

ORIGINAL ARTICLE

External validation of blood eosinophils, FE_{NO} and serum periostin as surrogates for sputum eosinophils in asthma

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ABSTRACT

Background Monitoring sputum eosinophils in asthma predicts exacerbations and improves management of asthma. Thus far, blood eosinophils and FE_{NO} show contradictory results in predicting eosinophilic airway inflammation. More recently, serum periostin was proposed as a novel biomarker for eosinophilic inflammation.

Objectives Quantifying the mutual relationships of blood eosinophils, FE_{NO}, and serum periostin with sputum eosinophils by external validation in two independent cohorts across various severities of asthma.

Methods The first cohort consisted of 110 patients with mild to moderate asthma (external validation cohort). The replication cohort consisted of 37 patients with moderate to severe asthma. Both cohorts were evaluated cross-sectionally. Sputum was induced for the assessment of eosinophils. In parallel, blood eosinophil counts, serum periostin concentrations and FE_{NO} were assessed. The diagnostic accuracy of these markers to identify eosinophilic asthma (sputum eosinophils $\geq 3\%$) was calculated using receiver operating characteristics area under the curve (ROC AUC).

Results In the external validation cohort, ROC AUC for blood eosinophils was 89% ($p < 0.001$) and for FE_{NO} level 78% ($p < 0.001$) to detect sputum eosinophilia $\geq 3\%$. Serum periostin was not able to distinguish eosinophilic from non-eosinophilic airway inflammation (ROC AUC=55%, $p=0.44$). When combining these three variables, no improvement was seen. The diagnostic value of blood eosinophils was confirmed in the replication cohort (ROC AUC 85%, $p < 0.001$).

Conclusions In patients with mild to moderate asthma, as well as patients with more severe asthma, blood eosinophils had the highest accuracy in the identification of sputum eosinophilia in asthma. The use of blood eosinophils can facilitate individualised treatment and management of asthma.

Trial registration NTR1846 and NTR2364.

INTRODUCTION

Asthma is a heterogeneous condition which includes several clinical phenotypes that differ in severity, natural history and responses to therapy.¹ There is recent evidence from prospective clinical studies that inflammatory (sub)phenotyping of patients can help to optimise therapy and disease outcome.² This suggests that biomarkers of inflammation should be considered in identifying patients and monitoring of asthma in clinical practice, such as the titration of steroid treatment.

Key messages

What is the key question?

- What are the mutual relationships of blood eosinophils, FE_{NO} and serum periostin with sputum eosinophils in a cohort of patients with mild to moderate asthma, and can the findings be replicated in a population with more severe asthma?

What is the bottom line?

- Previous studies on the diagnostic accuracy of blood eosinophils, FE_{NO} and serum periostin to assess eosinophilic airway inflammation have demonstrated conflicting results, and this triad of biomarkers has not been externally validated.

Why read on?

- This study shows that blood eosinophils is an accurate biomarker for eosinophilic airway inflammation in two independent cohorts of patients with asthma, which can have great practical advantages for guiding current and novel personalised therapies.

Sputum eosinophilia has been demonstrated to be a key marker in predicting asthma outcome.³ Whereas eosinophilic asthma responds well to anti-inflammatory treatment with steroids, non-eosinophilic asthma shows little or no response.⁴ Additionally, studies in which corticosteroids were withdrawn have consistently shown that a raised sputum eosinophil count is predictive of inducing an exacerbation.^{5,6} The strong evidence that monitoring sputum eosinophils improves outcome has come from randomised trials showing that normalising sputum eosinophil counts can lead to 60% reduction in asthma exacerbations.^{2,7,8}

Sputum induction by hypertonic saline is generally considered a reliable non-invasive method to assess and monitor eosinophilia.⁹ However, the use of sputum analysis is hindered by the requirement of lab facilities and the duration of the analyses. Furthermore, in patients with severe and uncontrolled asthma, induction of sputum can be problematic, because of hypertonic saline-induced

airway narrowing and/or failure to produce an adequate sputum sample in about a quarter of the patients.¹⁰

There is, therefore, a need for adequate surrogate markers of eosinophilic inflammation in asthma. The measurement of FE_{NO} has been considered a surrogate marker for eosinophilic airway inflammation. However, the correlation between FE_{NO} and sputum eosinophils appears to be only modest,¹¹ particularly in patients with steroid-dependent asthma.¹² This is in line with a Cochrane meta-analysis demonstrating insufficient benefit of monitoring steroid therapy by FE_{NO},² even though this was challenged by a recent positive result in primary care.¹³ Alternatively, blood eosinophil counts exhibit moderate to good correlation with sputum eosinophils in asthma,¹⁴ being associated with disease severity and asthma phenotypes.^{15–16} Blood eosinophils may, therefore, predict and direct anti-inflammatory therapy, for which there is preliminary evidence in asthma and COPD.^{17–20} Nevertheless, a very recent study demonstrated poor correlations of FE_{NO} and blood eosinophils with sputum eosinophils, both separately and combined,²¹ thereby raising controversy. Finally, serum periostin was proposed as a systemic biomarker of eosinophilic airway inflammation in asthma, by showing a significant correlation with sputum eosinophils and prediction of steroid responsiveness in asthma.^{22–23}

Based on international guidelines on STAndards for the Reporting of Diagnostic accuracy studies, it is mandatory to perform external validation when assessing diagnostic or phenotypic accuracy of disease markers.²⁴ This has not been done for sputum eosinophils with the triad of FE_{NO}, blood eosinophils and serum periostin. Therefore, we aimed to quantify the mutual relationships of FE_{NO}, blood eosinophils and serum periostin with sputum eosinophils in an external validation cohort of patients with mild to moderate asthma and to replicate findings in a population with more severe asthma.

METHODS

Subjects

For the external validation cohort, we recruited 200 patients with mild to moderate asthma in the outpatient clinic of the Academic Medical Center (AMC) in Amsterdam and two non-academic pulmonary second-line referral outpatient clinics. For the replication cohort, we recruited 40 patients with moderate to severe asthma in the outpatient clinic of the AMC. For both cohorts, the diagnosis of asthma was defined by a physician's diagnosis of asthma with reversibility in FEV₁ ≥ 12% of the predicted value and/or airway hyper-responsiveness (PC₂₀ methacholine < 8 mg/mL).

In the external validation cohort, smokers or ex-smokers with a smoking history > 10 pack-years were excluded if they did not show an improvement in FEV₁ of at least 12% after inhalation of 400 µg salbutamol with a normal diffusion capacity at the time of inclusion. In the replication cohort, all smokers or ex-smokers with a smoking history > 10 pack-years were excluded. At the time of the study visit, no patients had any symptoms of respiratory infection for at least 4 weeks.

Both studies were approved by the hospital medical ethics committee, and all patients gave their written informed consent. The external validation cohort was registered in The Netherlands trial register (<http://www.trialregister.nl>) under NTR1846 and the replication cohort under NTR2364.

Design

The studies had similar cross-sectional designs and included one hospital visit for all measurements. During this visit, inclusion and exclusion criteria were examined, lung function was

performed and sputum was induced by hypertonic saline. Inflammatory status in the external validation cohort was also measured by the assessment of blood eosinophils, FE_{NO} and serum periostin. In the replication cohort, blood eosinophils and serum periostin were measured in order to replicate findings in a population with more severe asthma.

Measurements

Lung function and allergy testing

Lung function was performed according to the European Respiratory Society (ERS) recommendations.²⁵ Atopic status was assessed by total and specific immunoglobulin E (IgE) to a panel of common aeroallergens. Patients were considered atopic if there was at least one serum-specific IgE > 0.34 kU/L.

Markers of inflammation

Sputum was induced by inhalation of hypertonic saline three times at intervals of 5 min, according to the ERS recommendations.²⁶ Before induction of sputum, patients inhaled 400 µg salbutamol. For the external validation cohort, the volume of the whole sputum sample was assessed and an equal volume of dithiotreitol (10 mM DTT in 135 mM Tris buffer, pH 8.0) was added. For the replication cohort, selected plugs were processed with 0.1% DTT. The processing of the sputum and cell counts was done by experienced laboratory analysts blinded to other results. Differential cell counts were expressed as the percentage of non-squamous cells, based on 500 non-squamous cells. Those with significant squamous contamination (> 80%) were excluded from analysis. According to previous studies, we used a sputum eosinophil count of 3% as the threshold for determining eosinophilic or non-eosinophilic airway inflammation.⁷

Peripheral blood eosinophil counts were obtained from standard complete blood counts done at the same centre, and FE_{NO} was measured using an online device at a constant flow of 50 mL/s (Niox Mino; Aerocrine AB, Solna, Sweden).²⁷ Serum was obtained by centrifugation of blood that coagulated for 30 min at room temperature, after which serum periostin levels were measured in an ELISA with the DuoSet Human Periostin/OSF-2 (R&D Systems) (see the Methods section in the Online Repository). This in-house ELISA for periostin was validated for measurement of periostin in serum by serial dilutions (10×, 20×, 40× and 80× diluted; ± 15.5% variation) and spike recovery (77.75% ± 11.69%; (mean ± SD)). The intra-assay and interassay coefficients of variability were 12.3% (9.08% ± 3.91%; (mean ± SD)) and 17.4% (12.69% ± 4.08%), respectively. Western blots were performed to determine which periostin isoforms were recognised by the antibody (see the Methods and the Results sections in the Online Repository). Furthermore, all blinded serum samples were analysed by a second and independent periostin assay (ElecSys Periostin, for use on the COBAS e601, under development by Roche Professional Diagnostics, Penzberg, Germany, using the same antibodies as previously described.²²

Statistical analysis

SPSS (V.18.0) was used for data analysis. The results for continuous variables were expressed as mean ± SD; skewed distributions were presented as medians with IQRs. Non-normally distributed variables were transformed to log or square root values. The relationship between sputum eosinophils and the surrogate markers were analysed using Pearson's correlation coefficient.

For the external validation cohort, receiver operating characteristic (ROC) curve analysis was performed for each variable individually or in combination, to determine the marker that best identified a sputum eosinophil count ≥ 3%. To analyse

whether the area under the curve (AUC) of different ROC curves differ significantly, comparisons of AUCs were performed using R (V2.15) and the pROC package.²⁸ The optimum cut-points were considered for each variable and sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. Additionally, sensitivity and specificity were calculated for alternative cut-points that were previously published: blood eosinophils $\geq 0.25 \times 10^9/L$ and $\geq 0.22 \times 10^9/L$; FE_{NO} levels >50, <24 and >20 ppb; serum periostin levels using the median of the biomarker as cut-off.^{16 22 29–31}

The diagnostic accuracy of the best predictive marker for sputum eosinophils in the external validation cohort was subsequently verified in the replication cohort using ROC curve analysis.

RESULTS

In the external validation cohort (recruitment: June 2009–June 2011) 110 out of 200 patients and in the replication cohort (recruitment: October 2010–June 2011) 37 out of 40 patients were able to produce adequate sputum samples. The patient characteristics of both cohorts are described in table 1, and characteristics stratified by sputum eosinophil counts of $\leq 3\%$ or $\geq 3\%$ are presented in online supplementary table E1 in the Online Repository.

External validity of blood eosinophils, FE_{NO} and serum periostin

Blood eosinophils and FE_{NO} correlated with sputum eosinophil percentages ($r=0.59$, $p<0.001$ and $r=0.52$, $p<0.001$, respectively). Using the in-house periostin ELISA, there was no significant correlation between serum periostin and sputum eosinophil percentages ($r=0.09$, $p=0.4$). Using the Elecsys Periostin assay, there was a weak but significant correlation between serum periostin and sputum eosinophil percentages ($r=0.32$, $p=0.001$).

The diagnostic accuracy of blood eosinophils, described as ROC AUC, was 89% ($p<0.001$, 95% CI 0.81 to 0.96) (figure 1). Using $\geq 0.27 \times 10^9/L$ blood eosinophils as a cut-point, eosinophilic and

non-eosinophilic inflammation was well differentiated with a sensitivity of 78% and a specificity of 91% (table 2).

The overall accuracy of FE_{NO} levels to differentiate eosinophilic and non-eosinophilic inflammation, described as ROC AUC, was 78% ($p<0.001$, 95% CI 0.66 to 0.89) (figure 1). This ROC AUC was not significantly different from the ROC AUC of blood eosinophils ($p=0.09$). A FE_{NO} level of ≥ 42 ppb provided a sensitivity of 63% and a specificity of 92% (table 2).

Serum periostin measured by the in-house ELISA was not able to distinguish eosinophilic from non-eosinophilic inflammation (ROC AUC=55%, $p=0.44$, 95% CI 0.43 to 0.67) (figure 1). Serum periostin analyses using the Elecsys Periostin assay showed similar results (see online supplementary results in the Online Repository).

When combining these three variables in the prediction of eosinophilic inflammation, no improvement was seen, resulting in an ROC AUC of 88% ($p<0.001$, 95% CI 0.79 to 0.97). Next, sensitivity, specificity, PPV and NPV for different criteria used in previous studies are presented in table 2.

Since others have reported 2% sputum eosinophils as an alternative criterion for the diagnosis of eosinophilic or non-eosinophilic asthma,⁸ additional ROC curve analyses were performed using 2% sputum eosinophils as threshold. The results were similar to those using 3% sputum eosinophils, with an ROC AUC of 88% ($p<0.001$) for blood eosinophils, an ROC AUC of 79% ($p<0.001$) for FE_{NO} and no significant diagnostic accuracy for serum periostin (see online supplementary table E2 in the Online Repository).

Replication

In the replication cohort as well, there was a significant correlation between blood eosinophils and sputum eosinophil percentages ($r=0.80$, $p<0.001$). Blood eosinophil levels were effective in assessing eosinophilic inflammation, with an ROC AUC of 85% ($p<0.001$, 95% CI 0.72 to 0.98) (figure 2). Using $\geq 0.27 \times 10^9/L$ blood eosinophils as reported in the external

Table 1 Patient characteristics

	External validation cohort	Replication cohort
Number of patients	110	37
Age (years)	49±13.8	53±11.4
Gender (% female)	51	51
BMI	28±5.2	30±7.5
Smoking history (py)*	4 (0–18)	0 (0–5.5)
Oral corticosteroids (%)	0	19
Inhaled corticosteroids (%)	85	100
Dose ICS (µg/day)*†	500 (250–500)	500 (500–1000)
Atopy (% positive RAST)	43	57
Total IgE (Ku/L)*	62 (26–235)	153 (42–288)
pb FEV ₁ (% predicted)	100±17.1	90±18.1
pb FEV ₁ /FVC (% predicted)	95±11.0	86±16
Sputum eosinophils, %*	0.6 (0.1–3.6)	2.1 (0.2–8.8)
Blood eosinophils, 10 ⁹ /L*	0.17 (0.11–0.29)	0.18 (0.09–0.32)
FE _{NO} level, ppb*	20 (13–40)	NA
Periostin (in-house), ng/mL*	25.5 (19.9–32.6)	36.3 (28.7–54.2)
Periostin (Elecsys), ng/mL*	47.7 (40.2–56.3)	50.8 (45.7–60.4)

Data expressed as mean±SD;

*Median (IQR).

†Fluticasone equivalent.

BMI, Body Mass Index; ICS, inhaled corticosteroids; IgE, immunoglobulin E; NA, not available; pb, postbronchodilator; py, pack-years; RAST, radioallergosorbent test.

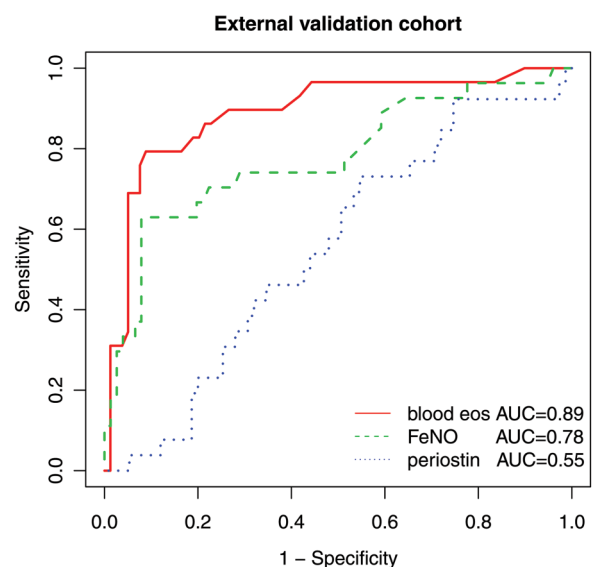


Figure 1 Receiver operating characteristics curve analyses of the sensitivity and the specificity of blood eosinophils (eos), FE_{NO} and serum periostin (in-house) for the diagnosis of eosinophilic inflammation. AUC, area under the curve.

Table 2 Sensitivity, specificity, PPV and NPV of different surrogate markers using alternative cut-points to diagnose eosinophilic airway inflammation (less than, more than or equal to 3% sputum eosinophils)

	Threshold	Sensitivity	Specificity	PPV	NPV
Blood eosinophils	$>0.22 \times 10^9/L$	86	79	60	93
Blood eosinophils	$\geq 0.25 \times 10^9/L$	79	84	64	91
Blood eosinophils	$\geq 0.27 \times 10^9/L$	78	91	79	91
FE _{NO} level	>20 ppb	74	57	40	87
FE _{NO} level	≥ 24 ppb	74	63	42	87
FE _{NO} level	≥ 42 ppb	63	92	74	89
FE _{NO} level	>50 ppb	56	92	67	84
Serum periostin (in-house)	>26 ng/mL	54	57	29	77

NPV, negative predictive value; PPV, positive predictive value.

validation cohort as best threshold, the sensitivity was 60% and specificity 90% (see online supplementary table E3 in the Online Repository). In line with the results of the external validation cohort, no correlation was found between serum periostin (using the in-house ELISA) and sputum eosinophils in the replication cohort ($r=0.13$, $p=0.46$), nor was periostin able to distinguish eosinophilic inflammation from non-eosinophilic inflammation (ROC AUC 54%, $p=0.79$, 95% CI 0.34 to 0.74) (figure 2). Independent analysis using the Elecsys Periostin assay provided similar results (see online supplementary results in the Online Repository).

DISCUSSION

This study shows that in patients with mild to moderate asthma, blood eosinophils is an accurate surrogate marker for sputum eosinophils. Next, we were able to replicate blood eosinophils as highly effective surrogate markers in a second independent cohort of patients with more severe asthma. FE_{NO} was second best, while serum periostin showed the lowest accuracy for eosinophilic asthma in both cohorts. These findings suggest that

blood eosinophil count can be used in mild, moderate and severe asthma as an easy-to-measure biomarker for sputum eosinophil percentage, which can have great practical advantages for guiding current or novel anti-inflammatory therapies. Periostin might provide different information than sputum eosinophils, which may be complementary in asthma phenotyping.

Interestingly, blood eosinophils and sputum eosinophils were highly correlated in both our cohorts and exhibited the highest diagnostic accuracy which validates previous data,^{31 32} and to a lesser extent a recent report.²¹ We were not able to show a role for periostin as diagnostic marker for sputum eosinophils in both populations. The present data are not in line with the single previous study investigating the relationship between airway eosinophilia and all three markers, which demonstrated the highest ROC AUC for serum periostin.²² However, the latter study used a combination of both high sputum and high-tissue eosinophils as definition of eosinophilic airway inflammation. Furthermore, they included patients with uncontrolled severe asthma only, whereas the present study included a larger cohort of mild to moderate patients and a somewhat smaller cohort of severe patients.

In our study, FE_{NO} appeared to be the second-best predictor for eosinophilic inflammation with an ROC AUC 0.78, which is nearly similar to previous studies^{21 22 31} although, surprisingly, the best combination of sensitivity and specificity was achieved at a rather high cut-point of 42 ppb in our cohort of patients with mild to moderate disease. Even though FE_{NO} was significantly associated with sputum eosinophils, when combining the three markers in the ROC analysis, neither FE_{NO} nor periostin had any additive value. Our data confirms a recent paper in which a weak correlation was found between blood eosinophils and FE_{NO},³³ suggesting that blood eosinophils and FE_{NO} relate to two different inflammatory pathways. This supports our main result that blood eosinophil count alone is the strongest independent predictor for eosinophilic airway inflammation.

To the best of our knowledge, this is the first study to externally validate serum periostin as surrogate marker for sputum eosinophils in a population with mild to moderate asthma, including replication in a second cohort with more severe disease. We believe that the strength of this study is that we have two independent well-characterised cohorts of varying asthma severity and treatment, though with similar stringent criteria for the diagnosis of asthma. Another strength is the size of the external validation cohort, which reassures the confidence of the analysis. However, the size of the replication cohort of patients with severe asthma was limited, which may require further analysis in large severe asthma cohorts, such as U-BIOPRED (Unbiased BIOMarkers in PREdiction of respiratory disease outcomes). The predictive accuracy of blood eosinophils is unlikely to be affected by treatment in our cohorts, since we recruited widely varying levels of therapy in mild, moderate and severe patients, including 19% of the severe patients using oral corticosteroids. Next, the sputum from both cohorts was processed in different standardised ways (whole sample vs selected plug). Nevertheless, the correlation with blood eosinophils was consistent, which may be due to careful quality control procedures. We used 3% sputum eosinophils as the threshold for eosinophilic or non-eosinophilic airway inflammation according to the literature. Because others have used 2% as the cut-point, we reanalysed the data with 2% blood eosinophils as threshold showing similar results. Finally, we used two independent periostin assays, thereby contributing to the validity of our data.

One of the potential weaknesses of our study is that we could not obtain adequate sputum in all patients. However, no

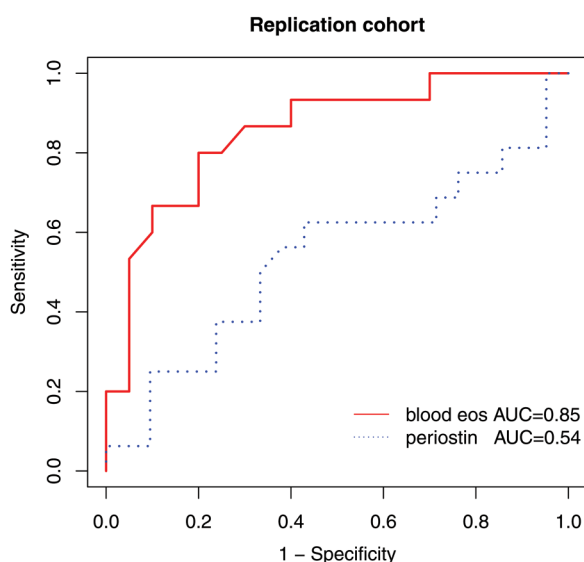


Figure 2 Replication of findings: receiver operating characteristics curve analyses of the sensitivity and the specificity of blood eosinophils (eos) and serum periostin (in-house) for the diagnosis of eosinophilic inflammation in a second cohort with more severe asthma. AUC, area under the curve.

significant differences were found in blood eosinophil counts and FE_{NO} level between the patients who successfully produced sputum and those who did not (data not shown). Therefore, we do not believe that the results of our study are biased by this limitation. Furthermore, the smoking status between the cohorts differed, as ex-smokers were included in the validation cohort and excluded in the replication cohort. In the validation cohort, patients with a smoking history, as compared with never-smokers, had borderline significantly higher sputum eosinophils ($p=0.05$), whereas no differences were found for blood or sputum neutrophils, blood eosinophils, FE_{NO} and periostin ($p=0.26$, $p=0.09$, $p=0.46$, $p=0.25$, $p=0.31$, respectively). As a result, smoking status does not seem to have affected our results. Finally, we used a different assay to measure serum periostin as compared with previous studies. Our in-house ELISA for periostin was validated as described in the Online Repository. It has been argued that some antiperiostin antibodies may not recognise all four isoforms of periostin in serum.^{22–34} Since it is unknown which isoforms are present in serum, we have extensively, but unsuccessfully attempted to determine which isoforms of periostin were present in (up to 10-fold concentrated) serum using western blotting with a goat polyclonal antibody (R&D; AF3548) affinity-purified on periostin (Asn22-Gln836; data not shown). Given that the amounts of periostin in serum reported here were similar to those reported by others,^{22–30} we consider it highly unlikely that our in-house ELISA failed to recognise the most abundant splice variants of periostin in serum. Moreover, the additional analyses by the Elecsys Periostin assay with antibodies aimed to recognise all known splice variants that showed similar results.

The correlation between blood and sputum eosinophils in asthma may not be biologically surprising. Eosinophils are produced in the bone marrow, and in case of inflammation, the formation is amplified and the eosinophils traffic into inflammatory sites, all under influence of a number of cytokines, such as interleukin (IL)-5.³⁵ Blood eosinophils of patients with asthma have a distinct phenotype, especially in relation to their adhesive properties,³⁶ which is involved in the transmigration across endothelium and epithelium. Increased eosinophils were observed in both the blood and sputum after allergen challenge.³⁷ Furthermore, several studies have demonstrated that the infusion of anti-IL-5 intravenously dramatically lowers eosinophil levels in both the blood and sputum or in bronchoalveolar lavage fluid.^{18–20 38–41} Hence, although the transport of eosinophils from the blood into the lung is a complex active process, in a chronic inflammatory disease such as asthma, the levels of eosinophils in the blood and sputum appear to be closely related.

What are the clinical implications of our study? Since the measurement of blood eosinophils is easy and quick in comparison with sputum eosinophils, our data support the opportunity to assess the presence or absence of eosinophilic airway inflammation and monitor treatment in asthma. This is supported by two very recent trials using anti-IL-5 (mepolizumab), resulting in a significant reduction in the daily requirement of oral glucocorticoid therapy, reducing exacerbations and improving asthma symptoms of patients with severe eosinophilic asthma, identified by a blood eosinophil count of ≥ 300 cells/ μ L during the year before screening or ≥ 150 cells/ μ L before randomisation.^{19–20} Additionally, in a large study using anti-IL-5 to target eosinophilic airway inflammation in patients with severe asthma, blood eosinophil count at baseline was predictive for the efficacy of reducing exacerbations.¹⁸ A follow-up analysis of this study

showed that blood eosinophil count in the placebo cohort was stable over time.⁴² Furthermore, several studies showed that anti-IL-5 treatment results in a significant decrease in both sputum and blood eosinophil counts, but not in FE_{NO},^{18–40} confirming the relevance of blood eosinophils in stratification studies for anti-IL-5. With regard to anti-IL-13 therapy, blood eosinophils were not successful in the stratification of patients responsive to treatment.⁴³ However, the latter study used a much lower cut-point for blood eosinophils ($\geq 0.14 \times 10^9/L$) as compared with our study, and used a combination of serum IgE and blood eosinophil counts to identify an IL-13 signature surrogate. A more recent study on anti-IL-4/IL-13, using a higher cut-point for blood eosinophils for the stratification of patients ($\geq 0.30 \times 10^9/L$), did show significant improvements after treatment, thereby supporting blood eosinophil count as biomarker.⁴⁴ Obviously, this needs replication.

Regarding periostin, this study shows that this biomarker is not associated with sputum eosinophilia. This does not exclude complementary information to sputum eosinophils by periostin as a biomarker in asthma. Indeed, it is likely that periostin can play a meaningful role in the identification of specific phenotypes based on a Th2-high cytokine profile, since serum periostin was demonstrated to be a successful biomarker for predicting effectiveness of anti-IL-13 therapy⁴³ and was associated with airway eosinophilia in patients with uncontrolled severe asthma.²²

In our study, the diagnostic accuracy of blood eosinophils to distinguish eosinophilic from non-eosinophilic asthma in the replication cohort was equal to the validation cohort. However, the best cut-point was different in both cohorts with a lower cut-point in the replication cohort (see table 2 and online supplementary table E3). This may be explained by the difference in disease severity between the cohorts. Therefore, when using blood eosinophil count as biomarker for eosinophilic airway inflammation, the optimum cut-point may differ per population and per study question.²⁴

In conclusion, we showed a meaningful relationship between blood eosinophils and sputum eosinophils in two independent cohorts with varying asthma severity. FE_{NO} was a second-best predictor for eosinophilic airway inflammation, though FE_{NO} did not demonstrate additive value to blood eosinophils. Serum periostin was not related to sputum eosinophils in mild to moderate asthma, and this finding was replicated in the population with more severe disease. This suggests that periostin might capture other asthma phenotypes than those represented by sputum eosinophils per se. Our data indicate that blood eosinophils represent an accurate biomarker for sputum eosinophils in asthma, which can facilitate effective guidance of individualised asthma treatment.

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Contributors AHW and SBdN collected and analysed the data, and wrote the paper. RL, ARS, EJMWW, EHB and PJS were responsible for the design of the study and revised the article critically. All approved this version to be published.

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TITLE PAGE

**External validation of serum periostin, FeNO, and blood eosinophils as
surrogates for sputum eosinophils in asthma**

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METHODS

In-house periostin assay set up and quality

Serum periostin was measured by ELISA (duoset DY3548: R&D systems) using poly-HRP (Sanquin, Amsterdam, the Netherlands) for amplification. In short, capture antibody (100 μ l/well; 1 μ g/ml) was incubated overnight in a NUNC 96-well ELISA plate at room temperature. After 3 washes with phosphate-buffered saline (PBS) pH 7.4 and 0.2% Tween-20 (PBST), remaining binding sites were blocked using 0.5% non-fat milk in PBS (150 μ l/well) for 30 min. After 3 washes with PBST, standard curve (10,000 pg/ml till 39 pg/ml; 1 to 1 dilutions), samples (1 in 40 and 1 in 80 dilution) and internal controls were added (100 μ l/well) and incubated for 2h, followed by three washes with PBST. Subsequently, detecting antibody (100 μ l; 2 μ g/ml) was added and left for 1h. After another 3 washes with PBST, 100 μ l of a 1 in 10,000 dilution of poly-HRP (Sanquin, the Netherlands) in PBS with 0.5% non-fat milk in PBS was added and incubated for 30 min. After 4 washes with PBST the plates were developed using tetra-methyl benzidine and stopped with sulphuric acid. Incubations were at 500 rpm, at room temperature and in the dark, unless indicated otherwise. This in-house ELISA for periostin was validated for measurement of periostin in serum by serial dilutions (10x, 20x, 40x and 80x diluted; \pm 15.5% variation) and spike recovery (77.75% \pm 11.69%; (mean \pm SD)). The intra- and inter-assay coefficients of variability were 12.3% (9.08% \pm 3.91%; (mean \pm SD)) and 17.4% (12.69% \pm 4.08%), respectively.

Western blot of periostin isoforms

Serum samples with high and low periostin were run on 10% polyacrylamide gels under reducing conditions with SDS. In some experiments serum proteins were concentrated by precipitation using 15 (w/v) TCA and carefully solubilized in Laemmli sample buffer before layering. After separation proteins were transferred to PVDF membranes, blocked with milk

powder in PBS tween-20 buffer and developed using a goat polyclonal periostin purified detecting antibody followed by an anti-goat secondary antibody (1:15000; LI-COR Biosciences). Membranes were scanned and quantified using the Odyssey Infrared Imaging system (LI-COR Biosciences).

Results

Western blot of periostin isoforms

No isoforms of periostin were detected in (up to 10-fold concentrated) serum using Western blotting with a goat polyclonal antibody (R&D; AF3548) affinity-purified on periostin (Asn22-Gln836).

Serum periostin analyses by Elecsys® Periostin

In conjunction with our data, serum periostin analyses using the Elecsys® Periostin assay showed similar results. In the external validation cohort there was a weak but significant correlation between serum periostin and sputum eosinophil percentages ($r=0.32$, $p=0.001$), whereas in the replication cohort there was no significant correlation ($r=0.28$, $p=0.1$).

The diagnostic accuracy of serum periostin to differentiate eosinophilic from non-eosinophilic airway inflammation using 3% sputum eosinophils as threshold, described as ROC AUC, was 62% ($p=0.09$, 95% CI: 0.48-0.75) in the external validation cohort and 55% ($p=0.6$, 95% CI: 0.35-0.75) in the replication cohort (Figure E1).

Table E1. Patient characteristics stratified by sputum eosinophil percentages

	External validation cohort		Replication cohort	
	Mild to moderate asthma		Moderate to severe asthma	
	EO \geq 3%	EO < 3%	EO \geq 3%	EO < 3%
	n=30	n=80	n= 16	n= 21
Age (years)	52 \pm 14.0	49 \pm 13.6	55 \pm 9.1	52 \pm 12.9
Gender (% female)	43	54	56	48
BMI	28 \pm 5.3	28 \pm 5.2	31 \pm 9.3	29 \pm 6.0
Smoking history (py) [#]	6 (0-17)	4 (0-19)	0 (0-7.5)	0 (0-5)
Dose ICS (μ g/day) ^{#1}	500 (250-500)	250 (250-500)	500 (500-1000)	625 (500-1000)
% positive RAST	60*	37*	50	62
Serum IgE (Ku/L) [#]	164 (34-262)*	54 (20-190)*	226 (35-383)	153 (44-267)
pb FEV ₁ , % pred	101 \pm 18.5	100 \pm 16.6	86 \pm 21.4	94 \pm 14.7
pb FEV ₁ /FVC, % pred	92 \pm 9.5	96 \pm 11.4	82 \pm 15.3	88 \pm 16.5
Blood eos, 10 ⁹ /l [#]	0.38 (0.29-0.61)**	0.14 (.09-0.20)**	0.32 (0.23-0.48)**	0.13 (.06-0.20)**
FeNO level, ppb [#]	55 (17-86)**	18 (13-32)**	NA	NA
Periostin (in-house), ng/mL [#]	27 (21.2-32.9)	25 (19.0-32.8)	42 (27.1-59.3)	36 (29.1-49.4)
Periostin (Genentech), ng/mL [#]	49.7 (42.4-62)	45.3 (39.4-54.6)	56.8 (45.5-61.2)	49.1 (45.6-58)

Data expressed as mean \pm SD; # median (interquartile range); *t-test p<0.05, **t-test p<0.001

Abbreviations: Dose ICS=fluticason equivalent; pb=postbronchodilator; pbb= parts per billion;

NA=not available

Table E2. Sensitivity, specificity, PPV and NPV of different surrogate markers using alternative cut-points to diagnose eosinophilic airway inflammation (less or more or equal to 2% sputum eosinophils)

	Threshold	Sensitivity	Specificity	PPV	NPV
Blood eosinophils	> 0.22 10 ⁹ /L	83	82	70	90
Blood eosinophils	≥ 0.25 10 ⁹ /L	74	86	72	88
Blood eosinophils	≥ 0.27 10 ⁹ /L	69	92	80	86
FeNO level	> 20 ppb	76	60	49	85
FeNO level	≥ 24 ppb	76	67	52	85
FeNO level	≥ 42 ppb	58	94	83	84
FeNO level	> 50 ppb	48	94	80	80
Periostin (in-house)	> 26 ng/ml	56	57	37	73

PPV= positive predictive value; NPV= negative predictive value

Table E3. Replication cohort: sensitivity, specificity, PPV and NPV of different surrogate markers using alternative cut-points to diagnose eosinophilic airway inflammation (less or more or equal to 3% sputum eosinophils)

	Threshold	Sensitivity	Specificity	PPV	NPV
Blood eosinophils	> 0.22 10 ⁹ /L	80	80	75	84
Blood eosinophils	≥ 0.25 10 ⁹ /L	67	85	77	77
Blood eosinophils	≥ 0.27 10 ⁹ /L	60	90	83	78
Periostin (in-house)	> 36 ng/ml	56	67	50	65

PPV= positive predictive value; NPV= negative predictive value

Figure legends

Figure E1. ROC curve analyses of the sensitivity and the specificity of serum periostin, using the Elecsys® Periostin assay, for the diagnosis of eosinophilic inflammation. **AUC** = area under the curve.

Elecsys Periostin assay

