Practical Laboratory Medicine 17 (2019) e00138

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Development of an automated ImmunoCAP research assay for eosinophil derived neurotoxin and its use in asthma diagnosis in children

Niclas Rydell [a,*], Mizuho Nagao [b], Helena Ekoff [a], Anders Sjölander [a], Magnus Borres [a], Takao Fujisawa [b]

[a] Thermo Fisher Scientific, Uppsala, Sweden
[b] Allergy Center of Mie National Hospital, Tsu, Japan

ARTICLE INFO

Keywords:
Eosinophil derived neurotoxin
Asthma
Biomarker
Automated immunoassay

ABSTRACT

Background: Eosinophil Derived Neurotoxin (EDN) is an eosinophil granule protein that is released during eosinophil activation. EDN has proven to be a promising marker for eosinophilic inflammation both in asthma and in wheezing symptoms in children.
Objectives: Here we present a novel ImmunoCAP^{TM} automated immunofluorescence research assay for measurement of EDN and its potential use in diagnosis of asthmatic children.
Methods: We report the analytical performance of the assay in serum, heparin- and EDTA-plasma in terms of precision, linearity, sensitivity, interfering substances and specimen handling. We also compared the EDN research assay with established methods for asthma diagnosis: fraction of exhaled nitric oxide (FeNO) and blood eosinophil fraction (EOS%) to demonstrate the diagnostic value of EDN in childhood asthma.
Results: The total precision (measured using percentage CV) was ≤5.8% for both serum and plasma. The dilution analysis yielded linear results across the dynamic range of the assay for both serum and plasma. No notable interferences of endogenous substances were observed.
The median concentration of EDN was significantly higher in the asthma group compared to the healthy controls and the EDN correlates well with EOS%.
Conclusions: we have shown that EDN can be measured reliably and robustly with the ImmunoCAP platform in both serum and plasma samples. EDN can be used to distinguish asthmatic from healthy children and correlates well with EOS% and could be a valuable complement to both EOS % and FeNO.

1. Introduction

Eosinophil Derived Neurotoxin (EDN) is an eosinophil granule protein that is released during eosinophil activation. EDN has proven to be a promising marker for eosinophilic inflammation both in asthma and wheezing symptoms in children [1–4] as well as in other inflammatory diseases such as rhinosinusitis, atopic dermatitis, inflammatory bowel disease (IBD) and eosinophilic GI disorders [1,5–8].

EDN is a single-chain polypeptide of approximately 18.6 kDa and it shares approximately 70% sequence homology with Eosinophil Cationic Protein (ECP). EDN has antimicrobial, antiviral and chemotactic properties [9,10] and is mainly expressed in eosinophils but

* Corresponding author. Thermo Fisher Scientific, Box 6460, 75137, Uppsala, Sweden.
E-mail address: niclas.rydell@thermofisher.com (N. Rydell).

https://doi.org/10.1016/j.plabm.2019.e00138
Received 11 January 2019; Received in revised form 29 August 2019; Accepted 17 September 2019
2352-5517/© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

can be found to a lesser degree in basophils and neutrophils [11]. Changes in blood EDN concentrations are therefore likely to depend mostly on changes in eosinophil inflammation.

Asthma is characterized by chronic airway inflammation in which eosinophils are thought to be central for the pathophysiological changes and airway remodeling [12].

In most individuals with asthma, the levels of eosinophils are elevated both in the airways and in the blood and there is a positive correlation between eosinophil counts and uncontrolled or severe asthma [13,14]. When the asthma is controlled, the eosinophil counts are usually lower [15].

Activated eosinophils release EDN, ECP, major basic protein (MBP) and eosinophil peroxidase (EPO) which all to different extent may be important for the inflammatory response seen in asthma [9]. Understanding the secretory activity in eosinophils may be crucial for the understanding of the disease [16].

Reliablie biomarkers for both diagnosis and management of asthma and wheezing symptoms are needed, especially in young children, who sometimes may have difficulties providing exhaled NO and accurate pulmonary function tests.

Here we present a novel ImmunoCAP™ automated immunofluorescence research assay for measurement of EDN and its potential use in diagnosis of asthmatic children.

We report the analytical performance of the assay in serum, heparin- and EDTA-plasma in terms of precision, linearity, sensitivity, interfering substances and specimen handling.

We also compared the EDN research assay with established methods for asthma diagnosis: fraction of exhaled nitric oxide (FeNO) and blood eosinophil fraction (EOS%) to demonstrate the diagnostic value of EDN in childhood asthma.

2. Materials and methods

2.1. EDN immunoassay description

The EDN immunoassay was developed for use with the automated ImmunoCAP technology and the Phadia instrument systems (Thermo Fisher Scientific, Uppsala, Sweden).

Briefly, a pair of complementary in-house monoclonal antibodies (mAbs) were used where the capture mAb was covalently coupled to the ImmunoCAP solid phase (a cellulose derivative enclosed in a capsule) which captures EDN in the serum or plasma sample. After washing away non-specific components in the serum or plasma, the detection mAb, labelled with the enzyme β-galactosidase was added to form a complex. After incubation, unbound enzyme-labelled anti-EDN was washed away and the bound complex were then incubated with a development solution, which reacts with the enzyme and produces a fluorescent substance. After stopping the reaction, the fluorescence of the eluate was measured. The higher the fluorescence, the more EDN is present in the sample. Developer Solution, Stop Solution and Washing Solution are all generic standard ImmunoCAP reagents. Purified EDN (Athens Research and Technologies, Athens, GA, USA) was used to develop a calibrator curve consisting of 5 calibrator points with a measuring range of 2–200 μg/l (2, 5, 15, 50 and 200 μg/L). A new software method for EDN, was developed for the Phadia 250 instrument for running the assays, including calibrator curve fitting and conversion of assay responses to EDN concentrations. The sample and enzyme conjugate incubation times were 30 and 24 minutes, respectively. The incubations were performed at 37 °C and were terminated by extensive washing steps using ImmunoCAP Washing Solution. The fluorescence was measured by the Phadia 250 instrument at the end of the assay run and EDN concentrations were given in μg/l.

2.2. Assay performance

2.2.1. Precision testing

Five individual samples with five different EDN concentrations per sample matrix (serum, heparin- and EDTA-plasma) were assayed undiluted in three replicates in five separate assay runs and by using two different Phadia 250 instruments. The concentrations were selected to cover the measuring range (2–200 μg/L). Serum EDN (sEDN) concentrations were 154 μg/l, 102 μg/l, 66 μg/l, 21 μg/l and 5.4 μg/l. Heparin plasma EDN (hEDN) concentrations were 159 μg/l, 110 μg/l, 59 μg/l, 21 μg/l and 4.3 μg/l and EDTA plasma EDN (eEDN) concentrations were 162 μg/l, 109 μg/l, 50 μg/l, 23 μg/l and 4.6 μg/l. The intra- and inter assay coefficient of variation (%CV) was calculated for each sample.

2.2.2. Dilution linearity

The dilution linearity was evaluated by diluting three samples with three different EDN-concentrations 1/2, 1/3, 1/5, 1/10 and 1/20 in ImmunoCAP IgE/ECP/Tryptase sample diluent. The following undiluted concentrations (expected concentrations) were used: sEDN 146, 95 and 41 μg/l; hEDN 194, 83 and 60 μg/l; eEDN 61, 49 and 48 μg/l.

The observed (O)/expected (E) ratio was calculated for each of the three samples using the following formula: O/E = [(observed concentration x dilution factor)/(expected concentration)]x100. Regression analysis was also used to compare expected and observed EDN concentrations.

2.2.3. Spiked recovery

The recovery of EDN in serum and plasma samples was evaluated by spiking three samples of each of the sample matrices (serum, heparin- and EDTA-plasma) with each of three different known concentrations of EDN stock solution; sEDN: 100, 50 and 25 μg/l, hEDN and eEDN: 165, 78 and 36 μg/l. The recovery (percentage) was calculated using the following formula:

Recovery (%) = [a/(b + c)] x 100, where a = concentration of spiked sample, i.e. sample + EDN stock solution (9 + 1), b = concentration of unspiked sample, i.e. serum sample + ImmunoCAP IgE/ECP/Tryptase sample diluent (9 + 1) and c = concentration of dilution buffer spiked with stock solution, i.e. ImmunoCAP IgE/ECP/Tryptase sample diluent + EDN stock solution (9 + 1). All samples were analyzed in four replicates in one assay run.

2.2.4. Analytical specificity

Cross reactivity with other eosinophilic or neutrophilic proteins was evaluated. ECP (200 μg/l), MPO (1000 μg/l), Cathepsin (1000μ/l) and lysozyme (1000 mg/l) were spiked separately into ImmunoCAP IgE/ECP/Tryptase sample diluent and analyzed in three replicates respectively in one Phadia 250 assay run.

2.2.5. Interfering substances

Two individual samples of low and high EDN concentrations (sEDN 17 μg/l and 197 μg/l; hEDN 7.6 μg/l and 189 μg/l; eEDN 7.2 μg/l and 183 μg/l) were individually spiked with each of the interfering substances (final concentration) haemoglobin (510 mg/dl), chyle (1660 FTU), bilirubin C (conjugated)(19.8 mg/dl), bilirubin F (free) (19.1 mg/dl) and IgM rheuma factor (RF) 490 IU/ml to simulate hemolytic, lipemic and icteric samples using a commercial interference study kit (Interference Check A. Plus (Sysmex Corporation, Kobe, Japan). As reference, the samples were diluted with the same volume of buffer solution (unspiked). All samples were analyzed in duplicate and mean concentrations of EDN (μg/l) for the samples were calculated. The mean concentrations of the spiked samples (observed) were compared with the mean concentrations of the unspiked reference samples (expected) according to: quotient (%) = (observed/expected) x 100.

2.2.5.1. Heterophilic/human anti-mouse antibodies (HAMA). Three individual serum samples (EDN concentrations of 91, 68 and 21 μg/l respectively) were spiked 1:10 with three different sera with known concentrations of HAMA; Two HAMA controls from BioreclamationIVT (Baltimore, MD, USA) to a final HAMA concentration of 823 ng/ml and 2425 ng/ml respectively and one heterophilic antibody control sample from Scantibodies (Santee, CA, USA) to a final heterophilic antibody concentration of 246 ng/ml. As reference (expected values), the three unspiked serum samples were pretreated with heterophilic blocking tubes (Scantibodies, Santee, CA, USA).

All samples were analyzed in duplicate and mean concentrations of EDN (μg/l) for the samples were calculated. The mean concentrations of the spiked samples (observed) were compared with the mean concentrations of the unspiked reference samples pre treated with heterophilic blocking tubes (expected) according to: quotient (%) = (observed/expected) x 100.

2.3. Specimen handling

Three individual samples of different EDN concentrations (sEDN 6.1, 18 and 214 μg/l; hEDN 4.5 μg/l, 8 and 213 μg/l; eEDN 4.3, 7.8 and 192 μg/l respectively) were collected to assess sample stability. All blood samples were kept at ambient room temperature for 1 h before centrifugation and separation of serum and plasma and then kept at 2–8 °C for 7 d, at +30 °C for 6 h.

Three individual samples were also kept at −20 °C for at least 6 months (sEDN: 11, 41 and 95 μg/l; hEDN: 9.4, 31 and 57 μg/l; eEDN: 6.6, 15 and 21 μg/l respectively).

Fresh samples (i.e. samples analyzed immediately after centrifugation and separation of serum or plasma) were used as reference and were analyzed in quadruplicate. Samples from all other time points were analyzed in duplicate and mean concentrations of EDN (μg/l) for the samples were calculated. The mean concentrations of the samples stored at different conditions (observed) were compared with the mean concentrations of the fresh sample (expected) according to: quotient (%) = (observed/expected) x 100.

2.4. EDN concentration assessments and correlation to other biomarkers in healthy and asthmatic children

2.4.1. Study population

Voluntary children aged 5–18 years were recruited through the website of Mie National Hospital. They were to be excluded from the study if they had a non-allergic chronic disease or an acute infectious disease. All voluntary children completed the questionnaires of the International Study of Asthma and Allergies in Childhood [17]. In this study, we used its Japanese version [18]. Through the website, 227 children were recruited, of which 14 failed to show up. One hundred and two children had never experienced wheezing or whistling in the chest in the past and 106 children had. According to the answers to the questions, presence or absence of current (past 12 months) symptoms of asthma, rhinitis, atopic dermatitis and physician-diagnosed asthma were identified, see Fig. 1. Of the children with a history of wheezing, 43 had experienced wheezing in the past 12 months and out of these, 37 had at some point, experienced asthma (asthmatic children). Of the 102 children with no history of wheezing, 86 had never experienced asthma (healthy children). The children were classified into two groups: healthy children control group without any symptoms of allergic diseases; asthmatic children group with current symptoms of asthma and physician-diagnosed asthma. The median age for the healthy children (n = 86) was 12 years of age (range 5–16); males; n = 43, females; n = 43 and for asthmatic children (n = 37) 10 years of age (range 6–16); males; n = 22, females; n = 15.

2.4.2. EDN in serum, heparin- and EDTA plasma

Serum EDN, hEDN and eEDN concentrations were determined as the mean of duplicates from the asthmatic children (n = 37) and the healthy children (n = 86).

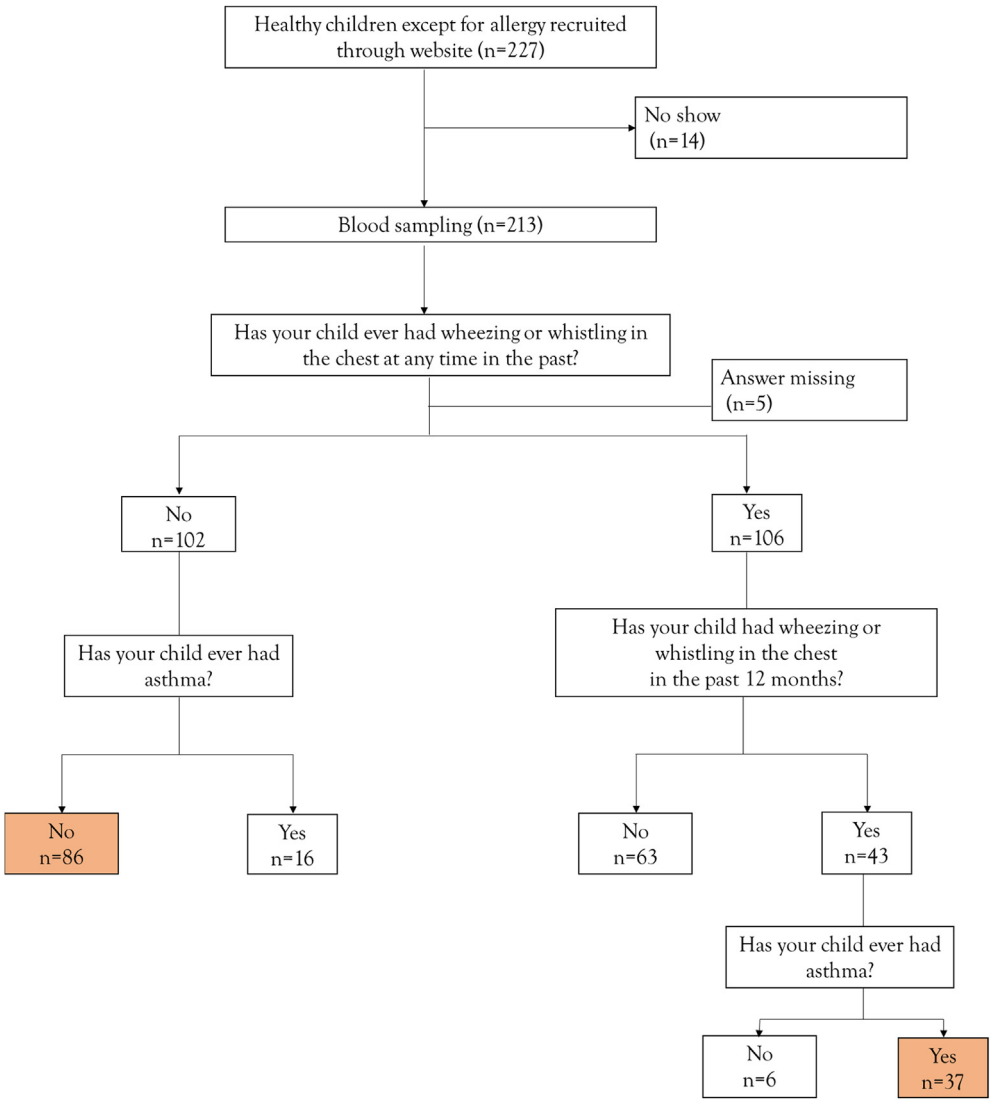

Fig. 1. Flow diagram of the patients included and excluded in the study. The children were classified into two groups: healthy children control group (n = 86) without any symptoms of allergic diseases, wheezing or asthma; asthmatic children group (n = 37) with current symptoms of asthma and physician-diagnosed asthma.

2.4.3. EOS% and FeNO

The eosinophil fraction (EOS%) was determined for all healthy and asthmatic children.by calculating the total number of eosinophils/total number of white blood cells using UniCel DxH (Beckman Coulter).

The fraction of exhaled nitric oxide (FeNO) was determined by NIOX MINO (NIOX MINO, Aerocrine, Stockholm, Sweden). FeNO determination was not done for one individual in the healthy children control group and one in the asthmatic children group due to inability to perform the procedure, thus reducing the number of healthy children for FeNO measurements to n = 85 and asthmatics to n = 36.

2.4.4. Ethical considerations

This clinical study was conducted at Mie National Hospital in accordance with the Declaration of Helsinki. Its protocol was reviewed and approved by the ethics committee of the hospital (#24-1). All patient's guardians provided written informed consent.

2.4.5. Statistical methods

The median concentrations, 95% percentiles and ranges were calculated for all biomarkers (EDN, EOS% and FeNO). Data was not normally distributed so differences between asthmatic and healthy children were evaluated using the Mann-Whitney test, p values < 0.05 were considered statistically significant. Correlations between EDN and the other biomarkers as well as correlations

Table 1
Serum EDN intra- and inter-assay precision.

Sample level (μg/l)	Intra %CV	Inter %CV	Total %CV
154	3.7	0	4.0
102	3.9	0	3.9
66	2.5	2–6	3.6
21	1.1	0.87	1.5
5.4	3.1	1.5	3.4

Intra (within assay variation), Inter (between assay variation), total (total assay variation), %CV (coefficient of variation). Samples were analyzed in three replicates in 5 different assay runs using 2 different instruments.

between EDN in serum, heparin and EDTA plasma were calculated using the Spearman's rank correlation coefficient (r). The diagnostic performance of all four biomarkers was evaluated by calculating the receiver operating characteristics (ROC) curve from which the cut-off concentration for each biomarker was obtained at the maximum combined sensitivity and specificity. The area under the curves served as means of comparing the different biomarkers regardless of cut-offs.

All statistical analyses were performed using GraphPad Prism 7.03, (La Jolla, CA, USA).

3. Results

3.1. Assay performance

3.1.1. Precision

The total precision (measured using percentage CV) was ≤5.8% for both serum and plasma samples (Tables 1–3) in five separate assay runs and using two different Phadia 250 instruments.

3.1.2. Dilution linearity

The dilution analysis yielded linear results across the dynamic range of the assay for both sEDN, hEDN and eEDN. The average correlation between observed and expected values was 0.99 for all matrices (Fig. 2). Residual plots were reviewed and no systematic biases were observed (data not shown). The observed/expected ratio was within ±10% for all dilutions (1/2-1/20) and sample matrices.

3.1.3. Spiked recovery

The mean (range) recovery of EDN spiked into serum and plasma was for sEDN 101% (95–106%), for hEDN 104% (98–109%) and for eEDN 102% (97–109%).

3.1.4. Analytical specificity

Neither ECP, MPO nor cathepsin could be detected with the EDN assay whereas lysozyme was detected at a concentration of 7 μg/l lysozyme (the concentration of lysozyme in the dilution buffer was 1000 μg/l). This corresponded to a cross reactivity of 0.7% with lysozyme.

(Lysozyme preparation likely contained small amounts of EDN since EDN and lysozyme were purified from the same source material).

3.1.5. Interfering substances

Neither of the substances bilirubin F, bilirubin C, hemoglobin, chyle and rheumatoid factor (RF) interfered notably with the assay, with observed/expected ratios for all sample matrices, ranging from 92 to 106%, Table 4.

3.1.5.1. HAMA/heterophilic antibodies. The three serum samples spiked with three different sera with known concentrations of HAMA/heterophilic antibodies had an average observed/expected ratio of 99% (range 94–105%), suggesting no assay interference by HAMA or heterophilic antibodies.

Table 2
Heparin plasma EDN intra- and inter-assay precision.

Mean (μg/l)	Intra %CV	Inter %CV	Total %CV
159	3.5	3.0	4.6
110	2.4	3.6	4.2
59	2.7	0.51	2.7
21	2.3	2.5	3.4
4.3	2.0	0	2.0

Intra (within assay variation), Inter (between assay variation), total (total assay variation), %CV (coefficient of variation). Samples were analyzed in three replicates in 5 different assay runs using 2 different instruments.

Table 3
EDTA plasma EDN intra- and inter-assay precision.

Mean (μg/l)	Intra %CV	Inter %CV	Total %CV
162	4.1	4.0	5.8
109	2.0	1.5	2.5
50	3.1	1.4	3.4
23	1.6	0.85	1.8
4.6	1.7	1.2	2.0

Intra (within assay variation), Inter (between assay variation), total (total assay variation), %CV (coefficient of variation). Samples were analyzed in three replicates in 5 different assay runs using 2 different instruments.

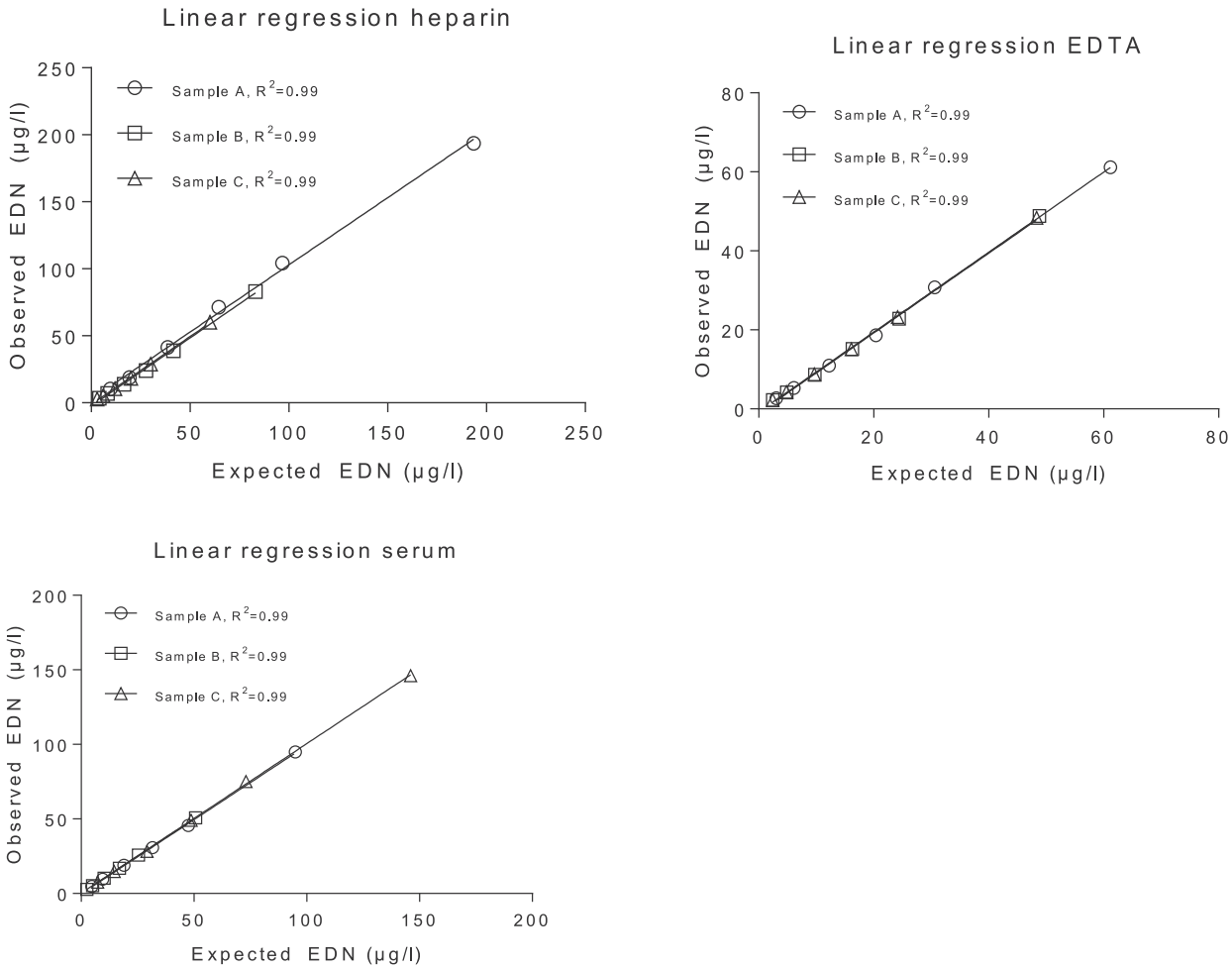

Fig. 2. Relationship (linear regression analysis) between expected and least squared mean observed EDN concentrations following dilution of samples 1 (circle), 2 (square) and 3 (triangle).

3.1.6. Specimen handling

EDN in all tested sample matrices met the specifications with an average O/E(%) between 80 and 120%, Table 5.

3.2. EDN in serum, heparin- and EDTA plasma in healthy and asthmatic children

The median concentration of EDN was significantly higher ($P < 0.0001$) in the asthma group compared to the healthy group for all three sample matrices (Fig. 3). The median EDN concentrations differed between the different sample matrices. Serum samples had higher EDN concentrations compared to heparin plasma while EDTA plasma had the lowest EDN concentrations (Table 6).

The receiver operating characteristics (ROC) curves showed similar performance between the three sample matrices. The areas under the curve (AUC) were 0.84, 0.82 and 0.81 respectively for eEDN, hEDN and sEDN (Fig. 5). There was no statistically significant differences between the AUCs.

Table 4
Mean observed/expected ratios after spiking with interfering substances into different sample matrix with low and high EDN concentrations.

	(observed/expected) x100 (%)					
	sEDN		hEDN		eEDN	
	low EDN	high EDN	low EDN	high EDN	low EDN	high EDN
Bilirubin F	106	101	96	106	103	102
Bilirubin C	100	97	103	98	99	98
Hemoglobin	93	109	105	92	108	102
Chyle	104	95	99	100	101	99
RF	96	97	101	103	101	100

RF (rheumatoid factor).

Table 5
Mean observed/expected ratios for different sample storage conditions; sEDN, hEDN and eEDN, n = 3 per sample matrix.

	(observed/expected) x 100 (%)		
	sEDN	hEDN	eEDN
RT (30 °C), 6 h	102	102	104
Refrigerated (2–8 °C), 7d	99	99	98
Frozen (−20 °C), 6 m	120	108	93

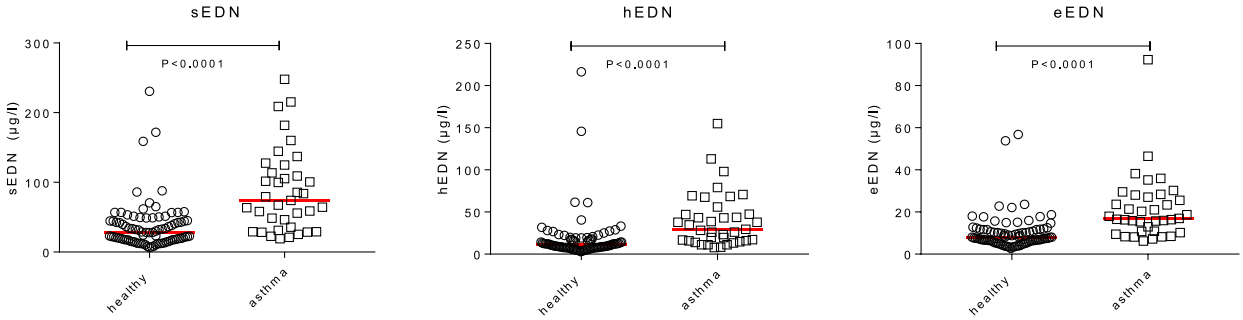

Fig. 3. EDN concentrations in healthy (n = 86) and asthmatic children (n = 37) in different sample matrices; sEDN (serum), hEDN (heparin plasma) and eEDN (EDTA plasma). The line indicates the median.

Table 6
Median EDN concentrations with 95% percentiles and ranges in healthy (n = 86) and asthmatic children (n = 37) in different sample matrices; sEDN (serum), hEDN (heparin plasma) and eEDN (EDTA plasma).

Biomarker	Asthma Median (n = 37)	95% percentile	Range	Healthy Median (n = 86)	95% percentile	Range
sEDN (µg/l)	74.1	218.6	19.0–247.9	28.1	87.2	6.5–230.5
hEDN (µg/l)	29.6	117.1	7.84–154.8	11.0	53.9	3.01–216.3
eEDN (µg/l)	17	51.0	6.24–92.3	7.83	22.6	2.71–56.8

The sEDN concentrations in all individuals (asthma and healthy) correlated with the hEDN concentration (r = 0.91) and eEDN concentration (r = 0.87) and the hEDN concentration correlated with the eEDN concentration (r = 0.93), P < 0.0001 (Fig. 4).

The ROC curves were used to find the EDN cut-off concentrations at the maximum combined sensitivity and specificity (Table 7).

3.3. Comparison of serum EDN, EOS% and FeNO in healthy and asthmatic children

The median concentrations of sEDN, EOS% and FeNO were significantly higher (P < 0.0001) in the asthma group compared to the healthy group for all three biomarkers (Fig. 6). Median concentrations with 95% percentiles and ranges are shown in Table 8.

The ROC curves showed similar performance between the three biomarkers. The areas under the curve (AUC) were 0.81, 0.78 and 0.73 respectively for sEDN, EOS% and FeNO (Fig. 8). There was no statistical differences between the AUCs.

The ROC curves were used to find the EDN, Eos% and FeNO cut-off concentrations at the maximum combined sensitivity and specificity (Table 9).

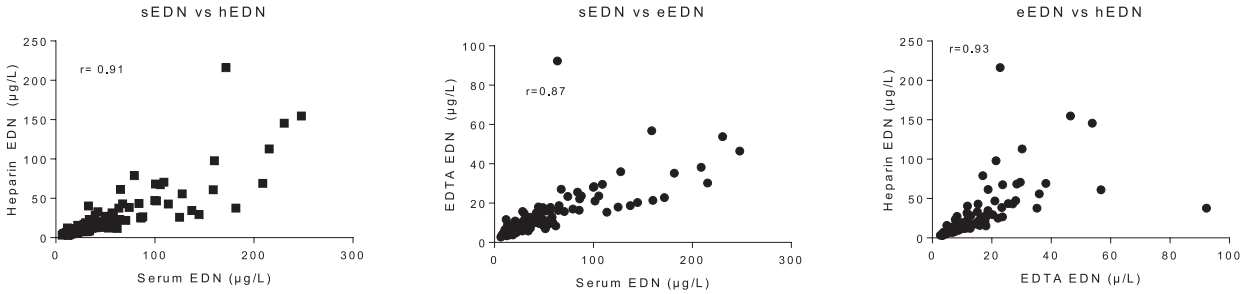

Fig. 4. Correlation plots and spearman correlation coefficients (r) for sEDN, hEDN and eEDN for all samples (healthy and asthmatic children).

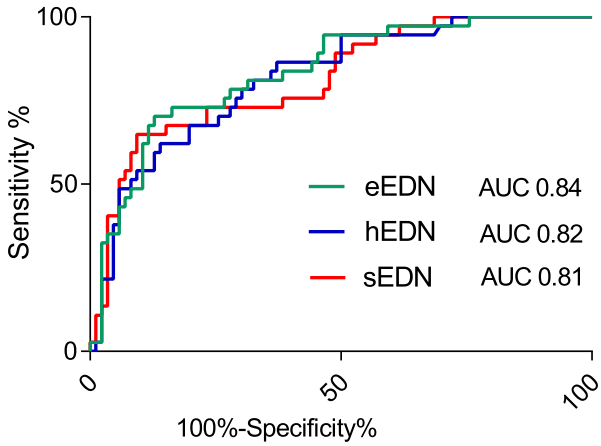

Fig. 5. ROC curves and area under curves (AUC) for eEDN, hEDN and sEDN.

Table 7
EDN cut-off concentrations at maximum combined sensitivity, specificity and area under curve (AUC) from receiver operator characteristics curves.

Biomarker	Cut-off	Sensitivity	Specificity	AUC
sEDN (µg/l)	46 µg/l	73%	77%	0.81
hEDN (µg/l)	16 µg/l	78%	70%	0.82
eEDN (µg/l)	13 µg/l	73%	84%	0.84

4. Discussion

We showed that the EDN immunoassay developed for use with the automated ImmunoCAP technology and the Phadia instrument systems (Thermo Fisher Scientific, Uppsala, Sweden) is a robust and reliable assay as indicated by: levels of precision ≤ 5.8% (including inter and intra assay variation) in serum, heparin plasma and EDTA plasma; a high sensitivity to EDN and no notable interference from haemoglobin, chyle, bilirubin (C and F) or IgM rheumatoid factor, nor from heterophilic or human anti-mouse antibodies.

EDN in all sample matrices tolerated +30 °C for 6 h as well as storage at 2–8 °C for 7 d and at −20 °C for at least 6 months, suggesting a general good sample stability.

There was a good dilution linearity covering the dynamic calibration range (2–200 µg/L) and good correlation between EDN concentrations measured in serum, heparin plasma and EDTA plasma.

The EDN concentrations from the same blood donors differed in different sample matrices according to: sEDN > hEDN > eEDN. The blood clotting prior to serum collection has been suggested by some to activate eosinophils, which makes them release more EDN thus adding to the free circulating EDN, much in the same manner as for ECP. Others claim that it might be a combined effect of eosinophils being primed to different extents in different individuals in combination with unknown factors in the different sample matrices [19–21]. Heparin prevents clotting by binding to plasma protein antithrombin and enhancing its effect against the coagulation system [22]. The mode of action of heparin most likely leaves the eosinophils in a less activated state compared to serum and thereby the hEDN concentration reflects the free circulating EDN, although this has not been proven to our knowledge. Ethylene diamine tetra acetic acid (EDTA) complexes metal ions in a 1:1 relationship. A lot of enzymatic processes are dependent on metal ions, e.g. calcium for blood coagulation. EDTA has shown optimal stabilization of blood cells since cells are inactivated and do not release any granules, thus possibly explaining the lower concentrations of EDN in this matrix [23].

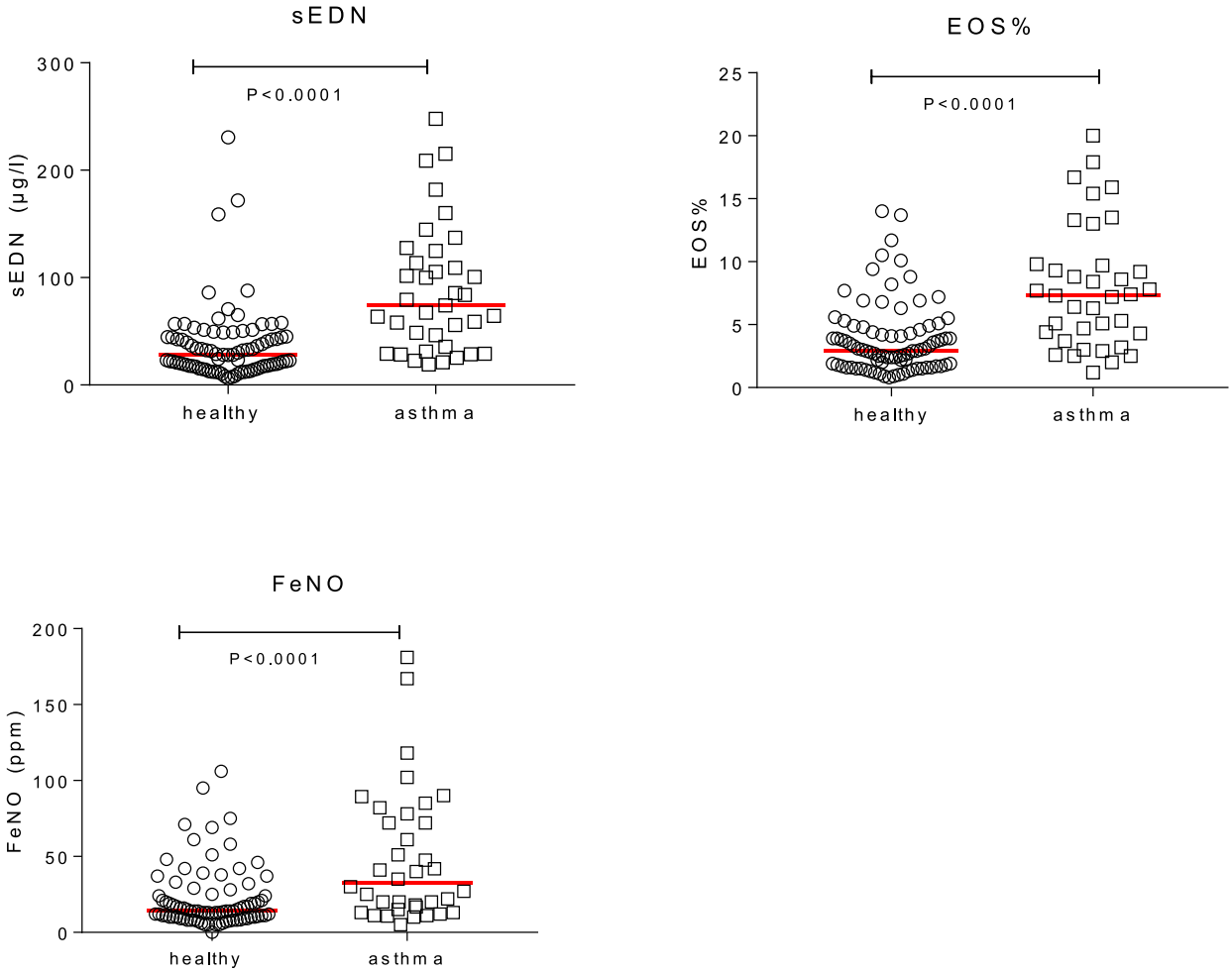

Fig. 6. Serum EDN, EOS% and FeNO concentrations in the asthmatic and healthy children. The line indicates the median concentration.

Table 8
Comparison of median biomarker concentrations with 95% percentiles and ranges for asthmatic and healthy children.

Biomarker	Asthma Median (n = 86)	95% percentile	Range	Healthy Median (n = 37)	95% percentile	Range
sEDN (µg/l)	74.1	218.6	19.0–247.9	28.1	87.2	6.5–230.5
FeNO (ppb)	32.5	169.1	5.0–181.0	14.0	70.4	0.0–106.0
EOS% (%)	7.3	18.11	1.2–20.0	2.95	10.36	0.8–14.0

Evaluation of the correlation between the serum EDN concentration and the EOS% and FeNO concentrations for all individuals (healthy and asthma) showed that serum EDN correlated with EOS% (r = 0.79), and to a lower degree with FeNO (r = 0.52) (Fig. 7).

Different sample matrices obviously results in different EDN concentrations but the mechanisms behind this discrepancy has not been fully described.

It is therefore not possible to directly compare EDN concentrations measured in different sample matrices.

Our findings show that EDN concentrations measured in serum, heparin- or EDTA-plasma were significantly higher in children with asthma compared to healthy children, which is consistent with other reports [1–4]. The maximum combined sensitivity and specificity differed somewhat between the different matrices, albeit not significantly. The sensitivity and specificity was 73 and 77% at 46 µg/l for sEDN, 78 and 70% at 16 µg/l for hEDN and 73 and 84% at 13 µg/l for eEDN with AUCs being 0.81, 0.82 and 0.83 respectively.

Serum being a commonly used sample matrix for EDN measurements was compared with EOS% and FeNO. As expected, the sEDN concentrations correlated well with EOS% but less with FeNO.

During inflammation in the airways, elevated levels of nitric oxide (NO) are released from epithelial cells of the bronchial wall [24]. Mechanistically, eosinophils and FeNO represent different pathways, which could partly explain the poor correlation [25–27]. Recently, it was shown that a combination of high FeNO and blood eosinophil count are related to impaired lung function and wheezing symptoms

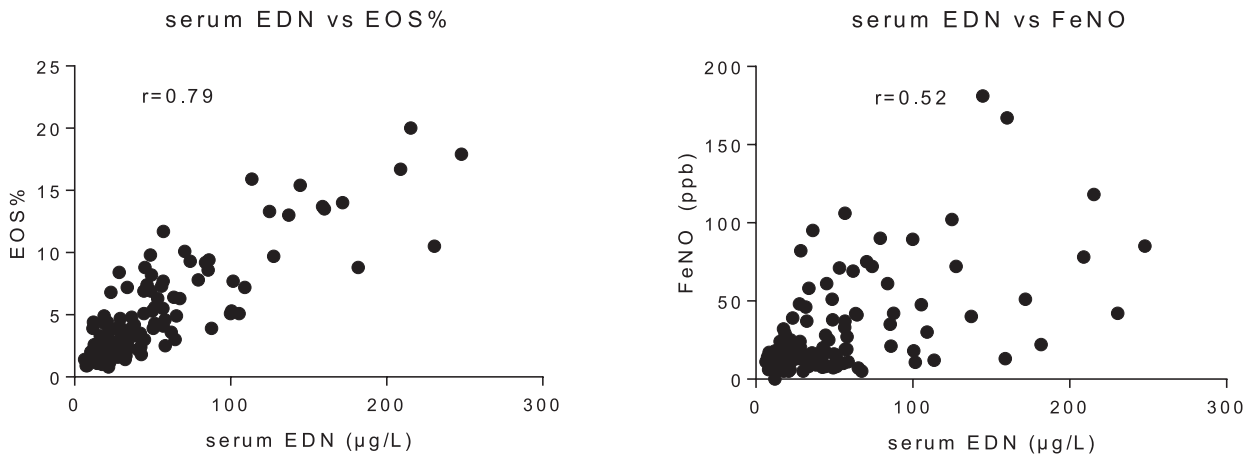

Fig. 7. Correlations and Spearman correlation coefficients (r) between EDN, EOS% and FeNO concentrations.

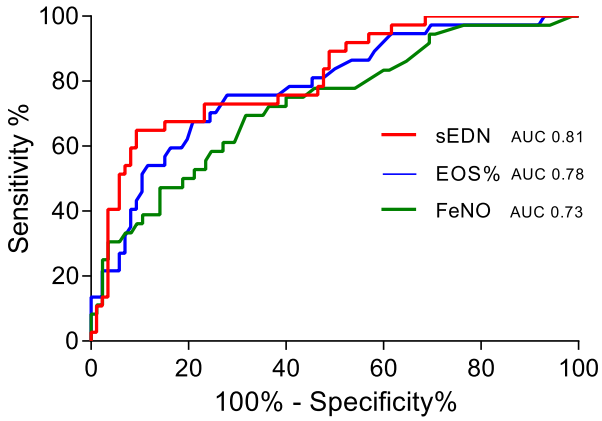

Fig. 8. ROC curves and area under curves (AUC) for sEDN, EOS% and FeNO.

Table 9
Sensitivity, specificity and area under curve (AUC) from receiver operator characteristics curves.

Biomarker	Cut-off	Sensitivity	Specificity	AUC
sEDN (µg/l)	46 µg/l	73%	77%	0.81
EOS%	4.25%	76%	72%	0.78
FeNO	19.5 ppb	69%	68%	0.73

but not to a history of asthma attacks [28]. This implies that the secretory activity of eosinophils (e.g. EDN) may be of great importance to measure in combination with other biomarkers.

Our data support this fact. In Fig. 9, the scatter plots depicting sEDN vs EOS% (Fig. 9A), sEDN vs FeNO (Fig. 9B) and EOS% vs FeNO (Fig. 9C) show that the number of children from the asthma group actually positive when using the cut-offs shown in Table 9, are indeed higher when combining the biomarkers rather than using either of the biomarkers alone.

In this study, EDN was shown to have similar diagnostic performance as EOS% and FeNO, both known biomarkers for asthma and used routinely in clinical practice.

The moderate correlation of EDN to FeNO likely reflect different aspects of asthma pathogenesis. EDN may therefore provide additional information for diagnosis and monitoring of asthma together with these markers.

Another important utility may be the application of EDN as a biomarker for preschool children in which FeNO measurement may be difficult to perform and EOS% has not been shown to be a sensitive marker.

Currently, we are working on the clinical utility of EDN in children with recurrent wheeze and preliminary results show that serum EDN may be a promising biomarker to predict the development of asthma.

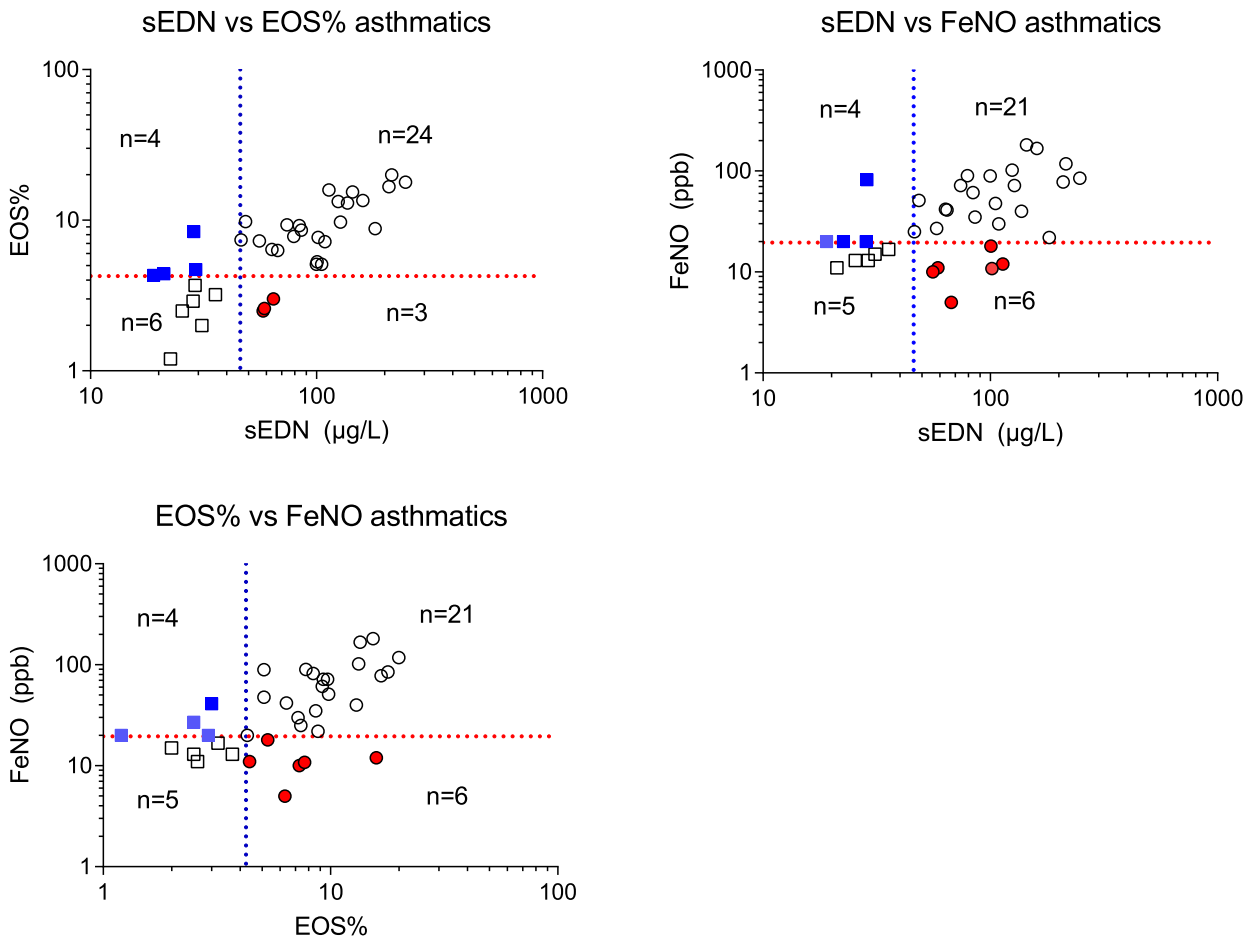

Fig. 9. Combination of biomarkers in the asthmatic group.
A-C: Open circles: asthma group samples positive for both biomarkers; Blue filled squares: asthma group samples positive for the biomarker on the y-axis, but not the x-axis; Red filled circles: asthma group samples positive for the biomarker on the x-axis, but not the y-axis. Open squares: asthma group samples negative for both biomarkers.
A: sEDN vs EOS%. Vertical dotted line shows cut-off for sEDN (46 µg/l), horizontal line shows cut-off for EOS% (4.25%), n = 37.
B: sEDN vs FeNO. Vertical dotted line shows cut-off for sEDN (46 µg/l), horizontal line shows cut-off for FeNO (19.5 ppb), n = 36, one FeNO sample not provided.
C: EOS% vs FeNO. Vertical dotted line shows cut-off for EOS% (4.25%), horizontal line shows cut-off for FeNO (19.5 ppb), n = 36, one FeNO sample not provided.

In conclusion, we have shown that EDN can be measured reliably and robustly with the ImmunoCAP platform in both serum and plasma samples. EDN can be used to distinguish asthmatic from healthy children and correlates well with EOS% and could be a valuable complement to both EOS% and FeNO in clinical practice.

Conflict of interest

Niclas Rydell, Anders Sjölander, Magnus Borres and Helena Ekoff are employees of Thermo Fisher Scientific.

Acknowledgements

We thank Lisa Thorell-Westberg, Frida Ekholm Pettersson for excellent technical and scientific support.
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2019.e00138.

References

[1] T. Goto, et al., Urinary eosinophil-derived neurotoxin concentrations in patients with atopic dermatitis: a useful clinical marker for disease activity, Allergol. Int. 56 (4) (2007) 433–438.
[2] K.W. Kim, et al., Serum eosinophil-derived neurotoxin (EDN) in diagnosis and evaluation of severity and bronchial hyperresponsiveness in childhood asthma, Lung 185 (2) (2007) 97–103.
[3] D.Y. Koller, et al., Assessment of eosinophil granule proteins in various body fluids: is there a relation to clinical variables in childhood asthma? Clin. Exp. Allergy 29 (6) (1999) 786–793.
[4] S. Taniuchi, et al., Serum eosinophil derived neurotoxin may reflect more strongly disease severity in childhood atopic dermatitis than eosinophil cationic protein, J. Dermatol. Sci. 26 (1) (2001) 79–82.
[5] H. Long, et al., A player and coordinator: the versatile roles of eosinophils in the immune system, Transfus. Med. Hemotherapy 43 (2) (2016) 96–108.
[6] M. Roca, et al., Fecal calprotectin and eosinophil-derived neurotoxin in healthy children between 0 and 12 years, J. Pediatr. Gastroenterol. Nutr. 65 (4) (2017) 394–398.
[7] H.F. Rosenberg, K.D. Dyer, P.S. Foster, Eosinophils: changing perspectives in health and disease, Nat. Rev. Immunol. 13 (1) (2013) 9–22.
[8] M.E. Rothenberg, Eosinophilic gastrointestinal disorders (EGID), J. Allergy Clin. Immunol. 113 (1) (2004) 11–28, quiz 29.
[9] S.P. Hogan, et al., Eosinophils: biological properties and role in health and disease, Clin. Exp. Allergy 38 (5) (2008) 709–750.
[10] D. Yang, et al., Eosinophil-derived neurotoxin (EDN), an antimicrobial protein with chemotactic activities for dendritic cells, Blood 102 (9) (2003) 3396–3403.
[11] R.I. Abu-Ghazaleh, et al., Eosinophil granule proteins in peripheral blood granulocytes, J. Leukoc. Biol. 52 (6) (1992) 611–618.
[12] J. Bousquet, et al., Airways remodelling in asthma: no doubt, no more? Int. Arch. Allergy Immunol. 107 (1–3) (1995) 211–214.
[13] J.D. Leuppi, et al., Predictive markers of asthma exacerbation during stepwise dose reduction of inhaled corticosteroids, Am. J. Respir. Crit. Care Med. 163 (2) (2001) 406–412.
[14] I.H. van Veen, et al., Consistency of sputum eosinophilia in difficult-to-treat asthma: a 5-year follow-up study, J. Allergy Clin. Immunol. 124 (3) (2009) 615–617, 617 e1-2.
[15] C. Lemiere, et al., Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes, J. Allergy Clin. Immunol. 118 (5) (2006) 1033–1039.
[16] P. Venge, Monitoring the allergic inflammation, Allergy 59 (1) (2004) 26–32.
[17] M.I. Asher, et al., International study of asthma and Allergies in childhood (ISAAC): rationale and methods, Eur. Respir. J. 8 (3) (1995) 483–491.
[18] M. Futamura, et al., Age-related prevalence of allergic diseases in Tokyo schoolchildren, Allergol. Int. 60 (4) (2011) 509–515.
[19] A. Björk, P. Venge, C.G.B. Peterson, Measurements of ECP in serum and the impact of plasma coagulation, Allergy 55 (2001) 442–448.
[20] Y.T. Wachtfogel, et al., Fibronectin degradation products containing the cytoadhesive tetrapeptide stimulate human neutrophil degranulation, J. Clin. Investig. 81 (5) (1988) 1310–1316.
[21] Y.T. Wachtfogel, et al., Human plasma kallikrein releases neutrophil elastase during blood coagulation, J. Clin. Investig. 72 (5) (1983) 1672–1677.
[22] E. Gray, J. Hogwood, B. Mulloy, The anticoagulant and antithrombotic mechanisms of heparin, Handb. Exp. Pharmacol. (207) (2012) 43–61.
[23] G. Banfi, G.L. Salvagno, G. Lippi, The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes, Clin. Chem. Lab. Med. 45 (5) (2007) 565–576.
[24] L.M. van den Toorn, et al., Airway inflammation is present during clinical remission of atopic asthma, Am. J. Respir. Crit. Care Med. 164 (11) (2001) 2107–2113.
[25] G.M. Gauvreau, et al., Effects of interleukin-13 blockade on allergen-induced airway responses in mild atopic asthma, Am. J. Respir. Crit. Care Med. 183 (8) (2011) 1007–1014.
[26] P. Haldar, et al., Mepolizumab and exacerbations of refractory eosinophilic asthma, N. Engl. J. Med. 360 (10) (2009) 973–984.
[27] M.J. Leckie, et al., Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response, Lancet 356 (9248) (2000) 2144–2148.
[28] I. Mogensen, et al., Simultaneously elevated FeNO and blood eosinophils relate to asthma morbidity in asthmatics from NHANES 2007-12, Clin. Exp. Allergy 48 (2018) 935–943.