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Diagnosing eosinophilic asthma using a multivariate prediction model based on blood granulocyte responsiveness

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Abstract

Background: The identification of inflammatory asthma phenotypes, using sputum analysis, has proven its value in diagnosis and disease monitoring. However due to technical limitations of sputum analysis, there is a strong need for fast and noninvasive diagnostics. This study included the activation state of eosinophils and neutrophils in peripheral blood to phenotype and monitor asthma.
Objectives: To (i) construct a multivariable model using the activation state of blood granulocytes, (ii) compare its diagnostic value with sputum eosinophilia as gold standard and (iii) validate the model in an independent patient cohort.
Methods: Clinical parameters, activation of blood granulocytes and sputum characteristics were assessed in 115 adult patients with asthma (training cohort/Utrecht) and 34 patients (validation cohort/Oxford).
Results: The combination of blood eosinophil count, fractional exhaled nitric oxide, Asthma Control Questionnaire, medication use, nasal polyposis, aspirin sensitivity and neutrophil/eosinophil responsiveness upon stimulation with formyl-methionyl-leucyl phenylalanine was found to identify sputum eosinophilia with 90.5% sensitivity and 91.5% specificity in the training cohort and with 77% sensitivity and 71% specificity in the validation cohort (relatively high percentage on oral corticosteroids [OCS]).
Conclusions: The proposed prediction model identifies eosinophilic asthma without the need for sputum induction. The model forms a noninvasive and externally validated test to assess eosinophilic asthma in patients not on OCS.

An estimated 334 million people worldwide suffer from asthma, while its prevalence is still rising (1). The majority of patients are well-controlled with beta-agonist combined with inhaled corticosteroids. However, 5–10% of the patients suffer from poorly controlled asthma, consume ~60% of total asthma-related healthcare costs and experience long-term side-effects of oral glucocorticoids use. This group needs better asthma treatment, and identifying inflammatory phenotypes is essential to choose the right treatment option.

Since the introduction of sputum induction to obtain cellular samples from the airways, it has been one of the most accepted methods to assess airway inflammation and thereby diagnosing the asthma inflammatory phenotype (2). Its clinical value in asthma management was established in three randomized controlled trials that tailored treatment based on sputum eosinophilia (3–5). These studies independently

Abbreviations
ACQ, Asthma Control Questionnaire; DA, discriminant analysis; FeNO, fractional exhaled nitric oxide; fMLF, formyl-methionyl-leucyl phenylalanine; ICS, inhaled corticosteroids; MARS, Medication Adherence Reporting Scale; NLPCA, nonlinear principal component analysis; OCS, oral corticosteroids; PC, principal component.

showed a reduction in asthma exacerbations after treatment adjustments that were based on sputum eosinophilia. In addition, the presence of eosinophils in airway epithelium (6) and sputum has been shown to correlate with exacerbation frequency (3–5). However, sputum induction is considered to be an invasive, time-consuming diagnostic test that needs to be performed only in specialized centres. Another disadvantage is the procedure fail rate (10–30%). These limitations restrict this type of adequate inflammatory phenotyping to a cohort of patients with severe asthma (7, 8).

Measuring peripheral blood eosinophil count is a promising alternative for sampling in the airways. In the past, cohort studies that focussed on relations between blood eosinophilia and asthma found correlations between blood eosinophilia and asthma diagnosis, asthma events, emergency department (ED) visits, sputum eosinophilia and wheeze (9, 10). In the DREAM study, blood eosinophilia correlated with a reduction in exacerbations after anti-IL5 (Mepolizumab) treatment and was a predictive indicator for reduction in sputum eosinophil count (11). Later on, blood eosinophilia was the basis for patient selection in two large phase III studies that looked into the effect of Mepolizumab on exacerbation frequency and glucocorticoid sparing (12, 13).

In contrast to sputum eosinophilia, it is yet unclear whether glucocorticoid treatment strategies based on blood eosinophilia can reduce exacerbation frequency or improve other outcome measures in asthma. Blood eosinophil count does not correlate perfectly with sputum eosinophilia. This stretches the importance to identify fast and accurate measures to predict airway eosinophilia. The blood compartment is favourable because it is easily accessible, already part of routine clinical workup and with technical advances in measurements such as multicolour flow cytometry has increased potential for inflammatory phenotyping.

In addition to eosinophil count, the activation state of eosinophils could be a promising biomarker. Johansson and colleagues indicated that priming and activation of eosinophils in the peripheral blood is deficient during episodes of tissue eosinophilia in severe and uncontrolled asthma (14). This hypothesis was partly founded on the upregulation of active FcgammaRII on activated blood eosinophils after segmental lung challenge in mild asthmatics (15). The latter seems to contradict the putative deficiency of primed or activated cells. However, long-term priming of eosinophils in the peripheral blood of severe asthmatics and the subsequent migration to the lung could lead to a deficiency of primed cells within the peripheral blood (16); an upregulation of active integrin receptors and activation-related receptors is found on blood granulocytes in mild-to-moderate asthma, and on the other hand, low expression profiles of these markers are found in severe inflammatory disease (17). These findings indicate relevance of granulocyte priming and activation for assessment of the inflammatory status of patients with asthma.

Not only additional biomarkers could improve asthma phenotyping; combined analyses of known clinical and biological characteristics provided important insights into airway disease mechanisms using the multivariate advantage (18). Multivariate advantage refers to classifications based on multiple, combined features that outperform the combined classifications on the separate features. In asthma, a key finding was the absence of correlation between eosinophilic inflammation and symptoms (19). Haldar and colleagues furthermore showed the value of inflammation-driven treatment decisions based on an unbiased approach for patient selection. Two other studies that made use of the multivariate advantage evaluated the power of blood eosinophil count, FeNO and periostin to predict sputum eosinophilia (20) and to predict the response to anti-IgE treatment (21). Both conclude that the combination of the three markers might be a good way to assess the inflammatory status of patients with asthma, while the value of the single parameters FeNO, blood eosinophils or total IgE to predict sputum eosinophilia has been regarded to be moderate. In a meta-analysis of 24 studies overall sensitivity and specificity in detecting sputum eosinophilia in adults were as follows: 0.66 and 0.76 for FeNO; 0.71 and 0.77 for blood eosinophils; and 0.64 and 0.71 for IgE (22).

We designed a cross-sectional study to investigate whether the classification accuracy of a multivariate prediction model for sputum eosinophilia benefits from including measurements of peripheral blood granulocyte activation status and whether such a noninvasive prediction model has sufficient diagnostic value to replace expertise-dependent sputum analysis. The multivariate prediction model is based on a training cohort (Utrecht, The Netherlands) and prospectively validated on independent data from a validation cohort (Oxford, UK). Sputum eosinophilia was set as gold standard.

Methods

Subjects

Training cohort

Patients with asthma aged 18–75 were recruited at the respiratory outpatient clinics of the University Medical Center Utrecht (UMCU), and the Central Military Hospital Utrecht (CMH), The Netherlands, between May 2012 and December 2013. Inclusion and exclusion criteria are provided in the Appendix S1 (Fig. S1, flow chart). Written informed consent was obtained, and the local ethics committee of the UMCU and CMH approved the study protocol.

Test/validation cohort

Adult patients with asthma were recruited at the respiratory outpatient clinic of the Churchill Oxford University Hospital between September 2014 and June 2015. The same inclusion and exclusion criteria were used as in the test cohort. The study protocol was ethically approved, and written informed consent was obtained from all patients.

Study design

Patients with asthma (see for demographics Table 1) underwent lung function measurement, sputum induction, blood withdrawal and fractional exhaled nitric oxide (FeNO)

Table 1 Baseline characteristics of subjects in Utrecht cohort

	Utrecht cohort n = 115		Oxford cohort n = 34	
Age (mean)	43		56	
Gender (M/F)	55/60		19/15	
BMI, kg/m^2	27		31	
Smoking ever (%)	27		41	
Pack years	1.28		4	
Aspirin sensitivity (%)	5		24	
Eczema	20			
Nasal polyposis (%)	19		29	
ACQ	1.4		1.6	
Proven allergy (anamnestic and spec. IgE) (%)	59		71	
History of allergy	77			
FeNO (ppb)	23†	16–36	18†	19–45
% predicted FEV1 (L)	86	82–89	68	60–75
Total eosinophil count in PB × 10^9/L	0.22†	0.13–0.41	0.25†	0.12–0.39
Sputum cell profile		%		%
Eosinophilic (>3% eosinophils)	21	18	11	32
Neutrophilic (>61% neutrophils)	14	12	8	24
Mixed (>3% eos. and >61% neutr.)	8	7	2	6
Paucigranulocytic	33	29	0	0
Epithelial (>80% epithelial cells)	39	34	13	38
Treatment		%		%
No medication (currently)	3	3	1	3
SABA	1	1	2	6
Low-dose ICS	5	4	1	3
Low-dose ICS + LABA or medium dose ICS	69	60	1	3
High dose ICS + LABA (and/or LTRA)	24	21	19	56
High dose ICS + LABA + OCS	13	11	10	30
MARS (nonadherence in percentage)	27			

BMI, body mass index; MARS, Medication Adherence Report Scale; ACQ, Asthma Control Questionnaire; FeNO, fraction of exhaled nitric oxide; FEV1, forced expiratory volume in 1sec; PB, peripheral blood; SABA, short-acting beta-agonist; LABA, long-acting beta-agonist; ICS, inhaled corticosteroids; LTRA, leukotriene receptor antagonist.
†Median and IQR.

measurement. Their medical history was taken, and both the Asthma Control Questionnaire (ACQ) (23) and the Medication Adherence Report Scale (MARS) (24) were filled out.

Sample size

For the sample size calculation, we refer to Appendix S1, Methods, sample size.

Measurements

Blood

Blood was obtained in 9-mL tubes containing sodium heparin, transported at room temperature and processed and analysed within 2 hours. Eosinophil and neutrophil priming was tested *in vitro*; four polystyrene tubes with 50 μL blood were incubated for 5 min at 37°C. Hereafter, two of the tubes were stimulated with 5 μL 0.001 mM N-formyl-methionyl-leucyl phenylalanine (fMLF) for 5 min. Subsequently, whole blood in all tubes was stained with fluorescein isothiocyanate (FITC)-labelled monoclonal phage antibodies (Abs) A17 or A27(31) and with phycoerythrin (PE)-labelled α_M (CD11b) and incubated for 30 min on ice. Hereafter, red cells were lysed in ice-cold isotonic NH_4Cl and cells were centrifuged at 1500 rpm for 5 min. The cell pellet was washed twice and resuspended in ice-cold PBS/1% human serum albumin. In the test cohort, cells were measured using a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA). In the validation cohort, cells were measured using a Cyan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Prior to the analysis, blood was stained with Krome Orange (KO)-labelled CD16 antibody. Eosinophils could be distinguished from neutrophils by low FcγRIII (CD16) expression. Data from individual experiments are reported as fluorescence intensity in arbitrary units (AU) or in *n*-fold change from baseline.

Lung function and FeNO

FEV1 measurements were performed using the PiKo-1 (nSpire™) device, and FeNO was determined using NIOX MINO® (Aerocrine, Solna, Sweden) with an expiration time of 10 s.

Sputum

Sputum induction was performed with hypertonic saline according to the ERS guideline (25). Cytospin slides of sputum cells were stained with May–Grünwald–Giemsa, and cells were differentiated and counted by an experienced technician. A cut-off value of 3% eosinophils in sputum was used to classify patients with eosinophilic or noneosinophilic inflammation. A cut-off value of 61% neutrophils in sputum was used to classify patients with neutrophilic inflammation. A mixed phenotype was assigned if >3% eosinophils and >61% neutrophils were counted. Eosinophils <3% and neutrophils <61% were regarded as a paucigranulocytic phenotype.

Statistical analysis

Nonlinear principal component analysis (NLPCA)

Principal component analysis (PCA) is a widely used unsupervised method to reduce dimensionality in data sets. However, PCA is only suitable to analyse data consisting of continuous variables. The majority of variables in our study were either categorical or nominal; therefore, we used nonlinear PCA (NLPCA). Linting et al. described a stepwise approach for NLPCA and applied the technique in clinical cohorts (26, 27). Applying this technique, we were able to take into account the correlated variance from 26 clinical and immunological parameters simultaneously. Briefly, the method applied entails transposition of all parameters to a linear scale, followed by reduction of the number of parameters by a two-step selection process based on correlation of variances and by PCA of the resulting data set to produce a simplified description of the data that retain as much variance as possible by a small number of principal components.

After creating a final model with NLPCA using the Utrecht cohort as a 'training set', the Oxford cohort was plotted in this PCA model as test set using >Data >Weight of 0.01 per patient. Thus, the Oxford cohort was used as validation set.

Discriminant analysis (DA)

Discriminant analysis was used on the NLPCA scores. A class for eosinophilic asthma and a class for noneosinophilic asthma were set (≥3 sputum eosinophils); this is a supervised step. External validation of the Oxford data was performed by weighting the NL-PCA scores of these patients by 0.01 in the DA. For an overview of both NLPCA and DA steps see Fig. 1.

Results

A total of 115 patients with asthma were recruited in The Netherlands (Fig. S2). In total, 76 patients could be classified by sputum analysis and 39 patients with asthma were not able to cough up or had sputum samples that showed >80% buccal squamous cells (34%). A total of 34 patients with asthma were recruited in the United Kingdom. Of these, 20 patients (59%) could be classified by sputum induction. Demographic details are presented in Table 1.

Multivariate diagnostic model

Of 26 parameters, NLPCA identified 12 important parameters that together described most variance within the cohort of patients in Utrecht (Fig. 1). Six of the final parameters were classical 'clinical' parameters, and the other 6 were peripheral blood parameters that describe responsiveness of eosinophils and neutrophils to fMLF. The six parameters with the highest variance accounted for (VAF) were as follows: aspirin sensitivity, CD11b response on eosinophils and neutrophils, nasal polyposis, ACQ and A17 response on neutrophils, in decreasing order. The remaining parameters explained less variance, being A27 response on eosinophils, medication, A17 response on eosinophils, FeNO, eosinophil count and A27 response of neutrophils. The stability of the NLPCA model was tested by performing a bootstrapping procedure on the test cohort (Utrecht). 10 cohorts were created that separately underwent NLPCA. The loadings from these NLPCA analyses were compared to the original loadings, and the RV-coefficient of this comparison was 0.84. This correlation coefficient indicates the NLPCA loadings are highly stable. Technical details of the performed NLPCA are supplied in paragraph I of the results section of the Appendix S1 'NLPCA' and in Fig. 1.

Interpretation of the model

The result of NLPCA is a set of 'scores' and 'loadings'. As there are four principal components in this model (see Appendix S1 for the origin of this number), each individual patient is represented by four scores. Figure 2 (middle) shows both the loadings of the 12 most relevant parameters and the scores of the patients on the first two principal components of the model. These two principal components together define the 2D projection of the data in which the most variability can be presented. The position of a patient indicates its score on PC1 (horizontal axis) and its score on PC2 (vertical axis). The 12 loadings each represent the contribution of a single parameter, such as eosinophil count, to the variability among the patients: The higher the correlated variance of a parameter, the higher the loading and the longer the vector in Fig. 2. Parameters pointing in the same direction are likely to be correlated.

The distribution of patients within the score/loading plot (Fig. 2) is largely determined by markers of eosinophilic inflammation as indicated by the direction of the markers; patients with sputum eosinophilia plot in the direction of the parameters FeNO, ACQ, eosinophil count, medication, nasal polyposis and aspirin sensitivity. On the contrary, these patients have low values of blood eosinophil responsiveness (A17, A27 and CD11b) and therefore plot in the opposite direction of these vectors. In short, if there is a high percentage of eosinophils present in sputum, a patient has blood eosinophilia with cells that are refractory to stimulation. On the other hand, patients with a neutrophilic and a paucigranulocytic sputum phenotype have lower values of for example FeNO and ACQ and higher values of responsiveness of eosinophils and neutrophils and therefore plot on the other side

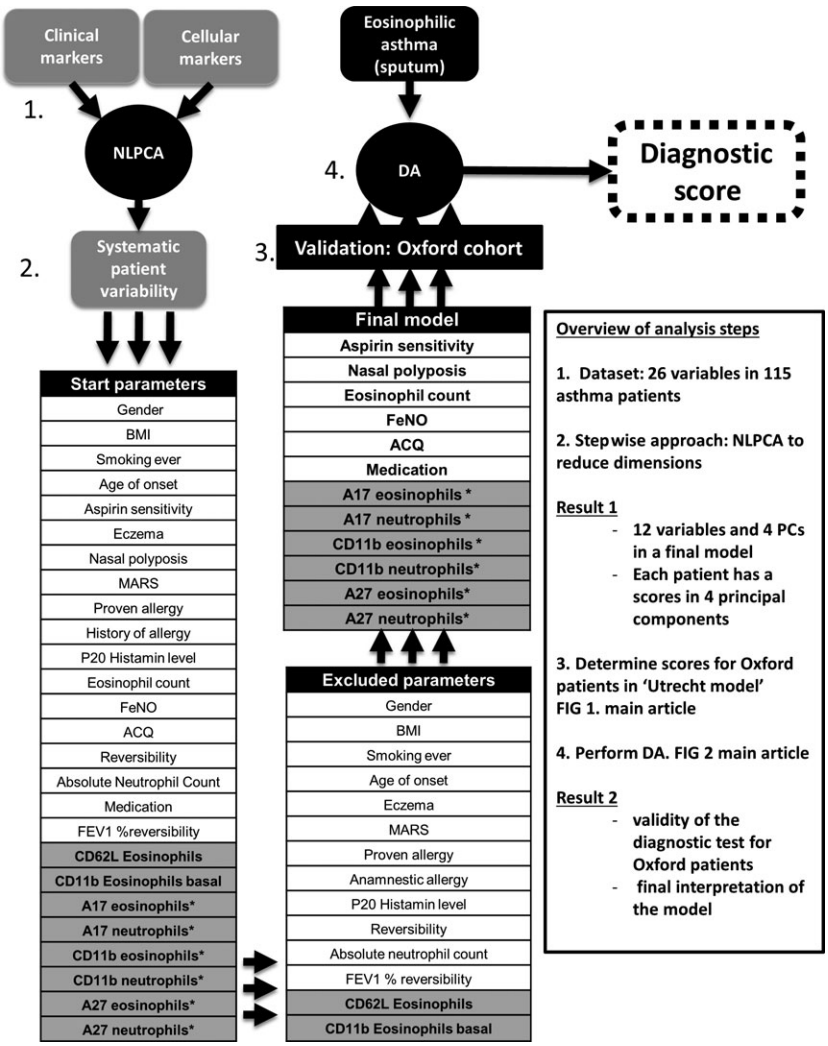

Figure 1 Overview data analysis. Steps 1 and 2: Clinical (in the white cells) and peripheral blood markers (in the grey cells) were combined to build a model using dimension reduction (NLPCA, unsupervised). After steps 1 and 2, the Oxford Cohort was added to the NLPCA model to validate the prediction model for airway eosinophilia. Subsequently, discriminant analysis was performed by setting a class for eosinophilic asthma and a class for noneosinophilic asthma (≥3 sputum eosinophils); this supervised step was required to obtain a diagnostic score.

of the graph. Notably, sputum characteristics were not part of the selection of parameters for the multivariate model (Fig. 1) and were used as a gold standard. The technical details of the performed DA are supplied in 'Statistical analyses, paragraph discriminant analysis' section of the Appendix S1.

Validation

An internal cross-validation was used to test classification accuracy of the NLPCA/DA hybrid model with sputum eosinophilia as the dependent variable. Based on a leave-one-out cross-validation of the Utrecht cohort, sputum eosinophilia could be predicted with a sensitivity of 90.5 and a specificity of 91.5 (Table S1), In the next step, using the 'Utrecht cohort' as a test set and subsequently adding the 'Oxford cohort' as a validation set it was possible to classify sputum eosinophilia with 77% sensitivity and 71% specificity (Table 2, tested by cross-validation).

The discriminant analysis results in four classes (Table 3) by means of positivity or negativity for sputum eosinophilia (gold standard) and positivity or negativity predicted by the model.

Four ROC curves (Fig. 3A–D) were created using the discriminant function and sputum eosinophilia as state variable. Notably, the fourth ROC curve (Fig. 3D) was created by leaving out patients who were taking oral steroid treatment.

Finally, the data were rerun without the granulocyte responsiveness data. The sensitivity dropped from 90.5 to

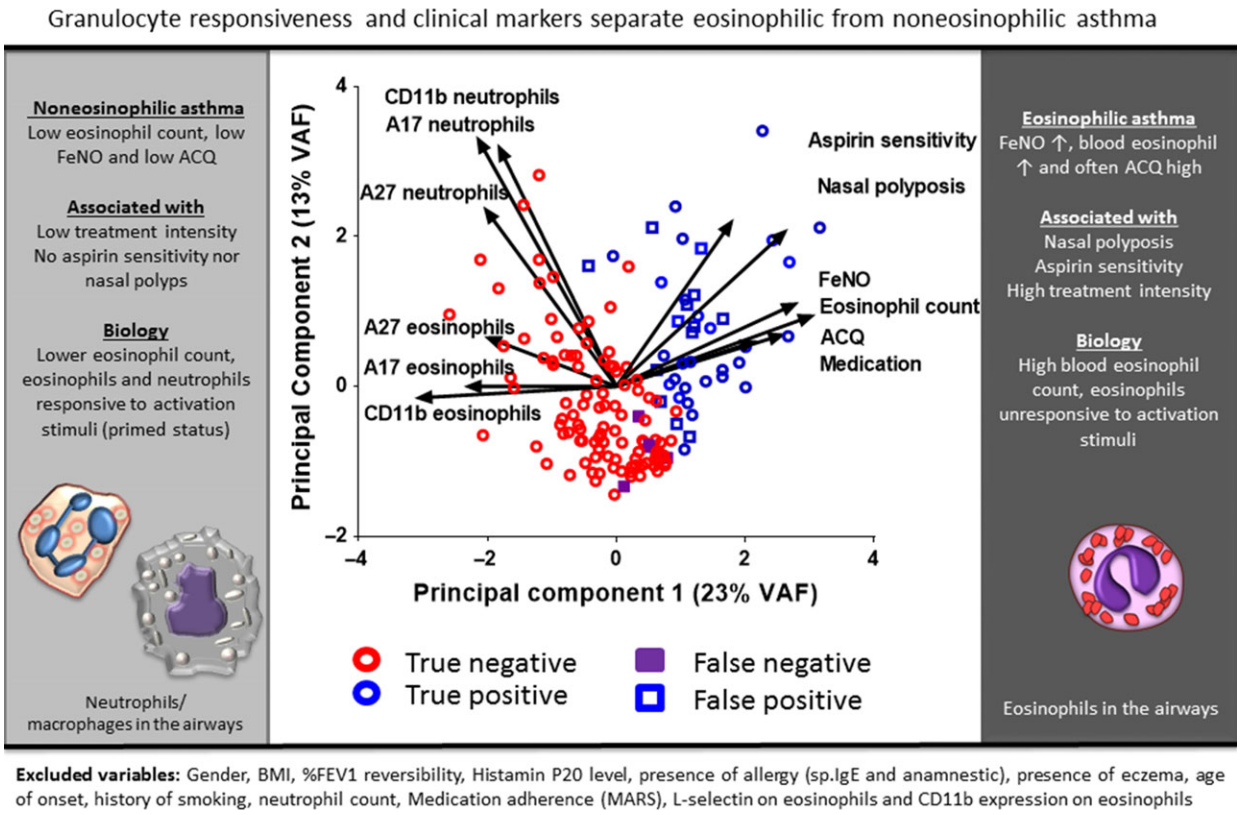

Figure 2 Combined scoring and loading plot after nonlinear principal component analysis. Each symbol represents a patient. Each vector represents a variable, in total 12 in this model. The more variability a variable has, the longer the vector. Each patients score depends on the value of these 12 variables. Therefore, eosinophilic patients (in blue) are high in FeNO, ACQ, eosinophil count and low in eosinophil responsiveness, while their noneosinophilic counterparts (in red and green) exhibit low values in these clinical markers, but higher values of granulocyte responsiveness and appear on the opposite side of the graph. CD11b, A17 and A27 on eosinophils and neutrophils represent fold-induction of receptor expression (fluorescence intensity after fMLP stimulation divided by baseline fluorescence intensity).

Table 2 2 × 2 contingency table with diagnostic score of the prediction model with respect to the Oxford Cohort

Prediction model for sputum eosinophilia		Predicted group membership		Characteristics
		Eos.	Non-eos.	
Sputum analysis	Eos.	10	3	Positive predictive value: 62.5%
	Non-eos.	6	15	Negative predictive value: 83.3%
Characteristics		76.9% Sensitivity	71.4% Specificity	Accuracy: 73.5%

The number of patients correctly classified with eosinophilic disease is 10 of 13 (76.9%). The number of patients with noneosinophilic disease is correctly identified in 15 of 21 (71.4%). On average, 73.5% of original grouped cases is correctly classified (leave-one-out cross-validation accuracy). Eos: eosinophilic asthma, Non-eos.: noneosinophilic asthma.

47.6%, and specificity increased slightly from 91.5 to 95.7% (Table 4 and Fig. 4A, B).

Discussion

The findings of this cross-sectional study in a 'training' cohort of 115 patients with asthma and a 'validation' cohort of 34 patients visiting university medical centres in, respectively, Utrecht and Oxford, underline the value of cellular markers in peripheral blood to classify asthma phenotypes. fMLF-induced upregulation of activation-associated receptors on eosinophils and neutrophils, together with a limited set of clinical parameters, can serve as an accurate read-out for eosinophilic asthma. Results of the unbiased analysis of both cellular and clinical parameters confirm the important role for already established measurements in asthma, such as eosinophil count, ACQ and FeNO. However in this study, adding measurements of blood granulocyte responsiveness

Table 3 Clinical characteristics of the four groups identified by the prediction model

	Eosinophilic by sputum and prediction model	Noneosinophilic by sputum and prediction model	Eosinophilic prediction model	Noneosinophilic prediction model
n	29	102	13	5
FeNO, median (IQR)	48 (215)	20 (81)	25 (120)	16 (21)
ACQ (CI)	2.71 (3.86)	1.29 (4.43)	1.0 (3.57)	1.29 (1.86)
Eosinophil count $\times 10^9$/L (CI)	0.49 (1.16)	0.16 (0.8)	0.27 (0.75)	0.18 (0.62)
Aspirin sensitivity % (*n*)	24 (7)	1 (1)	46 (6)	0
Nasal polyposis % (*n*)	66 (19)	6 (6)	54 (7)	0
Medication, % on OCS (*n*)	28 (8)	8 (8)	46 (6)	20 (1)

All values are represented in mean and 95% CI lower/upper limit, or in number (*n*) except for FeNO which is expressed in median and interquartile range.

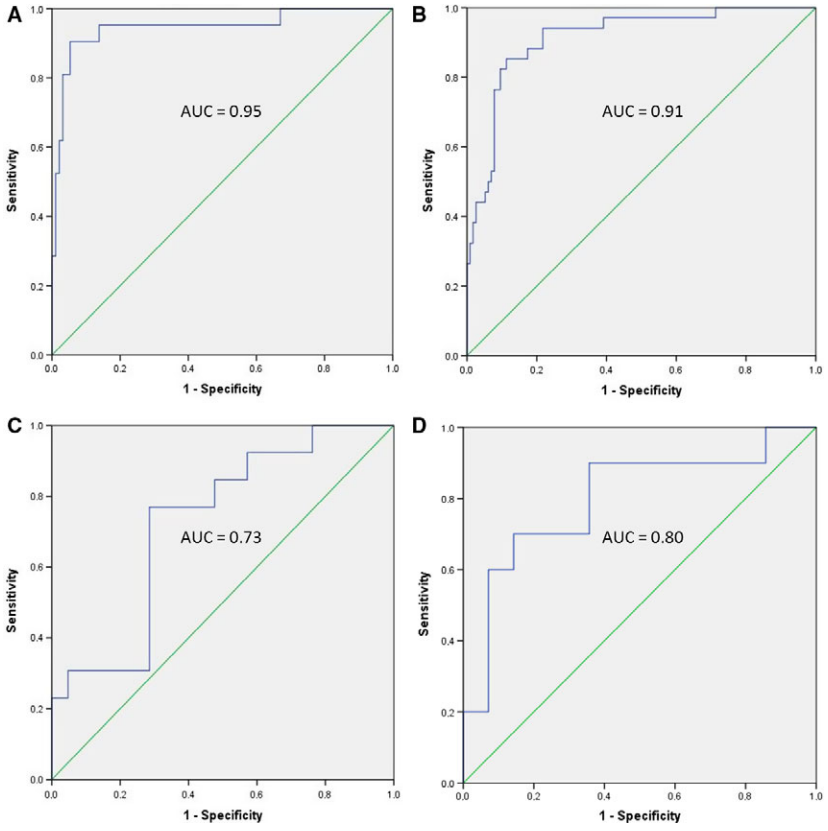

Figure 3 (A) ROC curve based on the Utrecht data set. This ROC curve has an AUC close to 1 as it is the base of the NLPCA model. AUC = 0.946, the reported *P*-value is <0.001. (B) ROC curve based on the combined Utrecht and Oxford data set. This combined set has a high AUC of 0.91. AUC = 0.914, the reported *P*-value is <0.001. (C) ROC curve based on the Oxford validation cohort only. The AUC is lower compared to the test set and indicates a difference between test set and validation set. AUC = 0.725, the reported *P*-value is 0.029. (D) ROC curve based on the Oxford validation cohort only without BTS treatment group 6. For this ROC curve, patients in BTS treatment group 6 (oral steroid treatment) were excluded. The AUC increased from 0.73 to 0.80. AUC = 0.800, the reported *P*-value is 0.014. [Colour figure can be viewed at wileyonlinelibrary.com]

significantly increased the predictive accuracy, improving the sensitivity from 47.6% to 90.5%.

Interestingly, the 'eosinophilic patients by prediction model' (i.e. patients without sputum eosinophilia) have distinct characteristics; these 13 eosinophilic patients have higher blood eosinophil counts, higher values of FeNO and a higher incidence of aspirin sensitivity and nasal polyposis compared to the noneosinophilic patients. More patients in this group are using oral glucocorticoids compared to the other groups (~46%, Table 3). Oral corticosteroids (OCS)

Table 4 2 × 2 contingency table with diagnostic score of the prediction model without blood markers, using sputum analysis as reference test

Prediction model for sputum eosinophilia		Predicted group membership		Characteristics
		Eos.	Non-eos.	
Sputum analysis	Eos.	10	11	Positive predictive value: 71.4%
	Non-eos.	4	90	Negative predictive value: 89.1%
Characteristics		47.6% Sensitivity	95.7% Specificity	Accuracy: 87.0%

The number of patients correctly classified with eosinophilic disease is 10 of 21 (47.6%). The number of patients with noneosinophilic disease is correctly identified in 90 of 94 (95.7%). On average, 87% of original grouped cases is correctly classified (leave-one-out cross-validation accuracy). Eos: eosinophilic asthma, Non-eos.: noneosinophilic asthma.

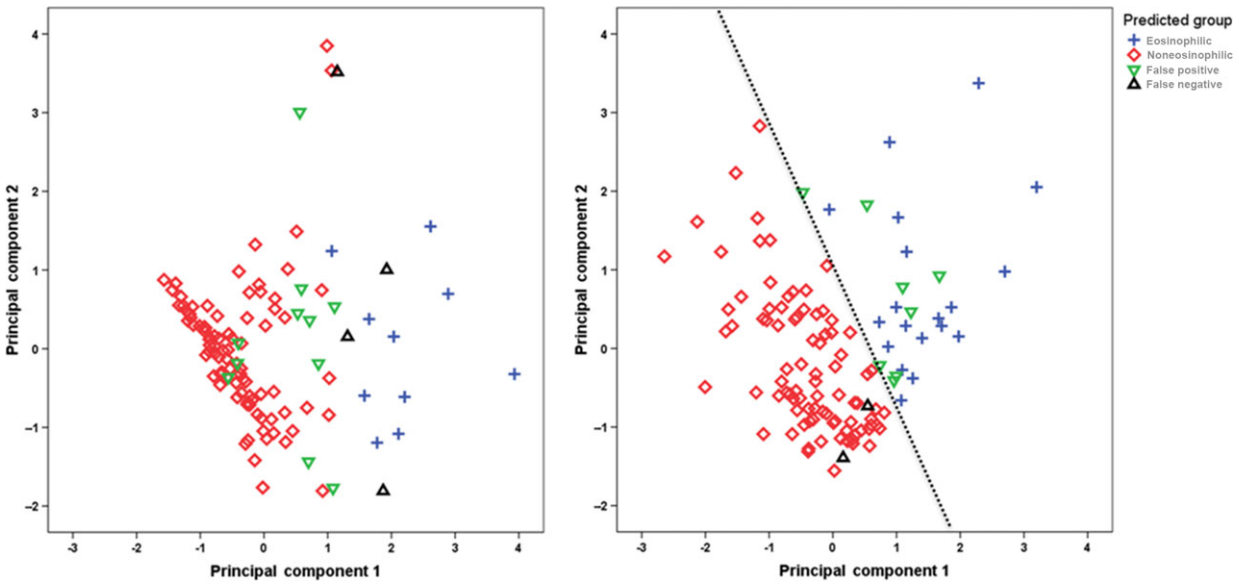

Figure 4 (A) Prediction model without blood markers has a poor diagnostic value. According to this model differences between groups of patients are not clear; true non-eosinophilic asthma patients (red diamonds), true eosinophilic asthma (plus sign), false-positive patients (green diamonds) and false negative (black triangles). (B) Prediction model based on blood markers discriminates accurately between eosinophilic and noneosinophilic asthma. The dotted line indicates the discrimination between eosinophilic (plus sign) and noneosinophilic (red diamonds) disease according to the prediction model based on clinical parameters and blood granulocyte measures. The two false negatives (black triangles) are not identified by the model; however, the eight false-positive cases (green diamonds) that have a high symptom and high eosinophilic inflammation profile illustrate the improved classification capability of the prediction model. These false positives would have been missed by sputum analysis only.

are known to induce apoptosis in eosinophils and can explain the 'false' low number of sputum eosinophils (28). To strengthen this, the Oxford cohort has a ~threefold higher percentage of patients on OCS compared to Utrecht. Therefore, these patients are particularly less likely to have sputum eosinophilia, leading to the 'false' conclusion they do not suffer from eosinophilic asthma. Based on known steroid effects and high OCS use in the group of 13 patients that were 'false positive', OCS use is the most likely explanation for the relatively low sensitivity and specificity of the prediction model in the Oxford cohort and suggests the prediction model as developed here is more suitable for asthma classification in patients not on OCS. This was validated by excluding patients on OCS from the Oxford cohort, which led to an increase in predictive power: 79.2% compared to 73.5% without this group. The sensitivity lowered from 76.9% to 72.7%, and the specificity increased extensively from 71.4% to 84.6%. The ROC curves with the full Oxford cohort (Fig. 3C) and the cohort with patients on oral steroids (Fig. 3D) also show a great improvement in AUC value.

Instead of using a single-parameter approach, an unbiased multidimensional approach was used to evaluate the experimental data. This is generally regarded as a promising analysis strategy for the understanding of heterogeneous diseases such as asthma (29, 30). Large asthma cohorts, such as SARP and the Leicester cohorts, already brought more insight into disease phenotypes using clustering techniques (19, 31, 32). One strong determinant of the quality of

multidimensional models is the included parameters. Therefore, it is important to include and test new parameters such as granulocytes responsiveness. In this study, we were able to improve the sensitivity of our prediction model from 47.6% to 90.5% by adding granulocyte responsiveness to the model. Using nonlinear principal component analysis, correlations between many of the measured clinical parameters were taken into account. These correlations may be clinically valuable and on the other hand may hamper multiple linear regression models. NLPCA provides a consistent, widely used and quantitative way to merge parameters measured on different levels.

Our prediction model is based on 12 clinical and cellular parameters and does not depend on several common asthma parameters such as atopy, gender and BMI. These latter parameters showed little discriminative value in our cohort. This finding is in agreement with findings in the larger Leicester and SARP cohorts (19, 31–34). The Leicester cohorts showed that atopy, gender and BMI were not significant determinants for the secondary care factor model. Similarly, the SARP cohorts also had low variability within the data sets for atopy, gender and BMI. These collective findings are also in line with insights from the DREAM cohort (11). Atopy in the DREAM cohort was not a predictor for the response to Mepolizumab, whereas peripheral blood eosinophil count and exacerbation frequency in the past year, both hallmarks of eosinophilic inflammation, had predictive value for the response. In summary, the model focuses attention on relevant parameters and is in line with data from earlier unsupervised multivariate models.

Flow cytometry analysis is the required technique to perform cell counts and in this study also to measure granulocyte responsiveness. State-of-the-art bench top flow cytometers are able to perform a stimulation step on whole blood, such as adding fMLF. A blood tube has to be loaded into the cytometer and the pipetting step is performed automatically by the cytometer, after which it measures fluorescence intensity. This important advancement makes it possible to use complex flow cytometry for clinical diagnostic tests, such as testing granulocyte responsiveness in patients with asthma.

In conclusion, the proposed prediction model identifies eosinophilic asthma with peripheral blood analysis, FeNO measurement and assessment of routine clinical data. Responsiveness of peripheral blood granulocytes was essential to come to a sensitive diagnostic test and adds to the ongoing scientific debate about the biological relevance of granulocyte responsiveness in asthma. The prediction model was prospectively tested in an independent patient population visiting a specialised asthma centre in Oxford (UK) and identified an important group of patients with potentially eosinophilic inflammation that rendered noneosinophilic in sputum most likely due to OCS use. Finally, this study underlines the potential of unbiased approaches to support clinical decision making in complex diseases such as asthma.

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Ethics approval

The AIR-study was conducted with the approval of the University Medical Centre Utrecht Medical Ethical Committee – protocol nr. 11-322 and the approval of the Medical Ethical Committee of the Central Military Hospital – protocol nr. 2013-021. Patients included in Oxford were part of the Brightling study, which was ethically approved by the Research Ethical Committee of the University Hospital in Leicester – protocol nr. 08/H0406/189.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Methods and additional results.

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