflammatory processes (90).

In contrast to eosinophilic asthma, which is mediated by IL-5 and GM-CSF, non-eosinophilic asthma may involve an alternative range of cytokines, including IL-1, IL-6, IL-8 and TNF-alpha. Irritant molecules bind to epithelial cells and macrophages, which elicit an inflammatory response partially through the NF-kB transcription factor (50).

3.4.4 Assessment of sputum eosinophilia
3.4.4.1 Measurement of eosinophils

Eosinophil counts can be obtained from several tissues and in induced sputum. Generally, measurement is done by obtaining a tissue sample, producing a standardised slide, and counting cells by microscopy. Either the absolute count (64) or the number of eosinophils as a percentage of total cells (differential count) (84) may be used as a measure, as the two agree statistically (88). Cutoffs for differential counts used to define eosinophilic asthma vary, but range between 2% and 4% (50). In this study, a cutoff of 3% was used.

Eosinophil levels in the sputum of asthmatics were found to be raised more than a century ago, although the difficulty of measurement reduced application of eosinophil counts as a biomarker until recently when techniques such as cytospins and fluid phase measurements allowed reproducible results to be obtained (85). Induced sputum collection requires the administration of nebulised hypertonic saline or a similar irritant in non-productive asthmatics (86).

In general sputum is used as a medium from which to count cells. Bronchial biopsy specimens have also been used as a source of tissue from which to evaluate eosinophil count (84) and bronchial lavage fluid has been used as a source for evaluating ECP level (86). Eosinophilia in bronchial biopsy specimens is a poorer marker than sputum eosinophil level in predicting asthma. In addition, many patients with high sputum eosinophil levels do not have corresponding high levels in bronchial biopsy specimens (84).

Bronchial biopsies are invasive and there are greater risks associated with this procedure (64). Easy-to-obtain sampling media such as saliva and urine are not generally usable for assessment of eosinophilia (86).
Asthma is associated with eosinophilia in peripheral blood, although blood eosinophils are also raised in conditions such as atopy, rhinitis, and eczema (85). Blood eosinophils and blood ECP are significantly poorer markers of airway inflammation than sputum eosinophils (85).

3.4.4.2 Detection by electronic nose

Due to the wide range of molecules released by eosinophils and their proximity to the airways, the exhaled breath of individuals with high levels of airway eosinophilia would be expected to be different in makeup from the exhaled air of non-eosinophilic individuals. The electronic nose may be able to detect such differences, and indeed may be better-suited to the task than direct breath analysis techniques due to the likely array of non-specific biomarkers associated with eosinophilia.
Methods and Materials

1 Study procedures
1.0 Overview

The design of the current study was based on a previous unpublished study by the department. Asthmatic participants in the study took a 2-week course of oral prednisone, preceded and followed by an AMP challenge, ACQ, and measurement of baseline spirometry. Before this, all patients currently on inhaled corticosteroid medication underwent a period of controlled steroid withdrawal lasting up to four weeks. Patients provided a sample of sputum immediately prior to beginning the steroid course.

For patients on inhaled corticosteroid, the study consisted of four visits (visits 1, 2, 3a, 3b, and 4), with the third visit split over two days. For patients not on inhaled corticosteroids, the study consisted of two visits (visits 1a, 1b, 2) with the first visit split over two days.

Healthy controls visited the lab once. At the visit, a set of airway tests and a single electronic nose measurement were conducted.

A flow diagram of the study is shown in figure 5.

A patient interview was conducted in all cases consisting of a standardised set of questions. Clinical tests were conducted according to current protocols. Electronic nose breathprints were obtained using the same protocol and apparatus as that used by earlier studies using electronic noses in diagnosis of airway pathology.

1.1 Protocols of simple study procedures
1.1.1 Initial procedures
1.1.1.1 Informed Consent

Informed consent was obtained from all patients prior to undertaking any study procedures. For asthmatic patients, separate informed consent was sought for AMP challenges. All informed consent forms were obtained at visit 1.
1.1.1.2 **Medical history**

All patients were asked a standardised set of questions covering demographics, medical history, and social history. All such data was collected prior to the commencement of any other study procedures. Demographic data included age, sex, and ethnicity. The medical history included a review of respiratory, autoimmune or atopic conditions, and a general review of current medical state and current medication list. The social history consisted of a brief review of smoking history, alcohol use, and exposure to environmental VOCs. A list of all questions asked as part of the medical interview is shown in table 2.

**Table 2. Summary of background data gathered.**

<table>
<thead>
<tr>
<th>Topic</th>
<th>Specific Questions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>Presence of condition</td>
</tr>
<tr>
<td></td>
<td>Prior admission to ICU due to asthma</td>
</tr>
<tr>
<td></td>
<td>Age at onset of asthma</td>
</tr>
<tr>
<td></td>
<td>Duration of asthma in years</td>
</tr>
<tr>
<td></td>
<td>General control of asthma</td>
</tr>
<tr>
<td>Other medical condition</td>
<td>Ear, nose, and throat conditions</td>
</tr>
<tr>
<td></td>
<td>Respiratory conditions</td>
</tr>
<tr>
<td></td>
<td>Cardiovascular conditions</td>
</tr>
<tr>
<td></td>
<td>Diabetes</td>
</tr>
<tr>
<td></td>
<td>Dyslipidaemias</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal conditions</td>
</tr>
<tr>
<td></td>
<td>Skin conditions</td>
</tr>
<tr>
<td></td>
<td>Inflammatory arthropathy</td>
</tr>
<tr>
<td></td>
<td>Other conditions</td>
</tr>
<tr>
<td>Adverse drug reactions</td>
<td>List of drugs and associated conditions</td>
</tr>
<tr>
<td>Medications</td>
<td>All current medications and doses</td>
</tr>
<tr>
<td>Volatile substance exposure</td>
<td>Smoking history (incl. pack-years)</td>
</tr>
<tr>
<td></td>
<td>Alcohol use</td>
</tr>
<tr>
<td></td>
<td>Environmental volatile substance exposure</td>
</tr>
</tbody>
</table>

1.1.1.3 **Physical data**

Heart rate, blood pressure, height, and weight were collected from all patients, prior to any spirometry, breath sampling, or allergen testing.

Heart rate was measured from the radial artery. Blood pressure was taken according to current regulations (91) using an aneroid manometer (size 14 x 45 cm, Acuson Corporation, Mountain View, CA, USA), inflatable cuff, and stethoscope (American Diagnostics
Corporation, Hauppauge, NY, USA). If necessary, blood pressure measurements were repeated by an independent observer.

Patient height was measured to the nearest centimetre and weight was recorded to the nearest 0.1 kg.

1.1.2 Airway tests
1.1.2.1 Spirometry

Spirometry was performed according to ERS standards (92). FEV₁, FVC, and FEF₂₅-₇₅ were measured using a rolling seal spirometer (Sensor – Medics, California, USA) or a KoKo PFT system, version 4 (PDS Instrumentation, Colorado, USA). A minimum of two measurements were made at each instance. Measurements then ceased if FEV₁ and FVC agreed between the measurements to less than 100 ml. If agreement was not attained for either variable, continued measurements were taken until the difference between the maximum value of the variable and the second-to-maximum value was at most 100 ml. For each measurement, an FEV₁/FVC ratio was calculated. The highest values of FEV₁, FVC, FEF₂₅-₇₅ and FVC were then recorded, even if they were from different measurements. When only FEV₁ was required, no measurement of FVC or FEF₂₅-₇₅ was made and patients were not required to exhale to residual volume.

Both spirometers were calibrated daily according to the manufacturer’s specifications. Prior to all spirometric measurements, patients were asked to withhold short acting beta-agonists for six hours. Note was taken of any long-acting beta agonist use in the previous sixteen hours, anticholinergic use in the previous 12 hours, or long-acting anticholinergic use in the previous 24 hours.

1.2.2.2 Bronchial challenge tests - Hypertonic Saline Challenge

Airway hyper-responsiveness to inhaled hypertonic saline was used as an inclusion criterion (as an alternative to reversibility) for asthmatics and an exclusion criterion for healthy controls. The procedure was conducted according to current specifications (72). An ultrasonic nebuliser (Ultra-neb 2000, DeVilbiss, Somerset, PA) and a Hans-Rudolph two-way non-rebreathing valve mouthpiece (No.2700 Hans Rudolph Inc., Shawnee, KA) were used to deliver 4.5% saline. Doses were given for 30 seconds, 1, 2, 4 and 8 minutes in succession,
with spirometry performed at baseline and one minute after each dose. The challenge was discontinued when a 15% fall in FEV$_1$ occurred, or after a cumulative inhalation time of 15.5 minutes. The cumulative provocation dose of hypertonic saline causing a 15% fall in FEV$_1$ from baseline (PD$_{15}$HS) was calculated according to the formula

$$PD_{15}\text{HS} = \begin{cases} \frac{X}{T_a} T_p \exp \left[ (\log(T_a) - \log(T_p)) \frac{0.85 V_B - V_p}{V_a - V_p} \right] & \text{if } T_p > 0 \\ \frac{X}{V_B} \frac{0.15 V_B}{V_B - V_a} & \text{if } T_p = 0 \end{cases}$$

where

- $V_B$ = baseline FEV$_1$
- $V_p$ = FEV$_1$ prior to dose causing 15% drop
- $V_a$ = FEV$_1$ prior to dose causing 15% drop
- $T_p$ = total saline inhalation time prior to dose causing 15% drop in FEV$_1$
- $T_a$ = total saline inhalation time after dose causing 15% drop in FEV$_1$
- $X$ = total dose of saline inhaled

Hyper-responsiveness to hypertonic saline was defined as $PD_{15}$HS < 12 ml

1.2.2.3 Bronchial challenge tests – AMP

Airway hyper-responsiveness to AMP was used as an end-point for steroid response assessment. Challenges were performed using a standardized protocol (72). AMP doses were delivered by a nebuliser connected to a breath-activated dosimeter (Morgan, Kent, UK). Initially, a dose containing 0.9% saline was given, followed by doses of AMP progressively doubling in concentration, ranging from 0.59mg/ml to 300mg/ml. Spirometry was performed at baseline, 1 minute after 0.9% saline and 1 and 3 minutes after each dose. The lower of the two FEV$_1$ measurements was recorded for each dose. The test was terminated on reaching a 20% fall in FEV$_1$ or after the maximum dose of AMP had been administered. The provocative concentration causing a 20% fall in FEV$_1$ (PC$_{20}$AMP) was derived according to the equation
\[ PC_{20}^{AMP} = \begin{cases} 
C_p \exp \left[ (\log(C_a) - \log(C_p)) \frac{0.8V_B - V_P}{V_a - V_p} \right] & \text{if } C_p > 0 \\
C_p \frac{0.2V_B}{V_B - V_a} & \text{if } C_p = 0 
\end{cases} \]

where

\( V_B = \) post - saline FEV\(_1\)
\( V_p = \) FEV\(_1\) prior to dose causing 20% drop
\( V_a = \) FEV\(_1\) prior to dose causing 20% drop
\( C_p = \) concentration of AMP dose immediately prior to 20% drop in FEV\(_1\)
\( C_a = \) concentration of AMP dose immediately after 20% drop FEV\(_1\)

1.1.2.4 **Reversibility**

Reversibility of airway obstruction was tested in all patients with a negative hypertonic saline challenge. Patients whose FEV\(_1\) dropped less than 10% during the hypertonic saline challenge were given 400 µg Salbutamol (Albuterol) by metered dose inhaler and volumatic spacer device (Allen & Hanburys, Middlesex, UK). Patients whose FEV\(_1\) dropped by more than 10% were given 5 mg salbutamol and 1 mg ipratropium by nebuliser (PulmoMATE, DeVilbiss, Somerset, PA, USA). Reversibility was defined as an increase of greater than 12% in FEV\(_1\) 15 minutes after bronchodilator administration, a definition identical to that used by earlier studies in the department (64).

1.1.3 **Exhaled breath tests**
1.1.3.1 **Exhaled nitric oxide**

Fractional exhaled nitric oxide (F\(_{E}NO\)) was measured prior to spirometric measurements or breath sampling. An electrochemical instrument was used (NIOX MINO, Stockholm, Sweden). Measurements were made in compliance with the recommendations of the manufacturer. The average of two acceptable tests was recorded.

1.1.3.2 **Breath sampling for electronic nose**

Breath samples were taken using the same methods and materials as a study in the Netherlands (35). Prior to any breath testing, patients were asked to withhold food and drink
(except water) for two hours, short acting beta-agonists for six hours, and alcohol for 24 hours. Breath samples were taken prior to any spirometric measurements but following measurement of exhaled nitric oxide.

Patients performed all manoeuvres while seated. Prior to the breath sample, patients underwent a 5-minute period of tidal breathing filtered air. The tidal breathing was performed through a small Hans Rudolph non-rebreathing valve (Hans Rudolph Inc., Shawnee, KA, USA) with a VOC filter (A168092, North Safety Products, Netherlands) attached to the inspiratory port and a chamber containing silica beads (Silica Gel, self-indicating blue). LabServ, Clayton, Vic., Australia). After the five-minute period patients were asked to inhale to total lung capacity, at which point a Tedlar Bag (5L Clear Tedlar with two Roberts and a second outer layer of black Tedlar, KeikaVentures/Eduwhere, PO Box 4704, Chapel Hill, NC 27515, USA) was attached to the expiratory port. Patients then expired one vital capacity through the silica reservoir into the bag. See figure 4 for a diagram of the apparatus.

At most 10 minutes prior to the breath sample, the same breathing apparatus was used to fill a second tedlar bag with VOC-filtered ambient air. This was accomplished by fitting the apparatus to a calibration cylinder.

Both tedlar bags were attached to the Electronic Nose (Cyranose 320, Smiths detection, Pasadena, CA, USA) and sampling was done in accordance with current protocol (6).
Figure 4. Breath sampling apparatus.

Patients breathed the mouthpiece. Inhaled air passed through the filter and through a one-way valve. Exhaled air passed through a one-way valve and through a silica reservoir into the tedlar bag. Patients were asked to breathe tidally through this apparatus without the tedlar bag attached. The bag was then attached as shown and patients were asked to inhale to TLC and exhale one vital capacity into the bag.

1.1.4 Asthma control monitoring
1.1.4.1 Questionnaires

The asthma control questionnaire (ACQ) by Juniper (70) was used as a study endpoint, with the final question (on FEV₁ as a percentage of predicted) left out. Patients were left alone to complete the questionnaire but were given the chance to ask questions about it. The ACQ was completed early in the visit before any spirometry or breath sampling. In addition, a ‘visual analogue scale’ was completed, consisting of a 10 cm line marked at each cm. Patients were
asked to mark the point on the line corresponding to their state of breathlessness over the past week, with one end of the line corresponding to ‘not breathless at all’ and the other to ‘absolutely breathless’.

1.1.4.2 Peak flow measurements

All asthmatic patients were asked to record morning and evening peak flow measurements for the duration of the study, using a technique compliant with current specifications (standardisation of spirometry). Patients were asked to take morning measurements immediately on waking, prior to taking any medications, and were asked to take evening measurements just before sleeping.

1.1.4.3 Diaries

Asthmatics were also asked to complete a symptom diary for the duration of the study. For each day, patients recorded morning and evening peak flow, the number of times they used a reliever inhaler during the day, and an 'asthma symptom score' grading the severity of their symptoms over the day. For each night, patients recorded whether they awoke because of asthma, and recorded the number of uses of reliever medication through the night. The diary was adapted from one used in previous studies in the department (64).

1.1.5 Other tests

1.1.5.1 Sputum induction and processing

Induced sputum was collected immediately after the hypertonic saline challenge where possible and safe. Patients were asked to rinse their mouths three times, then cough up any available sputum into a container. The sample was then processed in a standardised manner. The sample processed and a cell differential obtained from 400 non-squamous cells using a standardised method. The induction protocol, processing, and cell counting protocol were identical to those used in previous studies in the department (64).

1.1.5.2 Skin Prick Testing

Skin prick tests were conducted in accordance with earlier studies from the department (64).
Tests were conducted by pipetting a drop of each liquid allergen onto the skin of the volar aspect of the forearm. The skin under the drop was then scratched with a micro-lancet (STAllergenes, EBOS limited, New Zealand). House dust mite, 5-grass mix, and cat dander were used as allergens (Hollister-Steir Laboratories, Spokane, Washington, USA) along with a saline negative control and histamine positive control. A wheal of at least 2mm greater than that of the saline control constituted a positive test. Wheal and flare sizes were recorded as maximum diameters, and any ‘tracking’ along blood vessels was noted. Antihistamines were withheld for 72 hours prior to all skin prick tests. Skin prick readings using an identical protocol from up to 1 year prior to the study start were accepted.
Figure 5. Flow chart of study design.
1.2 Study design and chronology

1.2.1 Asthmatics

1.2.1.1 Recruitment

Asthmatic patients were recruited partially from departmental records of participants in previous studies, partially from referrals from the Respiratory Clinic at Dunedin Hospital, and partially from fliers and adverts placed around the hospital.

1.2.1.2 Inclusion/exclusion

Inclusion and exclusion criteria for asthmatic participants are shown in table 3.

Table 3. Inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 18-75</td>
<td>History of life-threatening asthma</td>
</tr>
<tr>
<td>Previous diagnosis of asthma</td>
<td>Presence of other significant respiratory disease</td>
</tr>
<tr>
<td>PD_{15} HS &lt; 12 ml OR reversibility of &gt;12%</td>
<td>(LRTI/URTI)</td>
</tr>
<tr>
<td>FEV\textsubscript{1} &gt; 50% predicted</td>
<td>Use of oral prednisolone in previous 1 month</td>
</tr>
<tr>
<td>Informed consent obtained</td>
<td>Presence of respiratory disease in previous 4 weeks</td>
</tr>
<tr>
<td></td>
<td>(LRTI/URTI)</td>
</tr>
<tr>
<td></td>
<td>Pregnant or breast-feeding</td>
</tr>
<tr>
<td></td>
<td>Other reason (at investigator’s discretion)</td>
</tr>
</tbody>
</table>

Table 3a. Inclusion and exclusion criteria for asthmatics

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 18-75</td>
<td>History of asthma or respiratory disease</td>
</tr>
<tr>
<td>Negative skin prick test to cat, grass, and house dust mite</td>
<td>PD_{15} HS &lt; 12 ml OR reversibility of &gt;12%</td>
</tr>
<tr>
<td>F_{3}NO &lt; 25 ppb</td>
<td>Presence of respiratory disease in past 2 months</td>
</tr>
<tr>
<td>Informed consent obtained</td>
<td>(LRTI/URTI)</td>
</tr>
<tr>
<td></td>
<td>&gt;10 pack years or smoked in past 3 months</td>
</tr>
<tr>
<td></td>
<td>Evidence of HS hyper-responsiveness</td>
</tr>
<tr>
<td></td>
<td>Evidence of reversibility</td>
</tr>
<tr>
<td></td>
<td>Pregnant or breast-feeding</td>
</tr>
<tr>
<td></td>
<td>Other reason (at investigator’s discretion)</td>
</tr>
</tbody>
</table>

Table 3b. Inclusion and exclusion criteria for healthy controls

1.2.1.3 Steroid withdrawal - visits 1-3a (non-steroid naïve asthmatics)

At visit 1, patients gave informed consent for the study. Baseline values of airway tests were
established (see figure 5). Medical and social history were taken, height, weight, heart rate and blood pressure were taken, a skin prick test was conducted, exhaled nitric oxide was measured, ACQ and VAS were completed, and spirometric measurements were made.

Patients were provided with a diary, peak flow meter, and emergency supplies, and instructed on how to complete the diary and how to take peak flows. Patients were asked to continue on all normal medication while keeping the diary for two weeks.

Visit 2 was scheduled for approximately 14 days after visit 1. At visit 2, diaries were retrieved and checked. Average morning and evening peak flow rates, number of 'puffs' of SABA per day, and number of night wakenings per week over the previous 14 days were calculated, all to the nearest whole number. Six loss of control (LOC) criteria were then computed, shown in table 4.

**Table 4. Loss of control criteria**

<table>
<thead>
<tr>
<th>Criteria</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Morning or evening peak flow less than 80% of the respective average for two consecutive days</td>
<td></td>
</tr>
<tr>
<td>2 Morning or evening peak flow less than 60% of the respective average on any day</td>
<td></td>
</tr>
<tr>
<td>3 Average morning peak flow over previous week less than 90% of morning average</td>
<td></td>
</tr>
<tr>
<td>4 SABA use per day over previous week (rounded to nearest whole number) of at least four puffs more than average</td>
<td></td>
</tr>
<tr>
<td>5 Night wakening per week (rounded to nearest whole number) of at least two more than average</td>
<td></td>
</tr>
<tr>
<td>6 Presence of distressing or intolerable asthma symptoms</td>
<td></td>
</tr>
</tbody>
</table>

Patients were informed of the peak flow rates corresponding to 80% and 60% of their morning and evening averages, and told to contact the research unit immediately if either of the first two criteria were met.

At the second visit, patients were also reviewed by a doctor to assess whether the steroid-withdrawal period could be undertaken safely.

Following visit 2, patients were asked to withdraw inhaled corticosteroids and all other preventer medication. Patients were contacted approximately every three days by telephone and diary entries were recorded.

As soon as one or more of the LOC criteria were met or 28 days had elapsed since visit 2, a third visit was booked. Patients were asked to withhold food and drink for two hours, SABAs
for six hours and alcohol for 24 hours prior to this third visit.

At the first part of the third visit, patients were screened for eligibility for the study and some pre-OCS tests were done. In all patients, $F_{E}NO$ was measured and ACQ and VAS were completed. A breath sample was taken and analysed using the electronic nose. If safe, a hypertonic saline challenge was then conducted. If $PD_{15}HS$ was found to be greater than 12 ml, reversibility was tested. Patients with no airway hyper-responsiveness ($PD_{15}HS > 12$ ml) and no significant reversibility (less than 12% improvement on baseline $FEV_{1}$ 15 minutes after bronchodilator) were withdrawn at this point.

See figure 5.

1.2.1.4 Visit 1a (steroid naïve asthmatics)

Patients not on inhaled corticosteroids or other preventer medications at the start of the study underwent a condensed version of visits 1-3. Prior to the first visit, patients were asked to withdraw food, alcohol and SABAs in the same way as for non-steroid naïve asthmatics. At the visit, informed consent was obtained. Height, weight, heart rate and blood pressure were measured, a skin prick test was done, $F_{E}NO$ was measured and ACQ and VAS were completed. A breath sample was taken and analysed by electronic nose. A hypertonic saline challenge was then performed, possibly followed by testing of reversibility, in the same manner as for non-steroid naïve asthmatics.

See figure 5.

1.2.1.5 Steroid trial

At visit 3b (non-steroid naïve asthmatics) or visit 1b (steroid-naïve asthmatics), an AMP challenge was conducted. Following this, patients were given 15 days supply of 30 mg oral prednisone, and instructed on how and when to take them. Patients were asked to continue keeping diaries throughout the steroid course.

Patients were contacted by telephone one week into the steroid course to check for adverse effects related to medication.
Prior to visit 4, patients were asked to withhold food, alcohol, and SABAs as for visit 3a. $F_{E}NO$ was measured, ACQ and VAS were completed, a breath sample was taken and analysed, and an AMP challenge was done. Following the visit, patients were re-started on their pre-study medication.

See figure 5.

1.2.2 Healthy controls
1.2.2.1 Recruitment

Healthy control participants were recruited partially from fliers placed around the hospital and university, partially from an e-mail sent to all university staff, and partially from acquaintances of the researchers.

1.2.2.2 Inclusion and exclusion criteria

Inclusion and exclusion criteria for healthy controls are shown in table 3.

1.2.2.3 Visit 1

Healthy controls visited the research unit once. Prior to the visit patients were asked to withhold food and drink and alcohol for the same periods as for asthmatics.

Initially, informed consent was obtained. Height, weight, heart rate, and blood pressure were measured. A skin prick test was done and $F_{E}NO$ was measured. If the patient was still eligible, a breath sample was then taken and analysed, and a hypertonic saline challenge was conducted. If PD$_{15}$HS was found to be greater than 12 ml, reversibility was tested. Patients with no airway hyper-responsiveness (PD$_{15}$HS greater than 12 ml) and no significant reversibility (less than 12% improvement on baseline FEV$_{1}$ 15 minutes after bronchodilator) were entered into the study.

See figure 5.

1.2.3 Ethical considerations and safety
Ethical approval was obtained from the Lower South Regional Ethics Committee. To ensure safety during the period of steroid withdrawal, asthmatic patients were briefed on LOC criteria, given prednisolone tablets for emergency use, and given an emergency contact details card. As well as telephone contact every three days patients had 24 hour access to medical professionals. LOC criteria included “presence of distressing or intolerable asthma symptoms” so that the steroid withdrawal period could be terminated at the patient’s request regardless of peak flow measurements if necessary.

2 Analysis of data
2.0 Overview

Demographic data was analysed using a standardised set of parametric statistical techniques. FeNO and eosinophil counts were transformed before prior to analysis. Paired tests were used to compare test values for the same group of patients before and after interventions (93).

Analysis of data from the electronic nose requires more complex techniques which are generally analogous to the neural processing which takes place in biological olfaction. There are three stages to the analysis of EN data: the initial data reduction; the assessment of between-group differences and correlations between EN output and other variables; and the construction of a discriminating function (15). The second step can be considered exploratory, and the third confirmatory. In the exploratory analysis, ‘unsupervised’ learning techniques were used, in which electronic nose output was considered without knowledge of the groups to which breathprints belong. In the confirmatory analysis, ‘supervised’ learning was used, in which knowledge of the group to which each breathprint belonged was used to propose a classifying algorithm.

The aim of confirmatory analysis of electronic nose output is to find a function which takes a patient’s electronic nose readings as input and outputs the group to which the patient most probably belongs (15). Such a function is termed a ‘discriminating function’. Because no pre-prescribed discrimination function exists, a candidate function must be constructed from the available data. The same data must also be used to test the function. The effectiveness of a classification function cannot be measured directly but can be estimated by techniques such as cross-validation or the bootstrap (94).

All procedures were implemented in the IDL programming language using double precision.
2.1 Analysis of steroid response data

2.1.1 Pre-processing of variables

For most measured variables (heart rate, height, FEV1 and similar), a normal distribution is assumed. Hypothesis tests on the differences in variables between groups are conducted under the null hypothesis that group means are equal.

FE\textsubscript{NO} and eosinophil counts are not normally distributed, showing significant positive skew (93). In order to use parametric statistical methods to analyse FE\textsubscript{NO}, it is necessary to perform a transformation on the data. Jones et al. (22) found that a logarithmic transform was appropriate for FE\textsubscript{NO} analysis (appendix 1d). The logarithm of FE\textsubscript{NO} values for a population is assumed to be normally distributed. Logarithmic transformation is mathematically equivalent to using the geometric mean (GM) and geometric standard deviation (GSD) (appendix 1d) in place of the usual arithmetic mean and standard deviation.

Eosinophil counts were transformed according to the equation.

\[ E' = \log(e + \frac{E}{T}) \]

where \( E' \) is the transformed eosinophil count, \( E \) is the original number of eosinophils on the slide, and \( T \) is the total number of cells counted on the slide.

2.1.2 Between-group comparisons

T-tests were used to compare differences in single variables between two groups. Bonferroni correction for multiple testing was used where appropriate. Analysis of variance (ANOVA) was used to analyse differences in a single variable over more than two groups. ANOVA was tested using the ‘F’ statistic. (93).

Paired t-tests and repeated measures ANOVA were used to compare differences in variables for the same patient group at different times, such as before and after a steroid course. The advantage of these tests is that variation between individuals in the group does not contribute
as much to the measurement (93).

2.2 Unsupervised analysis of electronic nose data

2.2.2 Processing and reduction of electronic nose data

2.2.2.1 Filtering and normalisation

Raw output from electronic nose sensors is in the form of a serial set of sensor resistances over a period of time. In general, each sensor will have a relatively constant ‘baseline’ resistance while in contact with ambient air, and resistance will increase when the sensor comes in contact with a gas sample (17). Sensor resistance will typically increase non-linearly with time, eventually reaching an asymptote at which time the sensor is in equilibrium with the sample (13). The resistance-time data tend to show high-frequency noise, the effect of which can be minimised by ‘filtering’ or smoothing the data. The onboard software of the Cyranose 320 uses the Savitzky-Golay method to accomplish this (17).

The raw output from the electronic nose was filtered using this method. Following filtering, the data from each sample \( i \) were reduced to single value \( R(i,s) \) for each sensor \( s \). The values from each sensor were then normalised according to the equation

\[
S(i,s) = \frac{R(i,s)}{\sum_{r=1}^{32} R(i,r)}
\]

where \( S(i,s) \) is the normalised sensor response for sensor \( s \) to sample \( i \). The values of \( R \) are located in a 32-dimensional ‘raw space’. The sensor space, in which the values of \( S \) are located, is a 31 dimensional subspace of the raw space. The reduced dimension is a consequence of the fact that, for all \( i \),

\[
\sum_{s=1}^{32} S(i,s) = 1
\]

Filtering and sensor response calculation were performed automatically by the Cyranose 320, with the un-normalised response saved as output (17). The sensor responses were then normalised manually.
2.2.2.2 Principal component extraction

Individual sensors responses from the electronic nose tend to show strong positive correlations. This is essentially the effect that swelling of one sensor in response to a sample corresponds to concurrent swelling of others. As a result, individual analyses on sensor responses are often superfluous and sample responses cannot be considered to be independent. This problem is overcome by the method of principal component analysis (PCA). PCA is utilised in most applications in which the Cyranose 320 is used; the onboard software begins analysis of samples with use of PCA (17) and PCA is a preferred initial step in electronic nose-based breath analysis (15).

The aim of PCA is to replace the 32 output variables with a set N linear combinations, or ‘components’, which collectively account for most of the variance of the sample. The components are chosen in sequence such that each component accounts for as much variance not already accounted for by previous components as possible. This means that components have pairwise zero correlation, and hence vary independently (93).

Because principal component reduction only produces linear combinations of variables, any non-linear correlations between sensor responses are not accounted for by PCA. In addition, it is an unsupervised learning technique, and all noise (within-group variation) in the data is retained. In this way, PCA is not a means of classification of groups in itself, but a way of reducing data to a point where they can be compared (15). Principal components can also be compared with other interval variables by correlation.

Differences in exhaled breath profiles between groups is likely to be in the form of subtle changes in a wide range of molecules, and as such are expected to be observed as small changes in readings from many sensors rather than large changes in few. Extraction of principal components allows a representation of such changes.

2.2.3 Exploratory analysis
2.2.3.1 Mahalanobis distance and comparison of principal components

Absolute separation of groups in sensor space can quantified using the Mahalanobis distance (M-distance) metric. The metric is a generalisation of the Euclidean distance which provides a
better distance measure in distributions with significant covariance between variables. Electronic nose sensor responses are highly correlated and the M-distance is widely used as a measure of separation in the field (15).

Principal components were compared between groups as a secondary indicator of between-group discrimination. Every two components are mutually non-covariant, so each captures a different 'direction' in which the data varies. Groups with insignificant M-distances between them which differ only in a subset of sensors may be differentiable when a particular principal component is considered. Because of the non-covariance of components, characteristics such as eosinophilia may only be exhibited in a single principal component (37).

The M-distance is computationally related to the extraction of principal components. The transformation of variables from sensor data to principal components in effect maps the 'sensor space' onto a 'component space' where components have zero covariance and unit variance. The M-distance between breathprints in sensor space is equal to the Euclidean distance in component space. In this sense, principal component extraction can be considered a 'distance preserving' transform (95) in which M-distances map to E-distances. The M-distance between two groups can be considered to be an average of between-group distance across all components.

If a significant M-distance is found between groups, it can be well-asserted that VOC profiles in the two groups differ significantly. However, because M-distance is a measure of distance between points in sensor space, it is a conservative marker of group discrimination. When groups are defined by subtle phenotypical differences, between-group differences may only be present on a small subset of the 32 sensors. The noise from the remaining sensors would generally cause the M-distance between groups to be small, even though they may be well-separated on the basis of one or two sensors. The comparison of principal components between groups was used to address this problem.

Each component accounts for a proportion of the total variance of the sensor values, allowing for covariance. A component accounting for more than 3.125% (100/32) of the total variance accounted for more variance than the average contributed by a single sensor. Components exhibiting at least 1.5% of the total variance were compared between groups, which resulted in around 6-10 comparisons in each case. Significances were then adjusted according to a Bonferroni correction.
Two groups were considered to be differentiated if either a principal component was significantly different between them or M-distance between them was significantly large.

2.2.3.2 Calculation of the Mahalanobis distance

If two groups A and B are to be compared, with \( n_A \) and \( n_B \) defined as the number of members of groups A and B respectively, and \( n = n_A + n_B \) the total number of samples, \( S_i = (S_{i1}, S_{i2} \ldots S_{i32})^T \) defined as the vector of normalised readings \( S_{is} \) from sensor \( s \) for sample \( i \), and \( C_{rs} \) defined as the covariance between sensors \( r \) and \( s \), the M-distance is given by

\[
m = \left[ (\mu_A - \mu_B)^T C^{-1} (\mu_A - \mu_B) \right]^{\frac{1}{2}}
\]

where

\[
(\mu_A)_s = \frac{\sum_{i \in A} S_{is}}{n_A} \quad \text{and} \quad (\mu_B)_s = \frac{\sum_{i \in B} S_{is}}{n_A}
\]

By contrast, the Euclidean distance between the groups is defined as

\[
E = \left[ (\mu_A - \mu_B)^T (\mu_A - \mu_B) \right]^{\frac{1}{2}}
\]

The inclusion of the covariance matrix in the calculation of M distance means that the contribution of variance in individual sensors to the total M-distance is normalised, reducing the contribution of sensors with large variances to the total variance estimate. Covariance between the sensors is also accounted for, allowing that sensors do not vary independently.

Because the true covariances of the sensor responses are not known, the true M-distance cannot be calculated. The ‘sample M-distance’ may be calculated using approximations to the covariance matrix based on the data. In practise the sample M-distance is treated as the true M-distance. The most obvious estimate of the covariance matrix is the sample covariance of all the sensor readings, which is appropriate when the sensor readings are not to be divided into groups. However, when M-distance is used as a measure between the means of two
groups, a better estimate of the covariance is given by the pooled sample covariances, defined as

\[
C = \frac{(n_A - 1)C_A + (n_B - 1)C_B}{n_A + n_B - 2}
\]

where \(C_A\) and \(C_B\) are the sample covariance matrices of groups A and B. This estimate of the covariance matrix is singular when \(n\) is less than the total number of variables, meaning that it cannot be used to calculate the M-distance. In this case, an alternative estimate of the covariance matrix based on a ‘shrinkage’ technique (96) is used.

### 2.2.3.3 Significance of M-distance separation

The M-distance can be used to test the separability of two groups, under the null hypothesis that the groups arise from the same distribution in sensor space.

Squared M-distances from random samples to a group mean follow a chi-squared distribution (95) and for \(n_A + n_B > 32\), distances between the means of two random samples follow an F-distribution (97), given by

\[
\frac{n_An_B}{n_A + n_B} \frac{n_A + n_B - 31}{32(n_A + n_B - 2)} M^2 \sim F(p, n_A + n_B - 2)
\]

M-distance is defined to have a distribution that depends only on the number of variables and the number of cases. When calculated for 32 variables between two groups of the same size, the M-distance can be used as a measure of relative separation of groups.

If \(n_A + n_B < 31\) the significance of an M-distance between two groups A and B may be approximated using a bootstrapping technique (94). If \(K\) is a set of \(k\) random \(n_A\)-element subsets of \(A \cap B\) and \(m(Q)\) is the sample M-distance between \(Q\) and the complement of \(Q\) in \(A \cap B\), let \(k'\) be the number of elements \(Q\) of \(K\) such that \(m(Q) > m(A)\). Then the approximate \(p\) value \(p'\) is given by

\[
p' = \frac{k'}{k}
\]
This procedure essentially involves repeatedly grouping samples randomly (as opposed to separating into groups A and B) and comparing the separation between random groups to the separation between A and B.

2.3 **Supervised analysis of electronic nose data**

2.3.1 **Difficulties in electronic nose-based discrimination**

2.3.1.1 **Training and testing**

The algorithm on which group membership is predicted based on raw electronic nose samples is unspecified prior to data collection. Because of this, the analysis of data of this type is approached differently to the testing of a known classifier between groups. There is little scope to assess between-group discrimination without first proposing a classification function.

Because of the small number of samples, the same data set must be used to design a classifying function and to test the group discrimination achieved by the function. However, designing a function to optimally separate samples from a set D into groups entails that the between-group discrimination for samples in D is maximised. Testing the function on the same data therefore assesses only the ability of the function to discriminate members of D, rather than giving any indication of how the function would perform in the population from which D is taken.

This gives rise to the phenomenon of overtraining. Functions can be produced which classify all samples in D arbitrarily well, and thus indicate arbitrarily good group discrimination when tested on D, although the functions model principally noise in the data and have no predictive power outside D.

For this reason, a classifying function designed to classify samples in a dataset D cannot be tested directly on D. A good approximation can be made of the desired proportion using the method of cross-validation (see 2.4.3.1).

Cross-validation requires that the algorithm used to produce a discriminating function \( f \) from a dataset \( D' \) is deterministic and unbiased; that is, the same function \( f \) will always be produced from the same dataset. If a discriminating function producing algorithm can be assumed to be adequate, then the cross-validation value so obtained approximates the true
error rate of the algorithm on test data (94). M-distance can be used to loosely assess between-group separation based only on raw data, but does not produce a usable classification function.

2.3.1.2 The curse of dimensionality

The curse of dimensionality is a phenomenon arising from the difficulty of analysing a high number of output variables simultaneously. When considering the 32-dimensional sensor space, it is likely that data clusters exist in lower-dimensional subspaces, which may or may not correspond to clusters in the sensor space. For instance, a particular phenotype could cause a marked increase in response from sensors 2, 10, and 20, but no response from other sensors. When looked at as a response in these space defined by these three sensors alone, a clear discrimination could be identified. However, when considered in 32-dimensional space, a far smaller discrimination would be seen due to noise from other sensors (15).

When more variables are used than cases are available, several conventional estimates of distribution parameters (such as covariance matrices) become unusable. This necessitates the use of techniques such as ‘shrinkage’ (96) or bootstrapping (94).

2.3.2 Estimation of error rate in group discrimination

2.3.2.1 Cross-validation (jack-knife method)

Cross validation is a highly effective unbiased technique for estimating the error in a proposed classification function (94). In the context of electronic nose analysis, cross validation is used to assess the effectiveness of the algorithm producing a classification function from a training set, and to estimate how well a classification function can discriminate groups of samples. Cross-validation also allows different predictors such as FeNO to be compared with the electronic nose (7).

A discriminant function is produced from a set of samples and knowledge of the group membership of each sample. If it is assumed that the set of samples are representative of those groups, the cross-validation value gives an estimate of the likelihood that the discriminant will correctly identify a random sample not used in the production of the discriminant. The global sensitivity and specificity of a classification function can also be estimated in this way (94).
In leave-one-out cross validation with n cases, a total of n discriminant function are constructed, each using all cases but one. Each discriminant function is then tested on the left-out case. The proportion of cases correctly classified by the corresponding discriminant function is termed the ‘cross-validation value’.

For a set U of n samples \( U = \{s_1, s_2, \ldots, s_n\} \) which are classified into subsets A and B, define
\[
g(s_i) = 1 \text{ if } s_i \in A \text{ and } g(s_i) = 0 \text{ if } s_i \in B.
\]
If \( G \) is an algorithm that, given a set \( U' \) of samples, produces a function \( \{G(U')(s_i)\} \) from U to \([0,1]\), the cross-validation value \( c \) for \( G \) and U is given by
\[
c = 1 - \frac{\sum_{s_i \in U}|G(U_i)(s_i) - g(s_i)|}{n}
\]
where \( U_i = \{s_1, \ldots, s_{i-1}, s_{i+1}, \ldots, s_n\} \). The cross validation value is typically expressed as a percentage rather than a proportion.

### 2.3.2.2 Wilks’s lambda

For a linear discriminant the Wilks’s lambda statistic \( \Lambda \) can be used as a measure of group separability (98). The statistic is calculated based on a ratio of between-group variance and total variance, and can be considered a generalisation of the F-distribution (97).

Significance tests are constructed under the null hypothesis that the discriminating function being tested is not correlated with the group number. The distribution of the lambda statistic is complex, but it can be approximated by a chi-square distribution (97).

Wilks’s lambda is not used to assess non-linear discriminants

### 2.3.3 Construction of the discriminant function

#### 2.3.3.1 Linear discriminant analysis

A linear discriminant is a means of distinguishing two groups using several variables. Linear discriminant analysis (LDA) is a method of constructing and testing an optimum linear discriminant. LDA was used in to calculate the linear discriminants best separating groups of
patients based on normalised sensor outputs from the electronic nose. Either normalised sensor scores or principal component scores can be used as inputs to the discriminant.

If \( \mathbf{V}_i = (V_{i1}, V_{i2}, \ldots, V_{ip})^T \) is defined as the vector of the \( p \) input variables \( V_{iv} \) from variable \( v \) for sample \( i \), and \( n_A, n_B \) are the number of members of groups A and B respectively, a linear discriminant is a function \( f \) defined by

\[
f(i) = c + w_1 V_{i1} + w_2 V_{i2} + \ldots + w_p V_{ip} = \mathbf{w}^T \mathbf{V}_i
\]

for some vector \( \mathbf{w} = (w_0, w_1, w_2, \ldots, w_p)^T \) and scalar \( c \). A good discriminant function between two groups A and B will have generally positive values when \( i \) is a member of group A and negative values when \( i \) is a member of group B, or vice versa.

If A contains \( n_A \) samples and B contains \( n_B \) samples, with group means \( \mu_A \) and \( \mu_B \) and grand mean \( \mu \) defined as

\[
(\mu_A)_v = \frac{\sum_{i \in A} V_{iv}}{n_A}, \quad (\mu_B)_v = \frac{\sum_{i \in B} V_{iv}}{n_B}, \quad (\mu)_v = \frac{\sum_{i=1}^{n_A+n_B} V_{iv}}{n_A+n_B}
\]

the optimum linear discriminant is considered to be the vector \( \mathbf{w} \) that maximises the objective

\[
\lambda = \frac{\mathbf{w}^T X_b \mathbf{w}}{\mathbf{w}^T X_T \mathbf{w}}
\]

where \( X_b \) is a measure of between-group scatter given by

\[
X_b = n_A (\mu_A - \mu)^T (\mu_A - \mu) + n_B (\mu_B - \mu)^T (\mu_B - \mu)
\]

and \( X_T \) is a measure of the total scatter defined by

\[
X_T = \sum_{i \in A} (\mu_A - V_i)(\mu_A - V_i)^T + \sum_{i \in B} (\mu_B - V_i)(\mu_B - V_i)^T
\]
$X_b$ and $X_T$ can only be calculated when the number of cases $(n_A + n_B)$ is greater than $p$.

The vector $w$ then corresponds to an axis through the space generated by the input variables along which the maximum difference between the groups is observed. The cutoff $c$ is defined as

$$c = \frac{1}{2} \left( \sum_{i \in A} f(i) + \sum_{i \in B} f(i) \right) = w^T (\mu_A + \mu_B)$$

The value of the objective $\lambda$ is used to test the separability. The Wilks’s Lambda statistic $\Lambda$ is computed according to (98)

$$\Lambda = \frac{\lambda}{1 + \lambda}$$

While PCA finds linear combinations of the variables which encompass as much of the total variance as possible, LDA finds linear combinations which maximises variance between groups. Unlike principal component analysis, LDA takes both the samples and the groups they are in as input.

In two dimensions LDA can be considered to produce an axis along which the two groups are well-separated. This can be used to produce a straight line which separates the members of one group from the members of the other (15).

LDA has the obvious disadvantage of being a linear technique, so non-linear separations between the data will not be found (1). Cross-validation is also used to estimate the utility of a linear discriminant as a predictor of group membership.

### 2.3.3.2 Multi-layer perceptrons

Multilayer perceptrons (MLPs) are a type of artificial neural network (ANN). ANNs are interconnected groups of simulated neurons which can learn to perform various tasks in a similar way to biological brains (15). The neural processing of EN data allows the EN to function in a way analogous to the human nose (13).
Each simulated neuron has a vector of inputs \( \mathbf{x} = (x_1, x_2, \ldots, x_n) \) (at least two) and a scalar output \( y \) (see figure 6). The output value \( y \), which is conventionally in the range \([0,1]\), is calculated as \( y = f(w, \theta) \), where \( w = w_1 x_1 + w_2 x_2 + \ldots + w_n x_n = \mathbf{w}^T \mathbf{x} \) is a weighted sum of the input values, \( f \) is a function of \( w \) with values in \([0,1]\) termed the ‘activation function’, and \( \theta \) is a scalar. The number \( n \) of inputs and the activation function \( f \) of a neuron are fixed, and the values of \( w_1, w_n, \theta \) are varied in order to ‘train’ the neuron (15). The activation function used in this application was the sigmoid function \( f(w, \theta) = \frac{1}{1 + e^{-(w+\theta)}} \).

Groups of neurons can be connected in several ways, which are referred to as ‘topologies’. ‘Inputs’ to the network are fed into the inputs of several neurons. The output of the network is given by the outputs of one or several neurons. A ‘feed-forward’ neural network is an architecture in which neurons can be assigned hierarchies or ‘layers’ such that the inputs to each neuron come from either neurons of earlier or inputs to the network as a whole. In an MLP topology, one such feed-forward arrangement, all input values are fed into a number of different neurons (layer 1), the outputs of which are fed into a number of neurons at layer 2, and so on, with the outputs from the perceptron coming exclusively from the highest layer of neurons (99). See figure 6.

The perceptron essentially evaluates a function of the inputs. As long as there are enough neurons in each layer, the ‘universal approximation theorem’ specifies that only two layers – one hidden, one output - are necessary in order to accurately approximate any discriminating function (100).
The upper part of this figure shows the activity of a single ‘neuron’. The lower part shows the architecture of a multilayer perceptron with three inputs, a single hidden layer containing six neurons, and a single output. All perceptrons used in the study had five neurons in the hidden layer.

Figure 6. MLP architecture
In the context of electronic nose analysis, the standard use of multilayer perceptrons is in the calculation of the classification function. Typically the extracted features are used as inputs to the perceptron, with the output of the perceptron giving likely group the feature belongs to. Training the perceptron consists of inputting known EN data and adjusting the weights of the constituent neurons according to the difference between the perceptron output and the expected output (101). Initially all weights are set to random numbers between 0 and 1.

Training of multilayer perceptrons is generally via a technique called back-propagation, in which the error between the perceptron output and the expected output is ‘propagated’ back through the network. There are a range of algorithms for this (15). The algorithm used was derived from Rojas (102), chapter 7.

Testing of multilayer perceptrons is by cross-validation. For each sample, a new MLP was trained using all other samples, and used to classify the original sample. The number of correct such classifications determined the cross-validation value. Each new MLP was initialised using the same set of random weights.
Results

1. Characteristics of study groups
1.1 General
1.1.1 Enrolment numbers and withdrawals

Seventy five patients gave consent to participate in the study, comprising 39 potential asthmatics and 36 potential healthy controls. Of these, 47 were enrolled, comprising 27 asthmatics and 20 healthy controls. Sputum samples were obtained from 26 asthmatics and 19 healthy controls.

One asthmatic patient was withdrawn due to a low baseline FEV$_1$ (which precluded airway challenge tests). Eleven potential asthmatic patients were withdrawn due to a negative hypertonic saline challenge and no subsequent reversibility.

Four potential healthy controls were withdrawn due to a F$_{E\text{NO}}$ of >25 ppb with negative skin prick tests. Four potential controls were withdrawn due to one or more positive skin prick tests with low F$_{E\text{NO}}$ (<25 ppb). Six potential controls were withdrawn due to concurrent positive skin prick tests and high F$_{E\text{NO}}$ (>25 ppb). One potential control was withdrawn due to a history of smoking, and one due to >12% reversibility post-bronchodilator.

1.1.2 Classification by steroid response

Asthmatic patients were classified as steroid responsive or non-steroid responsive on the basis of testing before and after a course of oral prednisone. Responsiveness was defined as a decrease in ACQ score of at least 0.5, an increase of at least 300% (two doubling doses) in PC$_{20}\text{AMP}$, or an increase of at least 15% in FEV$_1$.

Three asthmatics had insufficient FEV$_1$ to safely undergo an AMP challenge prior to the OCS course. In two of these cases, the AMP challenge was not required in order to establish steroid response (the patient exhibited a significant ACQ decrease or FEV$_1$ increase). The remaining patient exhibited no change in ACQ or FEV$_1$, and was classified as non-steroid responsive.

Of the 27 enrolled asthmatics 16 (59%) were classified as ‘steroid responsive’ (SR asthma) and 11 (41%) were classified as ‘non-steroid responsive’ (NSR asthma). Twelve patients
(44%) showed a drop in ACQ score of at least 0.5, and 15 patients (56%) did not. Eight patients (30%) showed an increase in FEV\textsubscript{1} of at least 15%, and 19 patients (70%) did not. Nine patients (38%) showed an increase in PC\textsubscript{20}AMP of at least two doubling doses, and 15 patients (62%) did not.

1.2 Characteristics of asthmatic and healthy control groups

1.2.1 Comparison of demographic and physical data

Comparisons between asthmatic patients healthy controls are shown in Table 5a.

Asthmatic patients were significantly older than healthy controls (p<0.001) and had significantly higher weight (p=0.001) and BMI (p=0.002). No difference was found in sex, height, or the proportion of ex-smokers between groups. Almost all asthmatics were atopic. Since inclusion in the healthy control group entailed absence of atopy a calculation of significance was not appropriate in this case.

1.2.2 Comparisons of respiratory data

Comparisons of respiratory data between controls and asthmatics are shown in Table 5b.

At visit 3 all asthmatics were steroid free (either they were not on inhaled steroids prior to the study or they had undergone a period of controlled inhaled steroid withdrawal). When compared to values for controls, FEV\textsubscript{1} (p<0.001), FEV\textsubscript{1} as a percentage of predicted value (p<0.001), FVC (p=0.012), FVC as a percentage of predicted value (p<0.001) and FEV\textsubscript{1}/FVC (p<0.001) were significantly higher among controls. Eosinophil count was significantly higher among asthmatics (p<0.001).

As expected, F\textsubscript{E}NO was higher among asthmatics, given the exclusion criteria for healthy controls. All healthy controls were required to have F\textsubscript{E}NO <25 ppb and PD\textsubscript{15}HS>12 ml. Although all healthy controls underwent a hypertonic saline challenge, none demonstrated a 15% fall. A calculation of significance was not appropriate for F\textsubscript{E}NO or PD\textsubscript{15}HS differences.

Table 5. Comparisons of non-electronic nose data between asthmatics and controls
Table 5a. Comparison of baseline variables between asthmatics and controls

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls</th>
<th>Asthmatics</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>30.6 (15.0)</td>
<td>51.0 (12.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number of males†</td>
<td>9 (45%)</td>
<td>11 (41%)</td>
<td>NS§</td>
</tr>
<tr>
<td>Height (m)*</td>
<td>1.7 (0.1)</td>
<td>1.7 (0.1)</td>
<td>NS§</td>
</tr>
<tr>
<td>Weight (kg)*</td>
<td>66.8 (10.7)</td>
<td>83.7 (21.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>23.4 (2.6)</td>
<td>29.0 (7.8)</td>
<td>0.002</td>
</tr>
<tr>
<td>Ex-smokers†</td>
<td>2 (10%)</td>
<td>6 (22%)</td>
<td>NS§</td>
</tr>
<tr>
<td>Atopy</td>
<td>0%</td>
<td>96%</td>
<td>NA</td>
</tr>
</tbody>
</table>

* - Arithmetic mean/standard deviation
† – Number/percentage of group
‡ – Geometric mean/standard deviation
§ – Not significant
|| – Not applicable
¶ - Transformed mean/transformed standard deviation

Table 5b. Comparisons between respiratory data for untreated asthmatics and controls.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls</th>
<th>Asthmatics</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁ (L)*</td>
<td>3.8 (0.9)</td>
<td>2.3 (0.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV₁ %predicted*</td>
<td>122 (24)</td>
<td>88 (29)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FVC (L)*</td>
<td>4.5 (1.0)</td>
<td>3.7 (1.1)</td>
<td>0.008</td>
</tr>
<tr>
<td>FVC %predicted*</td>
<td>104 (14)</td>
<td>91 (19)</td>
<td>0.012</td>
</tr>
<tr>
<td>FEV₁/FVC (%)*</td>
<td>83(7)</td>
<td>63(10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Eosinophil count¶</td>
<td>0.02 (0.16)</td>
<td>11.76 (2.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>F₂NO ‡</td>
<td>16.5 (1.34)</td>
<td>30.0 (1.66)</td>
<td>NA</td>
</tr>
<tr>
<td>PD₁₅ HS‡</td>
<td>3.6 (2.6)</td>
<td>ND**</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Arithmetic mean/standard deviation
† Number/percentage of group
‡ Geometric mean/standard deviation
§ Not significant
|| Not applicable
¶ - Geometric mean/standard deviation of offset values
** No data available

1.3 Characteristics of steroid responsive and non-steroid responsive asthma groups

1.3.1 Comparison of demographics

Comparisons of demographics between steroid responsive and non-steroid responsive asthmatics are shown in Table 6a

Non-steroid responsive asthmatics were older (p=0.046) and lighter (p=0.034) than steroid responsive asthmatics, although BMI difference was of borderline significance (p=0.058).
There was no significant difference in sex ratio, height, or number of ex-smokers between the steroid responsive and non-steroid responsive groups.

Of the 27 asthmatic patients enrolled, 24 were taking regular ICS. Of these 24, 18 were on ICS alone and 6 were concurrently on regular LABAs. The mean daily BDP equivalent dose across all asthmatics was 498 µg/day. 75% of SR asthmatics and 55% of NSR asthmatics were on ICS without concurrent LABA medication. 13% of SR asthmatics and 36% of NSR asthmatics were on ICS with concurrent LABA medication.

1.3.2 Comparison of baseline respiratory data

Comparisons of baseline respiratory data between steroid responsive and non-steroid responsive asthmatics are shown in Table 6b

Following the steroid withdrawal period several differences were identified between the steroid responsive and non-steroid responsive asthmatic groups (although patients were not classified at this point). FEV₁ (p=0.006), FEV₁ as a percentage of predicted value (p=0.003), FEV₁/FVC (p=0.01) and sputum eosinophil count (p=0.002) were significantly higher among steroid responsive asthmatics. However, ACQ was significantly lower among non-steroid responsive asthmatics (p<0.001). There were no significant differences in FVC, FVC as a percentage of predicted value, FeNO, or PD₁₅ HS.
Table 6. Comparison of non-electronic nose data between steroid responsive and non-steroid responsive asthmatics

Table 6a. Comparison of demographics between steroid responsive and non-steroid responsive asthmatics.

<table>
<thead>
<tr>
<th></th>
<th>SR-asthmatics</th>
<th>NSR-asthmatics</th>
<th>p</th>
<th>All asthmatics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>47 (11)</td>
<td>57 (11)</td>
<td>0.046</td>
<td>51 (12)</td>
</tr>
<tr>
<td>Sex†</td>
<td>7 (44%)</td>
<td>4 (37%)</td>
<td>0.073</td>
<td>11 (41%)</td>
</tr>
<tr>
<td>Height (m)*</td>
<td>1.71 (0.09)</td>
<td>1.69 (0.07)</td>
<td>NS§</td>
<td>1.7 (0.1)</td>
</tr>
<tr>
<td>Weight (kg)*</td>
<td>91 (23)</td>
<td>73 (14)</td>
<td>0.034</td>
<td>83 (21)</td>
</tr>
<tr>
<td>BMI (kg/m2)*</td>
<td>31 (9)</td>
<td>26 (4)</td>
<td>0.034</td>
<td>29 (7)</td>
</tr>
<tr>
<td>Ex-smokers†</td>
<td>3 (27%)</td>
<td>3 (19%)</td>
<td>NS§</td>
<td>6 (22%)</td>
</tr>
<tr>
<td>On ICS (no LABA)†</td>
<td>12 (75%)</td>
<td>6 (55%)</td>
<td>0.004</td>
<td>18 (67%)</td>
</tr>
<tr>
<td>On ICS/LABA†</td>
<td>2 (13%)</td>
<td>4 (36%)</td>
<td>0.034</td>
<td>6 (22%)</td>
</tr>
<tr>
<td>BDP equivalent (µg/day)*</td>
<td>459 (316)</td>
<td>555 (353)</td>
<td>NS§</td>
<td>498 (286)</td>
</tr>
</tbody>
</table>

Table 6b. Comparison of respiratory data between steroid responsive and non-steroid responsive asthmatics (pre-OCS)

<table>
<thead>
<tr>
<th></th>
<th>SR-asthmatics</th>
<th>NSR-asthmatics</th>
<th>p</th>
<th>All asthmatics</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁ (L)*</td>
<td>2.6 (0.9)</td>
<td>1.9 (0.4)</td>
<td>0.006</td>
<td>51.1 (12.1)</td>
</tr>
<tr>
<td>FEV₁ %predicted*</td>
<td>75 (18)</td>
<td>65 (11)</td>
<td>0.003</td>
<td>11 (41%)</td>
</tr>
<tr>
<td>FVC (L)*</td>
<td>3.8 (1.4)</td>
<td>3.4 (0.6)</td>
<td>NS§</td>
<td>1.7 (0.1)</td>
</tr>
<tr>
<td>FVC %predicted*</td>
<td>91 (21)</td>
<td>92 (16)</td>
<td>NS§</td>
<td>83.7 (21.5)</td>
</tr>
<tr>
<td>FEV₁/FVC (%)*</td>
<td>68 (7)</td>
<td>57 (10)</td>
<td>0.010</td>
<td>29.0 (7.8)</td>
</tr>
<tr>
<td>Eosinophil count¶</td>
<td>18.49 (2.5)</td>
<td>5.48 (1.8)</td>
<td>0.002</td>
<td>11.76 (2.6)</td>
</tr>
<tr>
<td>F₂NO ‡</td>
<td>37 (2.0)</td>
<td>28 (1.4)</td>
<td>NS§</td>
<td>6 (22%)</td>
</tr>
<tr>
<td>ACQ*</td>
<td>1.9 (1.0)</td>
<td>0.6 (0.6)</td>
<td>&lt;0.001</td>
<td>1.4 (1.1)</td>
</tr>
<tr>
<td>PD₃₂HS‡</td>
<td>4.4 (2.3)</td>
<td>2.9 (3.0)</td>
<td>NS§</td>
<td>3.6 (2.6)</td>
</tr>
</tbody>
</table>

* Arithmetic mean/standard deviation
† Number/percentage of group
‡ Geometric mean/standard deviation
§ Not significant
∥ Not applicable
¶ Geometric mean/standard deviation of offset values
** No data available
1.3.3 Comparison of respiratory data during OCS course

Comparison of respiratory variables before and after the OCS course are shown in Tables 7a and 7b. Changes in respiratory variables following the OCS course are shown in figure 7.

The three criteria used to define steroid responsiveness (ACQ, FEV\(_1\), and PC\(_{20}\)AMP) all improved significantly for the asthmatic group following the course of oral prednisone. Mean ACQ was reduced from 1.4 to 0.8 (p=0.004), mean FEV\(_1\) rose from 2.3 to 2.5 (p=0.013) and PC\(_{20}\)AMP rose from 16.6 to 79 (p<0.001). The increase in PC\(_{20}\)AMP corresponded to a mean increase of 2.25 doubling doses.

FEV\(_1\) as a percentage of predicted value (p=0.007), FVC (p=0.008), FVC as a percentage of predicted value (p=0.008), and F\(_E\)NO (p<0.001) all improved significantly. No difference was found in FEV\(_1\)/FVC.

**Table 7. Comparison of variables before and after OCS course**

*Table 7a. Comparison of study endpoints for all asthmatics before and after OCS course*

<table>
<thead>
<tr>
<th></th>
<th>Pre-steroid</th>
<th>Post-steroid</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACQ*</td>
<td>1.4 (1.1)</td>
<td>0.8 (0.6)</td>
<td>0.004</td>
</tr>
<tr>
<td>FEV(_1)*</td>
<td>2.3 (0.8)</td>
<td>2.5 (0.8)</td>
<td>0.013</td>
</tr>
<tr>
<td>PC(_{20})AMP‡</td>
<td>16.6 (8.7)</td>
<td>79 (6.6)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Table 7b. Comparison of other respiratory variables for all asthmatics before and after OCS course*

<table>
<thead>
<tr>
<th></th>
<th>Pre-steroid</th>
<th>Post-steroid</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV(_1)% predicted*</td>
<td>71 (16)</td>
<td>76 (17)</td>
<td>0.007</td>
</tr>
<tr>
<td>FVC*</td>
<td>3.7 (1.1)</td>
<td>3.9 (1.1)</td>
<td>0.008</td>
</tr>
<tr>
<td>FVC % predicted*</td>
<td>91 (19)</td>
<td>97 (17)</td>
<td>0.008</td>
</tr>
<tr>
<td>FEV(_1)/FVC*</td>
<td>63 (10)</td>
<td>64 (10)</td>
<td>NS</td>
</tr>
<tr>
<td>F(_E)NO ‡</td>
<td>43 (1.9)</td>
<td>27 (1.6)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Arithmetic mean/standard deviation
† Number/percentage of group
‡ Geometric mean/standard deviation
§ Not significant
|| Not applicable
¶ Geometric mean/standard deviation of offset values
** No data available
Figure 7. Changes in respiratory variables during steroid trial.
2 Group discrimination by electronic nose

2.0 Overview and repeatability

Healthy controls provided a single breath sample at their initial visit. Asthmatics provided a single breath sample prior to the OCS course and a single breath sample following it. Each breath sample was measured twice, with one measurement immediately following the other. Immediately prior to both measurements, an initial ‘fake’ measurement was performed in order to compensate for the ‘first-sniff effect’ (35) which specifies that data collected immediately after turning the nose on may be erroneous.

Because of the ‘first-sniff’ effect, it was not assumed that the two ‘real’ measurements would give identical results. The effect implies the presence of differences in sensor response between the ‘fake’ measurement and the first ‘real’ measurement (although the sensor is responding to an identical sample). This suggests that sensors may also respond differently to the same sample in the first and second ‘real’ measurements. For this reason, ‘first’ measurements were generally only compared with other ‘first’ measurements, and ‘second’ measurements with other ‘second’ measurements.

Both first- and second- analysis data was used in the comparison of samples from asthmatics and healthy controls and in the comparison of steroid responsive and non-steroid responsive asthmatics. Otherwise only first analysis data was used. See discussion section for details.

2.1 Discrimination of asthmatics and healthy controls

2.1.1 Separation of groups

2.1.1.1 Comparison of principal components

Comparisons of principal components between healthy controls and asthmatics are shown in Table 8a and Table 8b.

See methods section 2.2.3.1 for details on comparison of principal components.

Electronic nose readings were compared between healthy controls and asthmatics before and after steroid using principal component analysis on two data sets. One set comprised the samples from healthy controls and the samples from asthmatics prior to the OCS course (pre-
OCS). The second set was generated from samples from healthy controls and the samples from asthmatics following the OCS course (post-OCS). Analysis was conducted separately on data from first and second measurements, so for each data set two groups of principal components were extracted.

In each case, 47 samples were used to compute the principal components. Principal components accounting for at least 1.5% of the total variance were compared between groups.

In the comparison of healthy controls to pre-OCS asthmatics, three principal components from first analysis data (PC2, PC4, PC6, \( p=0.0015, 0.000012, 0.0015 \), adjusted \( p=0.0090, 0.000060, 0.0090 \) respectively) and one principal component from second analysis data (PC2, \( p=0.00041 \), adjusted \( p=0.00246 \)) were found to be significantly different between groups.

In the comparison of healthy controls to post-OCS asthmatics, one principal component from first analysis data (PC6, \( p=0.0016 \), adjusted \( p=0.0096 \)) and one principal component from second analysis data (PC4, \( p=0.0070 \), adjusted \( p=0.042 \)) were found to be significantly different between groups. No principal components from second analysis data were significantly different between groups.

A comparison between the first three principal components of first analysis data from pre-OCS asthmatics and controls is shown in figure 8. The upper two figures show pairwise comparisons between components and the lower figure shows a three-dimensional comparison, with a two-dimensional projection for reference.
Figure 8. Comparison of first three principal component scores (first analysis) between healthy controls and asthmatics
Table 8. Comparison of EN data between healthy controls and asthmatics

### Table 8a. Differences in individual principal components between asthmatics (pre-steroid) and healthy controls. Significant differences in bold.

<table>
<thead>
<tr>
<th>Principal component for 'first' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>52.98</td>
<td>52.98</td>
<td>0.40</td>
</tr>
<tr>
<td>PC2</td>
<td>20.16</td>
<td>73.14</td>
<td><strong>0.0015</strong></td>
</tr>
<tr>
<td>PC3</td>
<td>7.78</td>
<td>80.92</td>
<td>0.11</td>
</tr>
<tr>
<td>PC4</td>
<td>3.02</td>
<td><strong>83.94</strong></td>
<td><strong>0.000012</strong></td>
</tr>
<tr>
<td>PC5</td>
<td>2.62</td>
<td>86.56</td>
<td>0.28</td>
</tr>
<tr>
<td>PC6</td>
<td>1.62</td>
<td><strong>88.18</strong></td>
<td><strong>0.0015</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Principal component for 'second' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>53.48</td>
<td>53.48</td>
<td>0.22</td>
</tr>
<tr>
<td>PC2</td>
<td><strong>17.66</strong></td>
<td>71.14</td>
<td><strong>0.00041</strong></td>
</tr>
<tr>
<td>PC3</td>
<td>7.83</td>
<td>78.97</td>
<td>0.72</td>
</tr>
<tr>
<td>PC4‡</td>
<td>4.42</td>
<td>83.39</td>
<td>0.0091</td>
</tr>
<tr>
<td>PC5‡</td>
<td>2.26</td>
<td>85.65</td>
<td>0.90</td>
</tr>
<tr>
<td>PC6‡</td>
<td>1.79</td>
<td>87.44</td>
<td>0.028</td>
</tr>
</tbody>
</table>

### Table 8b. Differences in individual principal components between asthmatics (post-OCS) and healthy controls. Significant differences in bold.

<table>
<thead>
<tr>
<th>Principal component for 'first' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1‡</td>
<td>54.00</td>
<td>54.00</td>
<td>0.034</td>
</tr>
<tr>
<td>PC2‡</td>
<td><strong>19.64</strong></td>
<td>73.64</td>
<td>0.031</td>
</tr>
<tr>
<td>PC3</td>
<td>5.84</td>
<td>79.48</td>
<td>0.19</td>
</tr>
<tr>
<td>PC4‡</td>
<td>3.96</td>
<td>83.44</td>
<td>0.017</td>
</tr>
<tr>
<td>PC5‡</td>
<td>2.29</td>
<td>85.73</td>
<td>0.035</td>
</tr>
<tr>
<td>PC6‡</td>
<td><strong>2.27</strong></td>
<td><strong>88.00</strong></td>
<td><strong>0.0016</strong></td>
</tr>
<tr>
<td>PC7</td>
<td>1.90</td>
<td>89.90</td>
<td>0.84</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Principal component for 'second' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1‡</td>
<td>56.80</td>
<td>56.80</td>
<td>0.023</td>
</tr>
<tr>
<td>PC2‡</td>
<td><strong>18.11</strong></td>
<td>74.91</td>
<td>0.030</td>
</tr>
<tr>
<td>PC3</td>
<td>4.43</td>
<td>79.34</td>
<td>0.0093</td>
</tr>
<tr>
<td>PC4‡</td>
<td><strong>3.62</strong></td>
<td><strong>82.96</strong></td>
<td><strong>0.0070</strong></td>
</tr>
<tr>
<td>PC5</td>
<td>2.76</td>
<td>85.72</td>
<td>0.19</td>
</tr>
<tr>
<td>PC6</td>
<td>1.93</td>
<td>87.64</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* A Bonferroni correction is used due to multiple testing. A p value of less than 0.05/6 (=0.0083) is considered significant.
† A p value of less than 0.05/7 (=0.0071) is considered significant.
‡ Not significant due to multiple testing.
2.1.1.2 Mahalanobis distance

Mahalanobis distances between healthy controls and asthmatics are shown in Table 8c and Table 8d.

See methods sections 2.2.3.1-2.2.3.3 for details on calculation and interpretation of M-distance.

Mahalanobis distances were calculated between the healthy control and pre- and post-OCS asthmatics. Separate distances were calculated in the sensor spaces defined by the first and second breath analyses. The minimum significant M-distance between groups depends on the sizes of the groups.

The covariance matrix for the sensor readings was estimated in each distance calculation using a weighted mean of sample covariances from the two groups. Significances were calculated under the null hypothesis that groups were identically distributed in the sensor space.

A sample Mahalanobis distance of 4.66 was found between from healthy control and pre-OCS asthmatic group using first analysis data. The magnitude of the M-distance indicated significant separation between the asthmatic and healthy control groups (p=0.031). A distance of 4.16 was found between second analysis data from the same groups. The separation was not significant using a 95% confidence interval (p=0.065).

A distance of 3.80 was found between healthy controls and post-OCS asthmatics using first analysis data. This separation was not significant (p=0.16). A distance of 3.50 was found using second analysis data, which was also not significant (p=0.15).

\textit{Table 8c. Mahalanobis distances between healthy control and pre-OCS asthmatic groups. Significant distances in bold.}

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values from first analysis</td>
<td>4.66</td>
<td>0.031</td>
</tr>
<tr>
<td>Sensor values from second analysis</td>
<td>4.28</td>
<td>0.065</td>
</tr>
</tbody>
</table>
Table 8d. Mahalanobis distances between healthy control and post-OCS asthmatic groups. Significant distances in bold.

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values from first analysis</td>
<td>3.80</td>
<td>0.16</td>
</tr>
<tr>
<td>Sensor values from second analysis</td>
<td>3.53</td>
<td>0.15</td>
</tr>
</tbody>
</table>

2.1.2 Between-group discrimination

2.1.2.1 Linear discriminant analysis

Results of linear discriminant analysis between healthy controls and asthmatics are shown in Table 8e and Table 8f.

An example of a linear discriminant function on the first two principal components of samples from healthy controls and asthmatics is shown in figure 9.

See Methods sections 2.3.2.1, 2.3.2.2, and 2.3.3.1 for details on calculation and interpretation of linear discriminants.

Linear discriminant analysis was used to produce discriminating functions between samples from controls and pre-OCS asthmatics and between controls and post-OCS asthmatics. For each comparison eight separate discriminant functions were calculated, using different input variables for each.

For each discriminant a Wilks’s lambda ($\Lambda$) value was computed. Using the $\Lambda$ value, a significance value ($p$) was computed under the null hypothesis that discriminant values were not correlated with group membership.

The percentage of cases correctly grouped into classes by the discriminant ($%c$) was also calculated, as well as the cross-validation value (CVV). Sensitivity and specificity were calculated based on the cross-validation.

All functions calculated could discriminate significantly between controls and pre-OCS asthmatics except the function based on raw sensor outputs from the second analysis.
Discriminants calculated with a large number of input variables often displayed high levels of correct classification with low cross validation values (see Discussion). Similar cross-validation results were obtained when only the first two principal components were used as input variables instead of the first seven. The best CVV values were around 75%.

Again, all functions calculated could discriminate significantly between controls and post-OCS asthmatics except the function based on raw sensor outputs from the second analysis. In most cases, the value of $\Lambda$ was higher for discriminants between controls and post-OCS asthmatics than the corresponding discriminant between controls and pre-OCS asthmatics, indicating greater separation between controls and pre-OCS asthmatics. The best CVV values were around 70%.

Table 8c. Results of linear discriminant analysis for healthy control group against pre-OCS asthmatic group

| Input variables                  | n* | $\Lambda$ | p‡  | $\%c$§ | CVV|| | Sens. ¶ | Spec** |
|---------------------------------|----|-----------|-----|--------|------|--------|--------|
| Sensor outputs from first analysis | 32 | 0.185     | 0.028 | 93.6   | 74.5 | 70.0   | 77.8   |
| Sensor outputs from second analysis | 32 | 0.376     | 0.65  | 85.1   | 55.3 | 45.0   | 63.0   |
| PCs from first analysis         | 2  | 0.792     | 0.0059| 74.5   | 74.5 | 75.0   | 74.1   |
| PCs from first analysis         | 6  | 0.539     | 0.00023| 83.0  | 74.5 | 65.0   | 81.5   |
| PCs from second analysis        | 2  | 0.736     | 0.0012| 78.7   | 76.6 | 80.0   | 74.1   |
| PCs from second analysis        | 6  | 0.690     | 0.016 | 78.7   | 72.3 | 70.0   | 74.1   |
| Combined PCs                    | 4  | 0.687     | 0.0029| 78.7   | 74.5 | 75.0   | 74.1   |
| Combined PCs                    | 12 | 0.501     | 0.0079| 87.2   | 70.2 | 60.0   | 77.8   |

Table 8f: Results of linear discriminant analysis for healthy control group against post-OCS asthmatic group

| Input variables                  | n* | $\Lambda$ | p‡  | $\%c$§ | CVV|| | Sens. ¶ | Spec** |
|---------------------------------|----|-----------|-----|--------|------|--------|--------|
| Sensor outputs from first analysis | 32 | 0.221     | 0.081| 97.9   | 51.1 | 40.0   | 59.3   |
| Sensor outputs from second analysis | 32 | 0.259     | 0.18  | 93.6   | 57.4 | 55.0   | 59.3   |
| PCs from first analysis         | 2  | 0.836     | 0.019 | 72.3   | 70.2 | 70.0   | 70.4   |
| PCs from first analysis         | 6  | 0.517     | 0.0011| 89.4   | 74.5 | 70.0   | 77.8   |
| PCs from second analysis        | 2  | 0.828     | 0.016 | 70.2   | 70.2 | 70.0   | 66.7   |
| PCs from second analysis        | 6  | 0.695     | 0.018 | 72.3   | 66.0 | 65.0   | 66.7   |
| Combined PCs                    | 4  | 0.776     | 0.028 | 74.5   | 63.8 | 65.0   | 63.0   |
| Combined PCs                    | 12 | 0.505     | 0.0087| 85.1   | 63.8 | 55.0   | 70.4   |

* Number of variables used to construct discriminant
† Wilks’s Λ. A lower value indicates better separation, subject to number of variables
‡ Significance. Calculated from Λ and number of variables
§ Proportion of cases correctly classified by the linear discriminant
|| Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.
¶ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
** Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation

2.1.2.2 Multilayer perceptron

Results from multilayer perceptron (MLP) based discriminants between asthmatics and healthy controls are shown in Table 8g and Table 8h.

An example of an MLP-based discriminant on the first two principal components of data from pre-OCS asthmatics and controls is shown in figure 9.

See methods sections 2.3.2.1 and 2.3.3.2 for details on construction and interpretation of multilayer perceptrons.

Multilayer perceptrons (MLPs) with one hidden layer containing 5 neurons were used to produce discriminating functions between healthy controls and pre- and post- OCS asthmatics based on several sets of input variables. Eight such MLPs were generated, based on the same groups of input variables used to produce linear discriminants. Each neuron was initialised with a random set of weights and MLPs were trained by back-propagation using 50 iterations through the set of samples.

The error rates of each MLP in discriminating asthmatics and controls were estimated using cross-validation values. Sensitivities and specificities were similarly estimated.

When used to discriminate healthy controls and pre-OCS asthmatics, CVVs of 75-83% were found on most analyses. Similar CVVs were found for all sets of input variables except raw sensor values from the second analysis. The highest CVVs were found when raw sensor outputs from the first analysis (CVV=83.0%) and when the first six principal components from first analysis data (CVV=80.9%) were used as input variables.
When used to discriminate healthy controls from post-OCS asthmatics, CVVs of around 70% were found on most analyses. Similar prediction rates were found for all sets of input variables. Discrimination was generally poorer between controls and post-OCS asthmatics than between controls and pre-OCS asthmatics.

Table 8g. Multilayer perceptron based discrimination of healthy controls and pre-OCS asthmatics.

| Input variables                      | n* | CVV†   | Sens‡  | Spec|| |
|--------------------------------------|----|--------|--------|-------|
| Sensor outputs from first analysis   | 32 | 80.9   | 80.0   | 81.5  |
| Sensor outputs from second analysis  | 32 | 63.8   | 55.0   | 70.4  |
| PCs from first analysis              | 2  | 74.5   | 70.0   | 77.8  |
| PCs from first analysis              | 6  | 83.0   | 80.0   | 85.1  |
| PCs from second analysis             | 2  | 78.7   | 70.0   | 85.2  |
| PCs from second analysis             | 6  | 68.1   | 60.0   | 74.1  |
| Combined PCs                        | 4  | 76.6   | 70.0   | 81.5  |
| Combined PCs                        | 12 | 78.7   | 70.0   | 85.2  |

* Number of variables used to construct discriminant
† Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.
‡ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
|| Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation

Table 8h. Multilayer perceptron based discrimination of healthy controls and post-OCS asthmatics.

| Input variables                      | n* | CVV†   | Sens‡  | Spec|| |
|--------------------------------------|----|--------|--------|-------|
| Sensor outputs from first analysis   | 32 | 72.3   | 65.0   | 77.8  |
| Sensor outputs from second analysis  | 32 | 74.5   | 75.0   | 74.1  |
| PCs from first analysis              | 2  | 68.1   | 70.0   | 66.7  |
| PCs from first analysis              | 6  | 70.2   | 70.0   | 70.4  |
| PCs from second analysis             | 2  | 72.3   | 65.0   | 77.8  |
| PCs from second analysis             | 6  | 72.3   | 70.0   | 74.1  |
| Combined PCs                        | 4  | 66.0   | 65.0   | 66.7  |
| Combined PCs                        | 13 | 74.5   | 70.0   | 77.8  |
Figure 9. Examples of discriminants between healthy controls and asthmatics.

Note non-linear nature of MLP-based discriminant.
2.2 Discrimination of steroid responsive and non-steroid responsive asthmatics

2.2.1 Separation of groups

2.2.1.1 Comparison of principal components

Comparisons of principal components between steroid responsive and non-steroid responsive asthmatics are shown in Table 9a, Table 9b, and Table 9c.

See methods section 2.2.3.1 for details on comparison of principal components.

Electronic nose readings were compared between steroid responsive asthmatics and non-steroid responsive asthmatics using three sets of principal components. The first set was generated from electronic nose samples taken prior to the OCS course (pre-OCS). The second set was generated from electronic nose samples taken following the OCS course (post-OCS). The third set was generated from the differences in sensor readings between normalised post- and pre-OCS samples.

In each case, the principal components were generated from a set of 27 samples. Samples from healthy controls were not included in the data used to produce the principal component scores.

Analysis was conducted separately on data from first and second measurements. Principal components accounting for at least 1.5% of the total variance were compared between groups.

No principal components from any of the three analyses could discriminate significantly between the groups.
Table 9. Comparison of EN data between steroid responsive and non-steroid responsive asthmatics.

Table 9a. Differences in individual principal components between steroid responsive and non-steroid responsive asthmatics (pre-OCS).

<table>
<thead>
<tr>
<th>Principal component for 'first' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>54.72</td>
<td>54.72</td>
<td>0.11</td>
</tr>
<tr>
<td>PC2</td>
<td>19.08</td>
<td>73.81</td>
<td>0.72</td>
</tr>
<tr>
<td>PC3</td>
<td>7.83</td>
<td>81.63</td>
<td>0.29</td>
</tr>
<tr>
<td>PC4</td>
<td>3.20</td>
<td>84.83</td>
<td>0.084</td>
</tr>
<tr>
<td>PC5</td>
<td>2.62</td>
<td>87.46</td>
<td>0.53</td>
</tr>
<tr>
<td>PC6</td>
<td>1.98</td>
<td>89.43</td>
<td>0.39</td>
</tr>
<tr>
<td>PC7</td>
<td>1.81</td>
<td>91.24</td>
<td>0.70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Principal component for 'second' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>57.14</td>
<td>57.14</td>
<td>0.11</td>
</tr>
<tr>
<td>PC2</td>
<td>13.67</td>
<td>70.81</td>
<td>0.93</td>
</tr>
<tr>
<td>PC3</td>
<td>10.38</td>
<td>81.19</td>
<td>0.50</td>
</tr>
<tr>
<td>PC4</td>
<td>5.02</td>
<td>86.21</td>
<td>0.16</td>
</tr>
<tr>
<td>PC5</td>
<td>2.48</td>
<td>88.69</td>
<td>0.23</td>
</tr>
<tr>
<td>PC6</td>
<td>1.91</td>
<td>90.60</td>
<td>0.26</td>
</tr>
<tr>
<td>PC7</td>
<td>1.61</td>
<td>92.21</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 9b. Differences in individual principal components between steroid responsive and non-steroid responsive asthmatics (post-OCS)

<table>
<thead>
<tr>
<th>Principal component for 'first' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>54.48</td>
<td>54.48</td>
<td>0.51</td>
</tr>
<tr>
<td>PC2</td>
<td>19.72</td>
<td>74.20</td>
<td>0.81</td>
</tr>
<tr>
<td>PC3</td>
<td>5.42</td>
<td>79.62</td>
<td>0.93</td>
</tr>
<tr>
<td>PC4</td>
<td>3.83</td>
<td>83.45</td>
<td>0.80</td>
</tr>
<tr>
<td>PC5</td>
<td>3.05</td>
<td>86.51</td>
<td>0.39</td>
</tr>
<tr>
<td>PC6</td>
<td>2.77</td>
<td>89.27</td>
<td>0.12</td>
</tr>
<tr>
<td>PC7</td>
<td>1.77</td>
<td>91.04</td>
<td>0.74</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Principal component for 'second' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>59.13</td>
<td>59.13</td>
<td>0.48</td>
</tr>
<tr>
<td>PC2</td>
<td>17.31</td>
<td>76.44</td>
<td>0.70</td>
</tr>
<tr>
<td>PC3</td>
<td>5.53</td>
<td>81.96</td>
<td>0.59</td>
</tr>
<tr>
<td>PC4</td>
<td>3.23</td>
<td>85.20</td>
<td>0.94</td>
</tr>
<tr>
<td>PC5</td>
<td>2.68</td>
<td>87.88</td>
<td>0.45</td>
</tr>
<tr>
<td>PC6</td>
<td>2.16</td>
<td>90.04</td>
<td>0.95</td>
</tr>
</tbody>
</table>
Table 9c. Differences in individual principal components between steroid responsive and non-steroid responsive asthmatics (difference pre/post OCS).

<table>
<thead>
<tr>
<th>Principal component for 'first' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>41.54</td>
<td>41.54</td>
<td>0.27</td>
</tr>
<tr>
<td>PC2</td>
<td>15.49</td>
<td>57.03</td>
<td>0.84</td>
</tr>
<tr>
<td>PC3</td>
<td>9.92</td>
<td>66.95</td>
<td>0.27</td>
</tr>
<tr>
<td>PC4</td>
<td>5.68</td>
<td>72.63</td>
<td>0.68</td>
</tr>
<tr>
<td>PC5</td>
<td>4.21</td>
<td>76.84</td>
<td>0.56</td>
</tr>
<tr>
<td>PC6</td>
<td>3.77</td>
<td>80.61</td>
<td>0.64</td>
</tr>
<tr>
<td>PC7</td>
<td>3.44</td>
<td>84.05</td>
<td>0.032</td>
</tr>
<tr>
<td>PC8</td>
<td>2.62</td>
<td>86.67</td>
<td>0.32</td>
</tr>
<tr>
<td>PC9</td>
<td>2.42</td>
<td>89.09</td>
<td>0.17</td>
</tr>
<tr>
<td>PC10</td>
<td>1.93</td>
<td>91.02</td>
<td>0.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Principal component for 'second' analysis data</th>
<th>Percentage of total variance</th>
<th>Cumulative percentage of total variance</th>
<th>P value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>35.64</td>
<td>35.64</td>
<td>0.15</td>
</tr>
<tr>
<td>PC2</td>
<td>14.08</td>
<td>49.72</td>
<td>0.62</td>
</tr>
<tr>
<td>PC3</td>
<td>10.47</td>
<td>60.18</td>
<td>0.79</td>
</tr>
<tr>
<td>PC4</td>
<td>8.61</td>
<td>68.79</td>
<td>0.38</td>
</tr>
<tr>
<td>PC5</td>
<td>6.26</td>
<td>75.05</td>
<td>0.96</td>
</tr>
<tr>
<td>PC6</td>
<td>4.02</td>
<td>79.07</td>
<td>0.32</td>
</tr>
<tr>
<td>PC7</td>
<td>3.56</td>
<td>82.64</td>
<td>0.18</td>
</tr>
<tr>
<td>PC8</td>
<td>3.26</td>
<td>85.90</td>
<td>0.54</td>
</tr>
<tr>
<td>PC9</td>
<td>2.45</td>
<td>88.35</td>
<td>0.83</td>
</tr>
<tr>
<td>PC10</td>
<td>1.83</td>
<td>90.18</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* A Bonferroni correction is used due to multiple testing. A p value of less than 0.05/6 (=0.0083) is considered significant.
† A p value of less than 0.05/7 (=0.0071) is considered significant.
‡ A p value of less than 0.05/10 (=0.0050) is considered significant.

2.2.1.2 Mahalanobis distance

Mahalanobis distances between steroid responsive and non-steroid responsive asthmatics are shown in Table 9d, Table 9e, and Table 9f.

See methods sections 2.2.3.1- 2.2.3.3 for details on calculation and interpretation of M-distance.

Mahalanobis distances were calculated between samples from steroid responsive and non-
steroid responsive asthmatics from before and after the OCS course. Separate distances were calculated in the sensor spaces defined by the first and second breath analyses.

Because the total number of samples was less than 32 (the number of sensors), sample covariance matrices were singular, and the distribution of M-distances could not be approximated by an F distribution. Covariance matrices were estimated using a technique described by Schafer and Strimmer (96) and p values were estimated using a bootstrap technique. 20,000 random groupings were used to approximate the p value.

All M-distances were less than 2. No differences between groups were significant.

### Table 9d. Mahalanobis distances between steroid responsive and non-steroid responsive asthmatic groups (pre-OCS).

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values from first analysis</td>
<td>1.66</td>
<td>0.22</td>
</tr>
<tr>
<td>Sensor values from second analysis</td>
<td>1.60</td>
<td>0.28</td>
</tr>
</tbody>
</table>

### Table 9e. Mahalanobis distances between steroid responsive and non-steroid responsive asthmatic groups (post-OCS).

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values from first analysis</td>
<td>1.49</td>
<td>0.77</td>
</tr>
<tr>
<td>Sensor values from second analysis</td>
<td>1.48</td>
<td>0.72</td>
</tr>
</tbody>
</table>

### Table 9f. Mahalanobis distances between steroid responsive and non-steroid responsive asthmatic groups (difference pre/post-OCS).

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values from first analysis</td>
<td>1.72</td>
<td>0.20</td>
</tr>
<tr>
<td>Sensor values from second analysis</td>
<td>1.61</td>
<td>0.56</td>
</tr>
</tbody>
</table>

### 2.2.2 Between-group discrimination

#### 2.2.2.1 Linear discriminant analysis

Results of linear discriminant analysis between steroid responsive and non-steroid responsive
asthmatics are shown in Table 9f, Table 9g, and Table 9h.

See methods sections 2.3.2.1, 2.3.2.2, and 2.3.3.1 for details on calculation and interpretation of linear discriminants.

Linear discriminant analysis was used to produce discriminating functions between samples between steroid responsive and non-steroid responsive asthmatics before and after the OCS course. For each comparison six separate discriminants were calculated, using different input variables for each. No more than seven principal components were used as input so as to minimise overtraining effects. Raw sensor outputs were not used as input in this case as less than 32 samples were available for each analysis.

No function on any set of variables could significantly discriminate between steroid responsive and non-steroid responsive asthmatics. Cross-validation values were generally around 50%.

**Table 9g. Results of linear discriminant analysis between steroid responsive and non-steroid responsive asthmatics pre-OCS**

| Input variables          | n* | A†  | p‡ | %c§ | CVV|| Sens. ¶ | Spec** |
|--------------------------|----|-----|----|-----|-----|---------|--------|
| PCs from first analysis  | 2  | 0.904 | 0.30 | 66.7 | 63.0 | 72.7 | 56.3 |
| PCs from first analysis  | 6  | 0.751 | 0.39 | 70.4 | 40.7 | 45.4 | 37.5 |
| PCs from second analysis | 2  | 0.902 | 0.29 | 66.7 | 51.9 | 63.6 | 43.8 |
| PCs from second analysis | 6  | 0.775 | 0.47 | 59.3 | 51.9 | 45.4 | 56.3 |
| Combined PCs             | 4  | 0.890 | 0.61 | 66.7 | 51.9 | 63.6 | 43.8 |
| Combined PCs             | 12 | 0.977 | 1.00 | 55.6 | 63.0 | 54.6 | 68.8 |

**Table 9h. Results of linear discriminant analysis between steroid responsive and non-steroid responsive asthmatics post-OCS**

| Input variables          | n* | A†  | p‡ | %c§ | CVV|| Sens. ¶ | Spec** |
|--------------------------|----|-----|----|-----|-----|---------|--------|
| PCs from first analysis  | 2  | 0.981 | 0.79 | 51.9 | 37.0 | 36.4 | 37.5 |
| PCs from first analysis  | 6  | 0.705 | 0.26 | 77.8 | 55.6 | 45.4 | 62.5 |
| PCs from second analysis | 2  | 0.976 | 0.75 | 59.3 | 37.0 | 36.4 | 37.5 |
| PCs from second analysis | 6  | 0.924 | 0.94 | 66.7 | 25.9 | 27.3 | 25.0 |
| Combined PCs             | 4  | 0.948 | 0.87 | 55.6 | 29.6 | 18.2 | 37.5 |
| Combined PCs             | 12 | 0.924 | 1.00 | 66.7 | 40.7 | 18.2 | 56.3 |

**Table 9i. Results of linear discriminant analysis between steroid responsive and non-steroid responsive asthmatics (difference pre/post OCS).**
2.2.2.2 Multilayer perceptron

Results from multilayer perceptron (MLP) based discriminants between steroid responsive and non-steroid responsive asthmatics are shown in Table 9j, Table 9k, and Table 9l.

See methods sections 2.3.2.1 and 2.3.3.2 for details on construction and interpretation of multilayer perceptrons.

A multilayer perceptron (MLP) with one hidden layer containing 5 neurons was implemented and used to classify steroid responsive and non-steroid responsive asthmatics before and after the OCS course based on several sets of input variables. MLPs were trained on 50 iterations through the 27 samples. MLPs were initiated and tested as before.

Cross-validation values were generally around 40-60%. A perceptron using seven principal components from first analysis pre-OCS data as input had a CVV of 70.4%

Table 9j. Multilayer perceptron analysis of electronic nose data to separate steroid responsive and non-steroid responsive asthmatics pre-OCS.
Table 9k. Multilayer perceptron analysis of electronic nose data to separate post-OCS asthmatics with estimated sensitivities and specificities.

| Input variables                                      | n* | CVV†  | Sens‡ | Spec|| |
|-------------------------------------------------------|----|-------|-------|------|-----|
| Sensor outputs from first analysis                    | 32 | 33.3  | 9.1   | 50.0 |
| Sensor outputs from second analysis                   | 32 | 40.1  | 18.2  | 56.3 |
| PCs from first analysis                               | 2  | 59.3  | 36.6  | 75.0 |
| PCs from second analysis                              | 7  | 44.4  | 18.2  | 62.5 |
| PCs from first analysis                               | 2  | 40.1  | 18.2  | 56.3 |
| PCs from second analysis                              | 7  | 40.1  | 9.1   | 62.5 |
| Combined PCs                                          | 4  | 40.1  | 18.2  | 56.3 |
| Combined PCs                                          | 14 | 44.4  | 18.2  | 62.5 |

Table 9l. Multilayer perceptron analysis of differences in sensor values to separate steroid responsive and non-steroid responsive asthmatics.

| Input variables                                      | n* | CVV†  | Sens‡ | Spec|| |
|-------------------------------------------------------|----|-------|-------|------|-----|
| Sensor outputs from first analysis                    | 32 | 44.4  | 45.5  | 43.8 |
| Sensor outputs from second analysis                   | 32 | 40.7  | 36.4  | 43.8 |
| PCs from first analysis                               | 2  | 59.3  | 27.3  | 81.2 |
| PCs from second analysis                              | 7  | 37.0  | 9.1   | 56.2 |
| PCs from first analysis                               | 2  | 59.3  | 36.4  | 75.0 |
| PCs from second analysis                              | 7  | 51.9  | 36.4  | 62.5 |
| Combined PCs                                          | 4  | 59.3  | 45.5  | 68.8 |
| Combined PCs                                          | 14 | 48.1  | 45.5  | 50.0 |

* Number of variables used to construct discriminant
† Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.
‡ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
|| Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation

2.3 Relationship between sputum eosinophilia and electronic nose breathprint

2.3.1 Separation of low-eosinophil and high-eosinophil groups

Principal component analysis between low- and high-eosinophil asthma groups is shown in Table 10a. M-distance between low- and high-eosinophil asthma groups is shown in Table
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10b. Linear discriminant analysis between low- and high- eosinophil asthma groups is shown in Table 10c. Multilayer perceptron analysis between low- and high- eosinophil asthma groups is shown in Table 10d.

See methods sections 2.2.3.1 for details on comparison of principal components, sections 2.2.3.1-2.2.3.3 for details on calculation and interpretation of M-distance, sections 2.3.2.1, 2.3.2.2, and 2.3.3.1 for details on calculation and interpretation of linear discriminants, and sections 2.3.2.1 and 2.3.3.2 for details on construction and interpretation of multilayer perceptrons.

Electronic nose readings were compared between eosinophilic asthmatics and non-eosinophilic asthmatics using electronic nose samples taken prior to the OCS course (pre-OCS). Samples from healthy controls were not included in the data used to produce the principal component scores. Seven asthmatics had a differential eosinophil count of less than 3%. 19 had a count of greater than 3%.

M-distances were compared using shrinkage estimators of covariance matrices (96). Principal components were extracted and linear discriminant analysis was conducted using seven principal components as input. A multilayer perceptron with the same inputs was trained to discriminate the groups. Only the data from the first breath sample was used. In each case, the principal components were generated from a set of 26 samples.

One principal component (PC4) extracted from post-OCS samples could significantly discriminate between groups (p=0.00047, adjusted p=0.0033).

The M-distance between differences in sensor values was 2.00, which indicated significant separation between the groups (p=0.020)

The linear discriminant calculated based on the first six principal components had a significance of 0.051, indicating not-quite significant separation.

A multilayer perceptron based algorithm had a CVV of 76.9 in discriminating the groups.
Table 10. Comparison of electronic nose breathprints from eosinophilic and non-eosinophilic asthmatics

Table 10a. Significant differences in principal components between eosinophilic and non-eosinophilic asthma groups

<table>
<thead>
<tr>
<th>Raw data</th>
<th>Principal component</th>
<th>Percentage of total variance</th>
<th>P value*</th>
<th>Adjusted p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-OCS sensor values</td>
<td>PC4</td>
<td>3.18</td>
<td>0.00047</td>
<td>0.0033</td>
</tr>
</tbody>
</table>

* A p value of 0.05/7 (=0.0071) indicates significance in this case

Table 10b. M-distances between eosinophilic and non-eosinophilic asthma groups

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values (pre-OCS) from first analysis</td>
<td>2.00</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Table 10c. Linear discriminant analysis between eosinophilic and non-eosinophilic asthma groups

| Input variables                     | n* | A†  | p‡  | %c§ | CVV|| | Sens. ¶ | Spec** |
|-------------------------------------|----|-----|-----|-----|------|--------|--------|
| PCs from first analysis (pre-OCS)   | 6  | 0.551 | 0.052 | 76.9 | 65.4 | 71.4   | 63.2   |

* Number of variables used to construct discriminant
† Wilks’sΛ. A lower value indicates better separation, subject to number of variables
‡ Significance. Calculated from Λ and number of variables
§ Proportion of cases correctly classified by the linear discriminant
|| Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.
¶ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
** Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation

Table 10d. Multilayer perceptron analysis between eosinophilic and non-eosinophilic asthma groups

| Input variables                     | n* | CVV‡ | Sens¶ | Spec|| |
|-------------------------------------|----|------|--------|------|-----|
| PCs from first analysis (pre-OCS)   | 6  | 76.9 | 71.4   | 78.9 |
2.3.2 Correlation between principal components and sputum eosinophilia

Correlations between principal components and eosinophil counts are shown in Table 11a.

Based on data reported by Fens et al. (37) a comparison of principal component scores and sputum eosinophil counts was made using Spearman’s correlation coefficient $\rho$. Only asthmatic participants were included in the analysis. PCs were extracted from pre-OCS data, post-OCS data, and differences. Neutrophil counts were also compared.

Allowing for multiple comparisons, one principal component from pre-OCS measurements was significantly correlated with eosinophil count (PC4, $\rho=0.615$, $p=0.00082$, adjusted $p=0.0057$).

No significant correlations were found between any principal components and differential neutrophil counts.

A graph of the relationship between relevant principal component and eosinophil counts is shown in figure 10.

Table 11. Correlations between principal components and eosinophil levels (pre-OCS). Significant correlations in bold

<table>
<thead>
<tr>
<th>Principal component from pre-OCS data</th>
<th>Percentage of total variance</th>
<th>Correlation ($\rho$)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>54.87</td>
<td>-0.197</td>
<td>0.33</td>
</tr>
<tr>
<td>PC2</td>
<td>19.15</td>
<td>-0.214</td>
<td>0.29</td>
</tr>
<tr>
<td>PC3</td>
<td>7.98</td>
<td>0.204</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>PC4</strong></td>
<td><strong>3.18</strong></td>
<td><strong>0.615</strong></td>
<td><strong>0.00082</strong></td>
</tr>
<tr>
<td>PC5</td>
<td>2.46</td>
<td>0.082</td>
<td>0.69</td>
</tr>
<tr>
<td>PC6</td>
<td>2.02</td>
<td>0.175</td>
<td>0.39</td>
</tr>
<tr>
<td>PC7</td>
<td>1.77</td>
<td>0.131</td>
<td>0.52</td>
</tr>
</tbody>
</table>

* A p value of 0.05/7 (=0.0071) indicates significance in this case
Figure 10. Graph of eosinophil scores and relevant principal component.
2.3.3 Prediction of steroid response constituents

2.3.3.1 Prediction of ACQ improvement

Principal component analysis between asthmatics that showed ACQ improvement (ACQ-responsive) and asthmatics that did not show ACQ improvement (ACQ-unresponsive) is shown in Table 12a. M-distance between ACQ-responsive and ACQ-unresponsive groups is shown in Table 12b. Linear discriminant analysis between ACQ-responsive and ACQ-unresponsive groups is shown in Table 12c. Multilayer perceptron analysis between ACQ-responsive and ACQ-unresponsive asthma groups is shown in Table 12d.

See methods sections 2.2.3.1 for details on comparison of principal components, sections 2.2.3.1-2.2.3.3 for details on calculation and interpretation of M-distance, sections 2.3.2.1, 2.3.2.2, and 2.3.3.1 for details on calculation and interpretation of linear discriminants, and sections 2.3.2.1 and 2.3.3.2 for details on construction and interpretation of multilayer perceptrons. See methods section 1.1.4.1 for details on the ACQ questionnaire.

A comparison was performed between asthmatics showing improvement of at least 0.5 in ACQ score (ACQ-responsive) and asthmatics who did not show such an improvement (ACQ-unresponsive) following the OCS course. M-distances were compared using shrinkage estimators of covariance matrices (96). Principal components were extracted and linear discriminant analysis was conducted using seven principal components. A multilayer perceptron with the same inputs was trained to discriminate the groups. Only the data from the first breath sample was used.

No principal components generated from pre- or post- OCS sensor readings could significantly discriminate the groups (table not shown). One principal component generated from differences in sample readings showed significant separation (p=0.00041, adjusted p=0.0041) between the groups.

The M-distances between the groups did not indicate significant separation with 95% confidence, although the distance between differences in sensor values was close to significance (p=0.068).
A linear discriminant with difference in sensor values as input could significantly classify the groups (p=0.031). A cross-validation value of 74.1% was found. Discriminants based on pre- or post- OCS sensor values alone could not significantly classify groups.

Multilayer perceptron based discriminants with difference in sensor values as input were able to classify the groups with a CVV of 66.7%. Discriminants based on pre- or post- OCS sensor values alone were found to have CVVs of around 55%.

Table 12. Comparison of EN data from ACQ-responsive and ACQ-unresponsive asthmatics

Table 12a. Significant differences in principal components between ACQ-responsive and ACQ-unresponsive asthmatics

<table>
<thead>
<tr>
<th>Raw data</th>
<th>Principal component</th>
<th>Percentage of total variance</th>
<th>P value*</th>
<th>Adjusted p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-OCS sensor values*</td>
<td>(none)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-OCS sensor values*</td>
<td>(none)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difference in sensor values†</td>
<td>PC6</td>
<td>3.77</td>
<td>0.00041</td>
<td>0.0041</td>
</tr>
</tbody>
</table>

* A p value of 0.05/7 (=0.0071) indicates significance in this case
† A p value of 0.05/10 (=0.0050) indicates significance in this case

Table 12b. M-distances between ACQ-responsive and ACQ-unresponsive asthmatics

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values (pre-OCS) from first analysis</td>
<td>1.69</td>
<td>0.11</td>
</tr>
<tr>
<td>Sensor values (post-OCS) from first analysis</td>
<td>1.61</td>
<td>0.38</td>
</tr>
<tr>
<td>Difference in sensor values</td>
<td>1.77</td>
<td>0.068</td>
</tr>
</tbody>
</table>

Table 12c. Linear discriminant analysis between ACQ-responsive and ACQ-unresponsive asthmatics (significant results in bold)

| Input variables                | n* | A† | p‡ | %c§ | CVV|| Sens. ¶ | Spec** |
|--------------------------------|----|----|----|-----|------|--------|--------|
| PCs from first analysis (pre-OCS) | 6  | 0.708 | 0.27 | 74.1 | 51.9 | 53.3 | 50.0 |
| PCs from first analysis         | 6  | 0.810 | 0.59 | 74.1 | 51.9 | 46.7 | 58.3 |
Table 12d. Multilayer perceptron analysis between ACQ-responsive and ACQ-unresponsive asthmatics

| Input variables                        | n* | CVV† | Sens‡ | Spec|| |
|----------------------------------------|----|------|-------|------|
| PCs from first analysis (pre-OCS)      | 6  | 48.1 | 60.0  | 33.3 |
| PCs from first analysis (post-OCS)     | 6  | 48.1 | 46.7  | 50.0 |
| PCs from first analysis (difference)   | 6  | 66.7 | 73.3  | 58.3 |

* Number of variables used to construct discriminant  
† Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.  
‡ Specificity (%). Proportion of correctly classified healthy controls in cross-validation  
|| Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation

2.3.3.2 Prediction of FEV₁ improvement

Principal component analysis between asthmatics that showed FEV₁ improvement (FEV₁-responsive) and asthmatics that did not show FEV₁ improvement (FEV₁-unresponsive) is shown in Table 13a. M-distance between FEV₁-responsive and FEV₁-unresponsive groups is shown in Table 13b. Linear discriminant analysis between FEV₁-responsive and FEV₁-unresponsive groups is shown in Table 13c. Multilayer perceptron analysis between FEV₁-responsive and FEV₁-unresponsive asthma groups is shown in Table 13d.

See methods sections 2.2.3.1 for details on comparison of principal components, sections 2.2.3.1-2.2.3.3 for details on calculation and interpretation of M-distance, sections 2.3.2.1, 2.3.2.2, and 2.3.3.1 for details on calculation and interpretation of linear discriminants, and sections 2.3.2.1 and 2.3.3.2 for details on construction and interpretation of multilayer perceptrons. See methods section 1.1.2.1 for details on FEV₁.
A comparison was performed between asthmatics showing improvement of at least 15% improvement in FEV\textsubscript{1} (FEV-responsive) and asthmatics who did not show such an improvement (FEV-unresponsive) following the OCS course. Principal components were extracted and linear discriminant analysis was conducted using principal components as input. A multilayer perceptron with the same inputs was trained to discriminate the groups. Only the data from the first breath sample was used.

No principal components generated from pre- or post- OCS samples alone could significantly discriminate the groups. One principal component (PC6) based on differences in sensor readings could discriminate the groups significantly (p=0.0045, adjusted p=0.045).

The M-distances between the groups did not indicate significant separation at 95% confidence.

No linear discriminants were found which could significantly classify the groups at 95% confidence. A significance of 0.082 was found for the discriminant using differences in sensor values as inputs, with a cross-validation value of 70.4%.

An MLP-based discriminant with difference in sensor values as input could classify the groups with a CVV of 85.2%. A discriminant using pre-OCS samples had a CVV of 74.1%. A discriminant using post-OCS samples had a CVV of around 50%.

**Table 13. Comparison of EN data from FEV-responsive and FEV-unresponsive asthmatics**

<table>
<thead>
<tr>
<th>Raw data</th>
<th>Principal component</th>
<th>Percentage of total variance</th>
<th>P value*</th>
<th>Adjusted p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-OCS sensor values*</td>
<td>(none)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-OCS sensor values*</td>
<td>(none)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difference in sensor values†</td>
<td>PC6</td>
<td>3.77</td>
<td>0.0045</td>
<td>0.045</td>
</tr>
</tbody>
</table>

* A p value of 0.05/7 (=0.0071) indicates significance in this case.
† A p value of 0.05/10 (=0.0050) indicates significance in this case.
Table 13b. M-distances between FEV-responsive and FEV-unresponsive asthmatics.

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values (pre-OCS) from first analysis</td>
<td>1.76</td>
<td>0.68</td>
</tr>
<tr>
<td>Sensor values (post-OCS) from first analysis</td>
<td>1.73</td>
<td>0.43</td>
</tr>
<tr>
<td>Difference in sensor values</td>
<td>1.77</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Table 13c. Linear discriminant analysis between FEV-responsive and FEV-unresponsive asthmatics.

| Input variables                          | n* | A†  | p‡  | %c§ | CVV|| | Sens. ¶ | Spec** |
|------------------------------------------|----|-----|-----|-----|------|--------|--------|
| PCs from first analysis (pre-OCS)        | 6  | 0.693| 0.233| 74.1| 66.7 | 63.2   | 75.0   |
| PCs from first analysis (post-OCS)       | 6  | 0.805| 0.574| 74.1| 51.9 | 57.9   | 37.5   |
| PCs from first analysis (difference)     | 6  | 0.593| 0.074| 85.2| 70.4 | 79.0   | 50.0   |

* Number of variables used to construct discriminant
† Wilks’s Λ. A lower value indicates better separation, subject to number of variables
‡ Significance. Calculated from Λ and number of variables
§ Proportion of cases correctly classified by the linear discriminant
|| Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.
¶ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
** Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation

Table 13d. Multilayer perceptron analysis between FEV-responsive and FEV-unresponsive asthmatics.

| Input variables                          | n* | CVV† | Sens‡ | Spec|| |
|------------------------------------------|----|------|--------|------|
| PCs from first analysis (pre-OCS)        | 6  | 74.1 | 84.2   | 50.0 |
| PCs from first analysis (post-OCS)       | 6  | 51.9 | 68.4   | 12.5 |
| PCs from first analysis (difference)     | 6  | 85.2 | 94.7   | 62.5 |

* Number of variables used to construct discriminant
† Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.
‡ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
|| Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation
2.3.3.3 Prediction of PC$_{20}$AMP improvement

Principal component analysis between asthmatics that showed PC$_{20}$AMP improvement (AMP-responsive) and asthmatics that did not show PC$_{20}$AMP improvement (AMP-unresponsive) is shown in Table 14a. M-distance between AMP-responsive and AMP-unresponsive groups is shown in Table 14b. Linear discriminant analysis between AMP-responsive and AMP-unresponsive groups is shown in Table 14c. Multilayer perceptron analysis between AMP-responsive and AMP-unresponsive asthma groups is shown in Table 14d.

See methods sections 2.2.3.1 for details on comparison of principal components, sections 2.2.3.1-2.2.3.3 for details on calculation and interpretation of M-distance, sections 2.3.2.1, 2.3.2.2, and 2.3.3.1 for details on calculation and interpretation of linear discriminants, and sections 2.3.2.1 and 2.3.3.2 for details on construction and interpretation of multilayer perceptrons. See methods section 1.2.2.3 for details on PC$_{20}$AMP.

A comparison was performed between asthmatics showing improvement of at least 400% in PC$_{20}$AMP score (AMP-responsive) and asthmatics that did not show such an improvement (AMP-unresponsive) following the OCS course. Principal components were extracted and linear discriminant analysis was conducted using principal components as input. A multilayer perceptron with the same inputs was trained to discriminate the groups. Only the data from the first breath sample was used.

No principal components could significantly discriminate the groups.

The M-distances between the groups did not indicate significant separation at 95% confidence.

A linear discriminant using post-OCS samples as inputs could significantly discriminate groups (p=0.050) with a CVV of 72.7. Discriminants using pre-OCS samples or differences in sensor readings could not significantly discriminate the groups.

An MLP-based discriminant with post-OCS samples as input showed a CVV of 72.7. MLP-based discriminants based on pre-OCS samples or differences in sensor values showed CVVs
of 40.9% and 59.1% respectively

**Table 14. Comparison of EN data from AMP-responsive and AMP-unresponsive asthmatics**

**Table 14a. Significant differences in principal components between AMP-responsive and AMP-unresponsive asthmatics.**

<table>
<thead>
<tr>
<th>Raw data</th>
<th>Principal component</th>
<th>Percentage of total variance</th>
<th>P value*</th>
<th>Adjusted p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-OCS sensor values*</td>
<td>(none)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-OCS sensor values*</td>
<td>(none)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difference in sensor values†</td>
<td>(none)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A p value of 0.05/7 (=0.0071) indicates significance in this case
† A p value of 0.05/10 (=0.0050) indicates significance in this case

**Table 14b. M-distances between AMP-responsive and AMP-unresponsive asthmatics.**

<table>
<thead>
<tr>
<th>Variables used</th>
<th>M-distance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor values (pre-OCS) from first analysis</td>
<td>1.56</td>
<td>0.83</td>
</tr>
<tr>
<td>Sensor values (post-OCS) from first analysis</td>
<td>1.76</td>
<td>0.17</td>
</tr>
<tr>
<td>Difference in sensor values</td>
<td>1.68</td>
<td>0.56</td>
</tr>
</tbody>
</table>

**Table 14c. Linear discriminant analysis between AMP-responsive and AMP-unresponsive asthmatics (significant results in bold)**

| Input variables                                    | n* | A†  | p‡   | %c§  | CVV|| | Sens. ¶ | Spec** |
|----------------------------------------------------|----|-----|------|------|------|---------|--------|
| PCs from first analysis (pre-OCS)                  | 6  | 0.884 | 0.91 | 63.6 | 31.8 | 46.2    | 11.1   |
| PCs from first analysis (post-OCS)                 | 6  | 0.504 | 0.071| 86.4 | 72.7 | 61.5    | 88.9   |
| **PCs from first analysis (difference)**           | 6  | 0.697 | 0.41 | 68.2 | 54.5 | 53.8    | 55.6   |

* Number of variables used to construct discriminant
† Wilks’s A. A lower value indicates better separation, subject to number of variables
‡ Significance. Calculated from A and number of variables
§ Proportion of cases correctly classified by the linear discriminant
|| Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.
Specificity (%). Proportion of correctly classified healthy controls in cross-validation
Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation

Table 14d. Multilayer perceptron analysis between AMP-responsive and AMP-unresponsive asthmatics

| Input variables                      | n  | CVV† | Sens‡ | Spec|| |
|--------------------------------------|----|------|-------|------|
| PCs from first analysis (pre-OCS)    | 6  | 40.9 | 53.8  | 22.2 |
| PCs from first analysis (post-OCS)   | 6  | 72.7 | 84.6  | 55.6 |
| PCs from first analysis (difference) | 6  | 59.1 | 61.5  | 55.6 |

* Number of variables used to construct discriminant
† Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population.
‡ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
|| Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation

2.3.4 Comparison of predictors and markers of steroid response

2.3.4.1 Comparison of $\text{F}_\text{E}\text{NO}$, eosinophilia, and EN in predicting steroid response

Results of this comparison are shown in Table 15a, Table 15b, Table 15c, and Table 15d

An analysis was conducted on the relative abilities of $\text{F}_\text{E}\text{NO}$, sputum eosinophil count, and the electronic nose to predict steroid response, using similar techniques to Montuschi (7) in comparing diagnostic tests for asthma.

Optimised cutoff points have been published for predicting steroid response with pre-OCS $\text{F}_\text{E}\text{NO}$ (6). However, due to the non-existence of a predetermined criterion to measure steroid response by electronic nose there was no way to compare the predictive power of electronic nose readings to the predictive power of a predetermined cutoff on $\text{F}_\text{E}\text{NO}$ scores. Instead, the predictive power of $\text{F}_\text{E}\text{NO}$ was measured by producing a cross-validation value, in which the optimum cutoff for $\text{F}_\text{E}\text{NO}$ was repeatedly determined using results from all study samples but one, and tested on the remaining sample. For prediction by electronic nose a cross-validation value was calculated using an MLP-based discriminant with the first seven principal components as input.
Sensitivities (proportion of non-steroid responsive asthmatics correctly classified) and specificities (proportion of steroid responsive asthmatics correctly classified) were also approximated using cross-validation.

Eosinophil count (CVV=65.4%) performed best on prediction of composite steroid response (response according to one of the three criteria). F_{E}NO had a CVV of 59.3% and the electronic nose had a CVV of 51.9.

ACQ response was best predicted by eosinophil counts (CVV=76.9). F_{E}NO had a CVV of 59.3% and the electronic nose had a CVV of 48.1%.

FEV\textsubscript{1} response was best predicted by F_{E}NO (CVV=77.8%). Eosinophil counts had a CVV of 76.9% and the electronic nose had a CVV of 74.1%

AMP response was best predicted by eosinophil counts (CVV=76.9%). F_{E}NO had a CVV of 59.3% and the electronic nose had a CVV of 40.9%.

**Table 15. Comparison of F_{E}NO, eosinophilia, and the electronic nose in predicting steroid response**

*Table 15a. Comparison of pre-OCS F_{E}NO, eosinophilia, and electronic nose in predicting composite steroid response (one of ACQ improvement, FEV\textsubscript{1} improvement, or PC\textsubscript{20}AMP improvement).*

<table>
<thead>
<tr>
<th>Predictor</th>
<th>CVV†</th>
<th>Sens‡</th>
<th>Spec§</th>
</tr>
</thead>
<tbody>
<tr>
<td>F_{E}NO (pre-OCS)</td>
<td>59.3</td>
<td>45.4</td>
<td>68.8</td>
</tr>
<tr>
<td>Eosinophil count' (pre-OCS)</td>
<td>65.4</td>
<td>60.0</td>
<td>68.8</td>
</tr>
<tr>
<td>Electronic nose samples (pre-OCS)</td>
<td>51.9</td>
<td>18.2</td>
<td>75.0</td>
</tr>
</tbody>
</table>

*Table 15b. Comparison of pre-OCS F_{E}NO, eosinophilia, and electronic nose in predicting ACQ response (improvement of at least 0.5 in ACQ score).*

<table>
<thead>
<tr>
<th>Predictor</th>
<th>CVV†</th>
<th>Sens‡</th>
<th>Spec§</th>
</tr>
</thead>
<tbody>
<tr>
<td>F_{E}NO (pre-OCS)</td>
<td>59.3</td>
<td>73.3</td>
<td>41.7</td>
</tr>
<tr>
<td>Eosinophil count' (pre-OCS)</td>
<td>76.9</td>
<td>78.6</td>
<td>75.0</td>
</tr>
<tr>
<td>Electronic nose samples (pre-OCS)</td>
<td>48.1</td>
<td>60.0</td>
<td>33.3</td>
</tr>
</tbody>
</table>

*Table 15c. Comparison of pre-OCS F_{E}NO, eosinophilia, and electronic nose in predicting*
**FEV₁ response (improvement of at least 15% in FEV₁).**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>CVV†</th>
<th>Sens‡</th>
<th>Spec§</th>
</tr>
</thead>
<tbody>
<tr>
<td>FENO (pre-OCS)</td>
<td>77.8</td>
<td>89.5</td>
<td>50.0</td>
</tr>
<tr>
<td>Eosinophil count (pre-OCS)</td>
<td>76.9</td>
<td>88.9</td>
<td>50.0</td>
</tr>
<tr>
<td>Electronic nose samples (pre-OCS)</td>
<td>74.1</td>
<td>84.2</td>
<td>50.0</td>
</tr>
</tbody>
</table>

*Table 15d. Comparison of FENO, eosinophilia, and electronic nose in predicting PC_{20}AMP response (improvement of at least 400% in PC_{20}AMP).*

<table>
<thead>
<tr>
<th>Predictor</th>
<th>CVV†</th>
<th>Sens‡</th>
<th>Spec§</th>
</tr>
</thead>
<tbody>
<tr>
<td>FENO (pre-OCS)</td>
<td>59.3</td>
<td>73.3</td>
<td>41.7</td>
</tr>
<tr>
<td>Eosinophil count (pre-OCS)</td>
<td>76.9</td>
<td>78.6</td>
<td>75.0</td>
</tr>
<tr>
<td>Electronic nose samples (pre-OCS)</td>
<td>40.9</td>
<td>53.8</td>
<td>22.2</td>
</tr>
</tbody>
</table>

* Only 26 asthmatics had eosinophil counts, whereas 27 had valid breathprints and FENO
† Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population
‡ Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation
§ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
ǁ Only 22 asthmatics had PC_{20}AMP measurements

### 2.3.4.2 Comparison of FENO and EN in monitoring steroid response

Results of this comparison are shown in Table 16a, Table 16b, Table 16c, and Table 16d.

A comparison was made between the relative abilities of ratios of pre-and post- OCS (differential) FENO and differences in electronic nose scores to detect differences in pre- and post- OCS airway tests. The comparison was made analogously to the comparison above. Comparisons of differential predictors (difference in pre/post OCS samples) were made on all 27 asthmatics.

Differential FENO was a stronger predictor of composite steroid response (CVV=70.4%) than differential electronic nose samples (CVV=55.6%).

Differential electronic nose score was a stronger predictor of steroid response by ACQ (CVV=74.1%) than FENO (CVV=59.3%)
Table 16. Comparison of $F_{E}$NO and electronic nose readings in detecting steroid response

Table 16a. Comparison of differential values of $F_{E}$NO, and electronic nose in detecting composite steroid response.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>CVV†</th>
<th>Sens‡</th>
<th>Spec§</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{E}$NO (post-OCS / pre-OCS)*</td>
<td>70.4</td>
<td>87.5</td>
<td>45.4</td>
</tr>
<tr>
<td>Electronic nose samples (post-OCS-pre-OCS)</td>
<td>51.9</td>
<td>18.2</td>
<td>75.0</td>
</tr>
</tbody>
</table>

Table 16b. Comparison of differential values of $F_{E}$NO, and electronic nose in detecting steroid response measured by ACQ.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>CVV†</th>
<th>Sens‡</th>
<th>Spec§</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{E}$NO (post-OCS / pre-OCS)*</td>
<td>59.3</td>
<td>83.3</td>
<td>40.0</td>
</tr>
<tr>
<td>Electronic nose samples (post-OCS-pre-OCS)</td>
<td>66.7</td>
<td>73.3</td>
<td>58.3</td>
</tr>
</tbody>
</table>

Table 16c. Comparison of differential values of $F_{E}$NO, and electronic nose in predicting steroid response measured by $FEV_1$.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>CVV†</th>
<th>Sens‡</th>
<th>Spec§</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{E}$NO (post-OCS / pre-OCS)*</td>
<td>66.7</td>
<td>12.5</td>
<td>89.5</td>
</tr>
<tr>
<td>Electronic nose samples (post-OCS-pre-OCS)</td>
<td>85.2</td>
<td>94.7</td>
<td>62.5</td>
</tr>
</tbody>
</table>

Table 16d. Comparison of differential values of $F_{E}$NO, and electronic nose in predicting steroid response measured by $PC_{20}$AMP.

| Predictor || CVV† | Sens‡ | Spec§ |
|-----------|--------|-------|-------|
| $F_{E}$NO (post-OCS / pre-OCS)* | 31.8  | 11.1  | 46.2  |
| Electronic nose samples (post-OCS-pre-OCS) | 59.1  | 61.5  | 55.6  |

* $F_{E}$NO difference between post- and pre-OCS values is a ratio rather than a difference because values are typically log-transformed to a normal distribution
† Cross-validation value (%). For each sample in turn, a new discriminant is calculated based on all other samples, and tested on the original sample. The proportion of correctly classified ‘left out’ cases is the cross-validation value. The CVV is a ‘jackknife’ estimate of the correct classification rate in the general population
‡ Sensitivity (%). Proportion of correctly classified asthmatics in cross-validation
§ Specificity (%). Proportion of correctly classified healthy controls in cross-validation
|| Only 22 asthmatics had $PC_{20}$AMP measurements
Discussion

1 Interpretation of results
1.1 Overview of main results

The primary aim of this study was to attempt to distinguish steroid responsive from steroid unresponsive asthmatics using breath analysis by electronic nose. The results indicated that this was not possible. Breath prints could not be significantly differentiated using principal component analysis (no significant differences in principal components) or M-distance (all M-distances <2, all p>0.2). Predictive models, as expected, showed poor CVVs (all <63%). Analyses were conducted on pre- and post- steroid samples and on differences between the two (differential values).

Breath analysis by electronic nose was, however, able to differentiate between healthy controls and pre-OCS asthmatics. There were significantly different electronic nose readings between these two groups when analysed by principal component analysis (PC2, PC4, PC6, p=0.0090, 0.000060, 0.0090) and M-distance (m=4.66, p=0.031). Predictive models constructed using linear discriminants and multilayer perceptrons were associated with cross-validation values of up to 83%. Breath samples of healthy controls and post-OCS asthmatics were different when analysed by PCA (PC6, p=0.0096) and predictive models were associated with cross-validation values up to 74.5%.

A significant rank correlation (coefficient=0.615, p=0.0082) was found between a principal component extracted from pre-steroid electronic nose samples and sputum eosinophil counts. No significant correlations were found between the same principal components and sputum neutrophil counts. Samples from eosinophilic and non-eosinophilic asthmatics were significantly differentiated by principal components (PC4, p=0.0033) and M-distance (m=2.00, p=0.0020). Predictive models for eosinophilic-and non-eosinophilic groups were associated with cross-validation values up to 76.9%.

Improvement in ACQ (PC6, p=0.0041) and improvement in FEV₁ (PC6, p=0.045) following the OCS course could be detected based on principal components calculated from differences in electronic nose samples before and after the steroid course.
Pre-steroid electronic nose samples performed worse than pre-OCS $F_{E}NO$ or eosinophil count in predicting ‘composite’ steroid response (improvement in any of the three endpoints, CVV = 51.9% vs 59.3% and 65.4% respectively), ACQ improvement (48.1% vs 59.3% and 76.9%) and $PC_{20}$AMP improvement (40.9% vs 59.3% and 76.9%). The electronic nose performed slightly worse than $F_{E}NO$ and eosinophil count in predicting $FEV_1$ improvement (74.1% vs 76.9%, 77.8%) although CVVs were very similar in this case.

Differential (post-steroid minus pre-steroid) $F_{E}NO$ detected composite steroid response better than differential electronic nose samples (CVV=70.4% vs 51.9%). Differential electronic nose samples detected improvement in ACQ (CVV=66.7% vs 59.3%) and $FEV_1$ (CVV=85.2% vs 66.7%) better than $F_{E}NO$. Differential electronic nose samples also detected improvement in $PC_{20}$AMP better than $F_{E}NO$ (CVV=59.1% vs 31.8%) although both CVVs were very low.

The data obtained in this study confirm the possibility that the electronic nose could be developed into a clinical test for asthma diagnosis. The data also indicate that, with a larger number of patients, a test for eosinophilic asthma based on exhaled breath analysis could be developed. Such a test has the potential to be relevant, although this would have less clinical value than the ability to predict steroid responsiveness directly. Theoretically the ability to detect improvements in ACQ and $FEV_1$ allows the possibility of monitoring asthma control by serial electronic nose measurements.

1.2 Secondary results

1.2.1 Analysis techniques for electronic nose data

The analysis of discrimination between groups involved two phases. The first phase was exploratory in nature and sought to determine if any between-group differences were present. The second phase attempted to produce a predictive model between groups, and therefore provide a way to estimate which group (healthy or asthmatic, steroid responsive or non-steroid responsive) an unknown sample may belong to.

The first phase of analysis constituted comparison of principal components and analysis of M-distance. These two techniques can identify between-group differences in different ways. M-distance provides an estimation of ‘average’ discrimination provided by all principal components (see Methods section).
The second phase of analysis constituted the construction and analysis of linear discriminants (LDA) and the construction of multilayer perceptrons (MLPs). Although this second phase was largely redundant if no significant between-group discrimination was found in the first phase, in this study each analysis was conducted and a full range of comparisons was made. As expected, CVVs were around 50% when no discrimination was found in the first phase.

A more extensive second phase of analysis was conducted for the major comparisons of the study (healthy controls versus asthmatics, and steroid-responsive versus steroid-unresponsive asthmatics). For other analyses only a single linear discriminant and multilayer perceptron were produced, using the first six principal components as the input variables in each case. The choice of input variables was based on results from the large second-phase analyses.

In cases where discrimination was found to be present, MLPs performed considerably better than LDA in providing discriminating functions. When comparing controls and pre-OCS asthmatics, CVVs were significantly higher for MLPs than for LDAs using the same input variables (mean difference 4.0%, p=0.048, paired t-test on CVV values). When comparing controls and post-OCS asthmatics, CVVs were higher for MLPs, but not quite significantly so at 95% confidence (mean difference 6.7%, p=0.076).

In cases where no discrimination was found, MLPs did not perform any better than LDA. No significant difference in CVVs was found when comparing steroid-responsive and steroid-unresponsive asthmatics pre-OCS (p=0.33), when comparing steroid-responsive and non-steroid responsive asthmatics post-OCS (p=0.19), or when comparing steroid-responsive and non-steroid responsive asthmatics on difference in sensor readings (p=1.0).

The better performance of MLPs on groups known to be discriminated may be due to the ability of MLPs to model non-linear separation between groups or the different objectives maximised by LDA and MLPs. While LDAs maximise distance between group means, MLPs maximise the number of correctly predicted cases directly.

Overtraining of LDAs was evident when a large number of input variables were used. As an example the linear discriminant between healthy controls and pre-OCS asthmatics with raw second-analysis sensor values as input variables had a correct classification rate of 85.1% on
the training data but a CVV of only 55.1%.

1.2.2 First and second analysis data

In general, first analysis data was found to be more sensitive to between-group differences than second analysis data. For this reason, second analysis data was not used except in the two main comparisons (healthy controls vs asthmatics, steroid-responsive asthmatics vs non-steroid responsive asthmatics) and for assessment of eosinophilia. According to these results, second-analysis data do not need to be collected in future studies.

Healthy controls and pre-OCS asthmatics were separated more distinctly in the sensor space generated by first analysis data (M-distance=4.66) than in the space generated by second analysis data (M-distance=4.28). Healthy controls and post-OCS asthmatics were also further separated by first analysis data (M-distance=3.80) than by second analysis data (M-distance=3.53).

Several linear discriminants used principal components from both first and second analysis data as input. These discriminants did not perform noticeably better than discriminants based on first analysis data alone (see tables 8e-h, 9g-l).

2 Context of results

2.1 Implications

2.1.1 Significance of findings

The significant discrimination between the healthy control and asthmatic groups was important in that it validated the ability of the electronic nose to detect differences in phenotype when used according to the study protocol.

This may indicate that steroid responsive and unresponsive asthmatics, according to the definition used in the study, are unlikely to be genuinely differentiated on the basis of exhaled breath analysis. If no significant difference had been found between controls and asthmatics, the lack of difference between steroid-responsive and steroid-unresponsive asthmatics could be attributed to inadequate sampling or processing of the breathprints.
Healthy controls were separated to a greater degree from pre-OCS asthmatics (M-distance=4.66, p=0.031) than from post-OCS asthmatics (M-distance = 4.28, p=0.065). This indicates that VOC profiles of steroid-treated asthmatics are more like those of controls than profiles of untreated asthmatics, as expected.

The significance of the correlation between eosinophil counts and the relevant principal component score indicates that it is highly unlikely that the correlation arose by chance.

### 2.1.2 Clinical relevance of findings

The significant discrimination between asthmatics and controls affirms the results of earlier studies and furthers the possibility of adaptation of the electronic nose to a clinical tool for asthma diagnosis.

While it would be difficult to use the electronic nose in a clinical setting using the same methodology as was used in the study, the ability to take a non-invasive sample in under ten minutes invites the adaptation of the technique so that a more user-friendly methodology can be used.

Based on study results, it is very unlikely that the electronic nose could be used to predict steroid responsiveness among steroid-naive patients with lower respiratory tract symptoms such as cough wheeze or shortness of breath. This is disappointing given the importance of predicting steroid responsiveness as a clinical question. The detection of changes in ACQ and FEV₁ indicate that there may be scope for use of the electronic nose as a monitor of asthma control.

The strong correlation between the relevant principal component from pre-OCS electronic nose samples and eosinophil count indicates that the electronic nose may well be applicable as a rapid predictor of sputum eosinophilia. This could serve as an indicator for asthma therapies known to be effective in particular pathological phenotypes.

### 2.1.3 Relation to earlier studies

The study repeated the results of Fens et al. (35), Dragonieri et al. (32), and Montuschi et al.
(7) in successfully discriminating healthy controls and asthmatics. The best cross-validation results achieved in this study (MLP-based discriminant on six principal components) was CVV=83.0%. This was below the CVV range of 90-100% obtained by Dragonieri et al. (32), and the 95% found by Fens et al. (35), using identical apparatus and breath sampling methods to those used here. Montuschi et al. (7) obtained a CVV of 75% when a full exhaled breath was analysed and a CVV of 87.5% when only the first part of breath was analysed. The reasons for the lower CVVs obtained in this study are not clear.

The assessment of eosinophilia in the present study was analogous to the electronic nose-based analysis of eosinophilia in COPD by Fens et al. (37). A stronger correlation (coefficient=0.615, p=0.0057), was found between the relevant component and eosinophilia in asthma than was found between the relevant principal component and eosinophilia in COPD (coefficient=−0.50, p=0.03). No correlation between sputum neutrophil counts and principal components was found in the present study or that by Fens et al.

2.2 Alternative explanations of findings
2.2.1 Comparison of healthy controls and asthmatics

There were several demographic differences between healthy control and asthmatic groups, namely age and weight (or BMI). In addition, FeNO and atopy restrictions were present in the healthy control group. This introduced several sources of potential bias in the groups, and as such there can be no certainty that the differentiation between healthy control and asthmatic groups was due to asthma or due to differences in other variables. However, an analysis by Dragonieri (32) found that breathprints from ‘old’ and ‘young’ healthy individuals could not be distinguished, which suggests that age was not responsible for the VOC differences between controls and asthmatics.

2.2.2 Comparison of steroid-responsive and steroid-unresponsive asthmatics

It remains possible that complex separations existed between steroid-responsive and steroid-unresponsive asthmatics. More sophisticated analyses may have been able to differentiate the groups, although larger numbers of participants may have been needed.
The problem of the 'curse of dimensionality' was particularly prevalent in this study because the number of asthmatic participants was smaller than the number of variables being analysed (27 participants, 32 sensors). If the difference between steroid-responsive and steroid-unresponsive groups was only present on a small number of sensors, the random variation in the other sensors would mean that discrimination would not be evident when all 32 sensors were analysed together. It is not possible to predict prior to the study which sensors will be most sensitive to changes in the independent variable.

It is also possible that the inability to detect steroid unresponsiveness was because it is not associated with a specific enough pathophysiological phenotype. Alternatively, the methods used to detect steroid response may not have been sufficiently sensitive. Poor discrimination between steroid-responsive and steroid-unresponsive asthmatics could also have been due to differences which were too small to be detectable by the methodology.

2.2.3 Prediction of eosinophilic asthma

The correlation between eosinophil counts and a principal component score was significant and unlikely to have arisen by chance. Discrimination between eosinophilic and non-eosinophilic groups was also clear.

2.3 Limitations of the study
2.3.1 Comparison of healthy controls and asthmatics

Recruitment of healthy controls was from a population known to have no history of asthma or respiratory disease, and no known airway pathology, whereas asthmatics were recruited from participants in previous studies and from referrals from a respiratory clinic. A highly specific test was used to confirm asthma (the hypertonic saline challenge or reversibility to bronchodilator) and no patients with questionable or ambiguous diagnoses of asthma were included in the study. The ability of the electronic nose to diagnose asthma, rather than simply distinguish known asthma from known control, cannot be guaranteed from the results of this study. This would require a study of hitherto undiagnosed patients with asthma-like symptoms, in whom the diagnosis of asthma is prospectively validated, independently of the electronic nose data.
In addition, cross-validation rates for discriminant functions between controls and asthmatics were no higher than 83%, which is an approximation to the correct classification rate of the discriminant in the general population. CVVs were markedly lower than that achieved in earlier studies which used the same equipment and methodology (32, 35), suggesting that high discrimination rates between controls and asthmatics as obtained in the earlier studies may not be universally obtainable. The use of an electronic nose in a clinical setting would therefore require extensive validation of devices at different sites.

2.3.2 Comparison of steroid-responsive and steroid-unresponsive asthmatics

Although there was evidence that the electronic nose could be used to discriminate individuals exhibiting an increase in ACQ score post-OCS and to discriminate individuals exhibiting an increase in FEV\textsubscript{1} post-OCS, the discrimination was based on differences in electronic nose readings rather than pre-OCS readings alone. There was no evidence that improvement in the scores could be predicted prior to the steroid course.

2.3.3 Prediction of eosinophilia using the electronic nose

The correlation between eosinophilic and principal component reading, while significant, does not permit the prediction of eosinophil count from a breath sample. The correlation coefficient used (Pearson's rank correlation) does not fit a model to the data, and the coefficient indicates an association of ranks only. In order to be adapted to a test for eosinophilia, a predictive model must be developed, which would require significantly larger numbers of participants.

2.4 Areas for further research
2.4.1 Comparisons between healthy controls and asthmatics

The discrepancy between cross-validation values when comparing controls and asthmatics indicates the need for considerable validation of electronic nose apparatus and methods before any reliable clinical test could be applied. Research into the repeatability of measurements between different devices would be a necessary next step in the development of diagnosis of
asthma by electronic nose.

All studies on the topic to date (7, 32, 35) developed discrimination algorithms between controls and asthmatics based on available data only. If inter-device compatibility could be established, it may be possible to assimilate data from several studies and develop a database of electronic nose readings from various phenotypes. This could allow much better discriminants to be constructed. One of the great advantages of electronic nose based analysis is the ability of discriminating algorithms to continually adapt and improve as more data becomes available, and such an approach may allow improved discriminating algorithms to be produced.

2.4.2 Comparisons between steroid-responsive and steroid-unresponsive asthmatics

There was no evidence that steroid response or non-response can be predicted by electronic nose. While significantly larger study sizes may find differences in VOC profiles, this is not suggested by the data obtained in this study, and it is very unlikely that a significant enough difference would be found to justify the development of a clinical test.

However, the ability of the electronic nose to monitor asthma control may justify further investigation. The ability to detect an improvement in ACQ or FEV\textsubscript{1} based on differences in electronic nose data invites the investigation into how electronic nose readings for an individual patient vary. Methods such as PLS-DA can be used to compare electronic nose readings for the same patient over time (28).

3 Summary and conclusions

The results of this study showed no evidence that steroid-responsive and steroid-unresponsive asthma can be distinguished by analysis of exhaled breath using an electronic nose.

Healthy controls and asthmatics could be discriminated by electronic nose analysis of exhaled breath. Predictive models were associated with cross-validation values slightly lower than that of previous studies in the area.
A principal component generated from electronic nose samples was significantly correlated with eosinophil counts in sputum. Electronic nose analysis of breath samples from eosinophilic and non-eosinophilic asthma groups found them to be significantly different.

Improvements in FEV\textsubscript{1} and ACQ before and after steroid course were significantly associated with changes in electronic nose readings, suggesting that the electronic nose can detect changes in airway control when serial readings are taken.

The electronic nose is a less accurate predictor of steroid responsiveness than F\textsubscript{E}NO or sputum eosinophilia. Changes in electronic nose score before and after a steroid course are more strongly associated than changes in F\textsubscript{E}NO with improvements in FEV\textsubscript{1} and ACQ.

This study supports earlier evidence that the electronic nose may be used to differentiate asthma from controls and highlights the potential to develop electronic nose as a tool in clinical diagnosis. However, there is a need to validate reproducibility of electronic nose data.

Our results also suggest that the electronic nose may be developed into a tool to predict eosinophilic asthma. Further research would be needed to produce a predictive model. Results from differences in electronic nose readings suggest that the nose could be used as a monitor of asthma control.

In conclusion, the results of this study demonstrate the capability of the electronic nose to discriminate between healthy and diseased individuals by analysing exhaled breath. The ease of sampling is a significant advantage to the adaptation of electronic nose techniques to future clinical applications.
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