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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 µl/well; 1 µg/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 µl/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 µl/well) and incubated for 2h, followed by three washes with PBST. Subsequently, detecting antibody (100 µl; 2 µg/ml) was added and left for 1h. After another 3 washes with PBST, 100 µl 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; ± 15.5% variation) and spike recovery (77.75% ± 11.69%; (mean ± SD)). The intra- and inter-assay coefficients of variability were 12.3% (9.08% ± 3.91%; (mean ± SD)) and 17.4% (12.69% ± 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 ≥ 3%	**EO < 3%**	**EO ≥ 3%**	**EO < 3%**
	n=30	*n*=80	*n*= 16	*n*= 21
Age (years)	52 ± 14.0	49 ± 13.6	55 ± 9.1	52 ± 12.9
Gender (% female)	43	54	56	48
BMI	28 ± 5.3	28 ± 5.2	31 ± 9.3	29 ± 6.0
Smoking history (py)[#]	6 (0-17)	4 (0-19)	0 (0-7.5)	0 (0-5)
Dose ICS (µg/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 ± 18.5	100 ± 16.6	86 ± 21.4	94 ± 14.7
pb FEV_1/FVC, % pred	92 ± 9.5	96 ± 11.4	82 ± 15.3	88 ± 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 ± 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

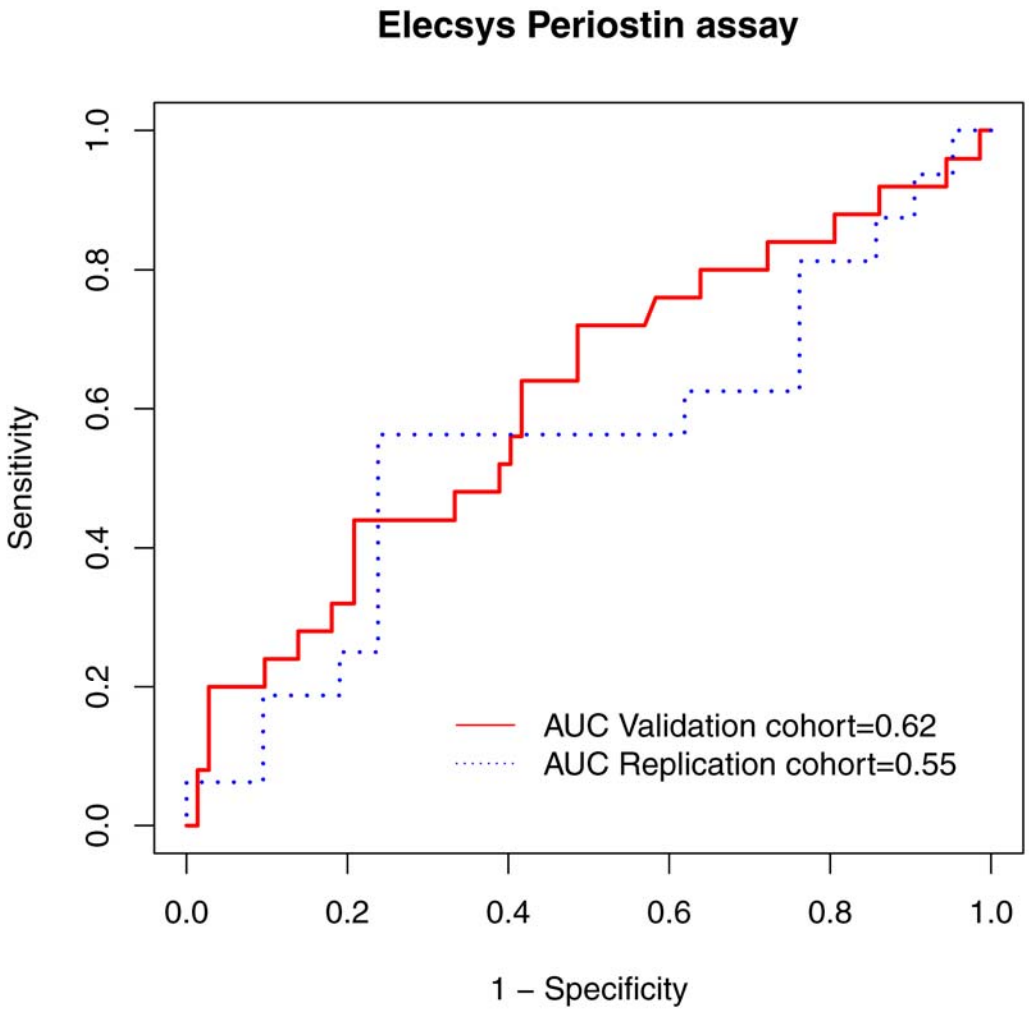
Table E3. Replication cohort: sensitivity, specificity, PPV and NPV of different surrogate markers using alternative cut-points to diagnose eosinophilic airway inflammation (less or more or equal to 3% sputum eosinophils)

	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
Sensitivity
1 – Specificity
0.0
0.2
0.4
0.6
0.8
1.0
AUC Validation cohort=0.62
AUC Replication cohort=0.55