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Article type : Original Article: Airway Diseases

Serum periostin relates to type-2 inflammation and lung function in asthma; data from the
large population-based cohort Swedish GA(2)LEN

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.13181

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Short title: Periostin in asthma and chronic rhinosinusitis

Abstract

Background

Periostin has been suggested as a novel, phenotype-specific biomarker for asthma driven by type-2 inflammation. However, large studies examining relationships between circulating periostin and patient characteristics are lacking and the suitability of periostin as a biomarker in asthma remains unclear.

Aim

To examine circulating periostin in healthy controls and subjects with asthma from the general population with different severity and treatment profiles, both with and without chronic rhinosinusitis (CRS), in relation to other biomarkers and clinical characteristics.

Methods

Serum periostin was examined by ELISA in 1091 subjects aged 17-76 from the Swedish GA(2)LEN study, which included 460 asthmatics with/without chronic rhinosinusitis (CRS), 97 individuals with CRS only, and 203 healthy controls. Clinical tests included measurement of lung function, FeNO, IgE, urinary eosinophil derived neurotoxin (U-EDN) and serum

eosinophil cationic protein (S-ECP), as well as completion of questionnaires regarding respiratory symptoms, medication and quality of life.

Results

Although median periostin values showed no differences when comparing disease groups with healthy controls, multiple regression analyses revealed that periostin was positively associated with higher FeNO, U-EDN and total IgE. In patients with asthma, an inverse relationship with lung function was also observed. Current smoking was associated with decreased periostin levels, whereas increased age and lower BMI related to higher periostin levels in subjects both with and without asthma.

Conclusion

We confirm associations between periostin and markers of type-2 inflammation, as well as lung function, and identify novel constitutional factors of importance to the use of periostin as a phenotype-specific biomarker in asthma.

Conflict of Interest Statement: None of the authors of this article have any conflicts of interest to disclose.

Author contributions: AJ wrote the manuscript and performed data interpretation. CJ and AM carried out data analysis. KA was responsible for biomarker measurements. KI devised and was responsible for periostin measurements, which were executed by JO and SO. CH was responsible for Elecsys[®] periostin measurements. AE was involved in analysis of clinical data. RM was project manager for Swedish GA(2)LEN. BD was responsible for bronchial

hyperresponsiveness testing. BD, BF, CJ, KA and SED were involved in the design of Swedish GA(2)LEN. KI and SED conceived of the current investigation. All authors participated in the drafting of the manuscript, revised it critically for content and all approve of its submission.

Introduction

Periostin has been proposed to be a novel phenotype-specific marker for asthma that is driven by type-2-mediated inflammatory processes. In humans, expression of this matricellular protein is especially high in the periosteum, hence its name (1), but more recent studies have shown that periostin is also secreted by structural cells of the airway including epithelial cells and fibroblasts where it is thought to be involved in airway fibrosis and remodeling (2). The involvement of periostin in type-2 mediated processes is suggested by its over-expression in epithelial cells from asthmatic patients, specific up-regulation by IL-4 and IL-13, and ability to determine response towards anti-IL-13 therapy in patients with asthma (3,4,5). Such “type-2-high” asthma patients have been described as being more atopic, having higher numbers of airway eosinophils, a greater degree of bronchial hyper-responsiveness and being more sensitive to corticosteroid therapy (6).

Despite the interest in periostin as a potential biomarker of type-2 inflammation in asthma, studies examining relationships between circulating periostin levels and airway eosinophilia, as well as other type-2 biomarkers, are few and have shown conflicting results. Wagener and colleagues report that periostin was unable to distinguish eosinophilic from non-eosinophilic airway inflammation, whereas Jia *et al.* reported that periostin was the most sensitive and specific predictor of airway eosinophilia (7,8). Thus, the involvement of periostin in type-2-mediated asthma remains unclear.

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It was therefore our aim to provide additional information regarding the usefulness of periostin as a biomarker by examining its levels in a comprehensive Swedish study including asthmatic subjects of varying severities from the general population, both with and without chronic rhinosinusitis. Subjects that did not fulfill the criteria for either asthma or CRS, but still reported a certain degree of respiratory symptoms were also investigated as a group named “other respiratory conditions”. This study was part of the Global Allergy and Asthma European Network (GA(2)LEN) survey, results from which have been published previously (9). Cross-sectional levels of periostin in these groups were compared with subjects without asthma, and relationships with other relevant clinical characteristics including urinary eosinophil derived neurotoxin (U-EDN) and serum eosinophil cationic protein (S-ECP), total IgE, lung function, bronchial hyper-responsiveness and exhaled NO were examined. Moreover, individual characteristics such as gender, age and BMI were also investigated in relation to periostin levels.

Methods

Study Design

Subjects in the current investigation were included from the follow-up arm of the Swedish GA(2)LEN study, which in turn is part of the European multicenter GA(2)LEN (Global Allergy and Asthma European Network) survey, both of which have been described in detail previously (9,10). Briefly, the first phase of the GA(2)LEN survey is a postal survey that aimed to examine the prevalence of asthma and chronic rhinosinusitis (CRS) symptoms in a random selection of the general population. Out of those 27,866 subjects who responded to the postal survey in Sweden, 1329 subjects took part in a second phase that consisted of a clinical follow-up and thereafter allocation to the five groups used in the current

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investigation; asthma, CRS, both asthma and CRS, other respiratory conditions that did not fulfill the criteria for either asthma or CRS, and finally, a group without respiratory conditions (9). The study was approved by the regional ethical review board in Stockholm (Dnr 2008/1100-31/4) and all subjects provided informed written consent.

Group Allocation

The participants were allocated into five groups based on self-reported symptoms in a structured interview at the clinical follow up: asthma without CRS, asthma with CRS, CRS without asthma, other respiratory disorders and controls (9). Asthma was defined by a self-reported diagnosis of asthma and either asthma symptoms (wheezing, attacks of shortness of breath and/or awakening at night with breathlessness) or by the use of asthma medication, all during the past 12 months. CRS was defined according to the European Position Paper on Rhinosinusitis and Nasal Polyps (EP₃OS) criteria (11), i.e. the presence of at least two of the following symptoms for at least 12 weeks in the past year: (i) nasal blockage, (ii) nasal discharge, (iii) facial pain or pressure, and (iv) a reduction in sense of smell with at least one of the symptoms being nasal blockage or nasal discharge.

Clinical tests

At the clinical visit, further information regarding symptoms of asthma and atopic disease, smoking, medication use and medical history was obtained during an oral interview. All patients completed the Euro Quality of Life (EQ-5D) health questionnaire and those fulfilling the criteria for asthma completed the Juniper Mini Asthma Quality of Life Questionnaire (12,13). Lung function and reversibility were assessed by spirometry (before and after

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bronchodilation with 200µg salbutamol) using the EasyOne™ spirometer (ndd Medizintechnik AG, Zurich, Switzerland). Height and weight was measured and body mass index (BMI) was calculated. The participants were categorised as never-, ex- and current smokers and pack years were calculated.

Methacholine challenge

Bronchial responsiveness was assessed by methacholine challenge in a subsample of asthmatics (n=55) from one of the centres (Stockholm). Inhalation challenge was performed using a dosimeter-controlled jet nebulizer (Spira Electro 2; Respiratory Care Center, Hameenlinna, Finland) and pulmonary function was measured as FEV₁. Challenges started with inhalation of the diluent. Provided that FEV₁ did not change by more than 10%, inhalation of methacholine commenced using the postdiluent FEV₁ value used as baseline. Methacholine was administered every third minute in doubling doses (14.2-7256 mg). The challenge was stopped when FEV₁ had fallen by at least 20%. The PD₂₀ value was derived by linear interpolation from the log cumulated dose-response curve.

Biomarker analyses

Serum for periostin analyses was available from patients included at three of the four Swedish centres (n=1091). Serum periostin levels were measured using an “in house” developed ELISA with 2 rat anti-human periostin mAbs (clones SS18A and SS17B) as described previously (14). In order to examine the periostin levels measured by this assay in relation to previously published serum periostin ranges, a comparison was performed between the periostin assay used in this study (14) and the Elecsys® periostin assay

developed by Palme *et al* (15), two of the most widely used assays to date. A strong correlation was found between the assays ($r=0.92$, $p<0.0001$), but systematically higher levels were found with the assay used in this study, and furthermore, care should be taken when comparing findings using these two assays as the difference between them was greater at higher concentrations. This analysis is reported in the online supplement.

Total IgE and serum eosinophil cationic protein (S-ECP) levels were measured using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Urinary eosinophil-derived neurotoxin (U-EDN), previously named eosinophil protein X (EPX), was measured by a sandwich ELISA (Diagnostics Development, Uppsala, Sweden), according to the manufacturer's instructions. Fraction of exhaled NO (FeNO) was measured using a NIOX MINO[®] (Aerocrine, Solna, Sweden).

Skin prick testing was performed using a standardized set of allergens for the GA(2)LEN network including timothy grass, mixed grass, birch, olive, cat, dog, cockroach, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, *Alternaria*, *Artemisia*, *Parietaria*, positive and negative controls (histamine and diluent). The number of positive prick test findings was used as a marker for the degree of IgE sensitization.

Statistics

Statistical analyses were performed using STATA 13 (STATA Corp, College Station, TX, USA) and GraphPad Prism 6 software (GraphPad Software Inc. La Jolla, CA, USA). The following variables were log-transformed prior to analyses: FeNO, S-ECP, U-EDN and total

IgE. Basic between-group comparisons were performed using Mann-Whitney tests and multiple between-group comparisons using Kruskal Wallis tests with Dunn's correction for multiple testing. Univariate correlations between periostin levels and clinical characteristics were examined by simple linear regression, and factors found to be significant were included in multiple linear regression analyses. The statistical tests used for the comparison between the two different periostin assays are described in the online supplement. A p-value of <0.05 was considered statistically significant.

Results

Cross-sectional periostin levels in GA(2)LEN subject groups

Basal circulating periostin levels were similar in the five groups examined (median (IQR), ng/ml); no respiratory condition (66 (54-78), n=206), CRS (66 (51-81), n=98), asthma (62 (50-80), n=363), asthma with CRS (66 (50-80), n=100) and those with any other respiratory condition (66 (53-83), n=333), figure 1. No significant differences were observed between any of these groups.

Relationships between periostin levels and subject characteristics in patients with and without asthma

In order to further explore relations between periostin levels and phenotypic characteristics, subjects were grouped into those with asthma (with and without CRS) and without asthma (healthy controls, other respiratory conditions and CRS) for further analyses. Relationships between periostin levels and categorical personal characteristics in these two groups are shown in figure 2. Periostin levels showed no association with gender in either asthmatic or

non-asthmatic subjects. When patients' age was grouped into 15 year intervals, those in the highest age group of 60-75 tended to display the greatest periostin levels, in both asthmatic and non-asthmatic patients. Decreasing levels of periostin were found with increased BMI along different BMI categories in both asthmatic and non-asthmatic subjects. Current smokers displayed lower periostin levels than ex-smokers and never smokers, yet the differences between these reached statistical difference in subjects without asthma only ($p=0.004$). Periostin levels were not different in atopic compared to non-atopic individuals (non-atopic 65 (52-79) vs atopic 67 (54-83), $p=0.15$, in the non-asthmatic group, and non-atopic 63 (49-83) vs atopic 63 (51-79) ng/ml, $p=0.99$, in the asthmatics). ICS use during the last week did not influence periostin levels in asthmatic subjects (63 (52-80) no ICS vs 63 (49-80) ng/ml with ICS, $p=0.47$).

Univariate correlations between periostin levels with disease-related variables were also examined in subjects with and without asthma as shown in table 1. In both groups, significant positive correlations were found with age, total IgE and FeNO. BMI showed a negative correlation with periostin levels in both asthmatic and non-asthmatic patients. Furthermore, in patients with asthma, higher periostin levels were associated with lower measures of lung function (FEV_1 and FVC). There was also a correlation between U-EDN levels and serum periostin in patients with asthma. No significant association was found between periostin and pack years, S-ECP, AQLQ and EQ-5D scores or bronchial responsiveness.

Periostin levels in "type-2-high" patients

In order to examine periostin levels in subjects that may display signs of ongoing eosinophilic inflammation, participants were grouped according to whether urinary U-EDN and exhaled NO levels were above the 3rd quartile (73 μ g/mmol creatinine and 25 ppb, respectively),

resulting in 4 groups of individuals with either low U-EDN and low FeNO, high U-EDN and low FeNO, low U-EDN and high FeNO or high U-EDN and high FeNO. Compared to those with low U-EDN and low FeNO, periostin levels were significantly higher in those with high U-EDN and high FeNO only, both in participants with asthma ($p=0.054$) and participants without asthma ($p=0.037$), figure 3.

Multiple regression model

In order to examine which subject characteristics show relationships with periostin following adjustment, a multiple regression model was designed including the variables that showed associations with periostin in the univariate analyses (table 2). Those variables that were found to be associated after adjustment with higher periostin levels in all subjects, with or without asthma, were old age (>60 years), having a lower BMI, a higher total IgE, as well as having both high FeNO and high urinary U-EDN levels. Current smoking was associated with lower periostin levels. In patients with asthma, poorer lung function was also associated with higher periostin levels. No association was found between periostin and pack years smoking, and including pack years in the models did not change any of the other associations.

Sensitivity analyses

The analyses above were also performed in healthy subjects alone, $n=203$. The association between periostin and BMI ($p=0.001$) and periostin and total IgE ($p=0.004$) remained significant when analyzing this subsample, while all other associations became statistically non-significant.

Discussion

In the present study, serum periostin levels were not elevated in 460 subjects with asthma and/or chronic rhinosinusitis or other respiratory diseases compared to 203 healthy controls in subjects selected from a large, population-based sample. Periostin was, however, independently and positively associated with higher FeNO, urinary U-EDN and total IgE levels, and specifically in patients with asthma, also with measures of lung function. Constitutional factors, such as increased age and lower BMI, were related to higher periostin levels in subjects both with and without asthma. Current smoking was associated with decreased periostin levels in subjects both with and without asthma. No associations were observed between periostin levels and atopic status or quality of life.

Despite observations of increased tissue expression of periostin, both mRNA and protein, in airway epithelial cells from patients with asthma and nasal tissue from patients with chronic rhinosinusitis (4,16,17,18), we do not observe differences in circulating periostin levels in these two disease groups compared to controls. Relatively few studies have examined cross-sectional blood periostin levels in patients with asthma compared to healthy controls, but our findings are in line with those of Caswell-Smith *et al* and Fingleton *et al* who observe similar serum periostin levels in 480 subjects without respiratory disease and 386 patients with obstructive airway disease in two separate investigations utilizing the same periostin assay (19, 20). Likewise, no between-group differences in serum periostin were observed in two smaller studies of patients with asthma as compared to healthy controls (21, 22). Matsusuka and colleagues did however report significantly elevated serum periostin levels in 190 asthmatic patients of varying severities, but this was compared to 11 healthy subjects (23).

Differences regarding periostin levels observed when comparing studies of patients with asthma could relate to the inflammatory phenotype of the patients included. Asthma is now widely recognized as a heterogeneous disorder consisting of several sub-phenotypes, often classified as being mediated by type-2 or non-type-2 inflammatory processes (6). Periostin levels have been suggested to reflect a type-2 inflammation due to upregulation of the *POSTN* gene by type-2-specific cytokines IL-4 and IL-13 (4), and by the finding that high serum periostin levels are associated with improved response towards anti-IL-13 treatment (5). Weak, yet significant correlations between periostin and exhaled NO and blood eosinophils, or increased periostin levels in groups of asthmatic subjects characterized by eosinophilic airway inflammation are also common (20,21,24,25). We demonstrate that periostin levels are independently related to higher FeNO, urinary U-EDN and total IgE levels (although interestingly, not specific IgE sensitization), which is in line with higher periostin levels reflecting a more eosinophilic airway inflammation, although a limitation of the current study is that neither blood nor sputum eosinophils were measured. However, urinary U-EDN (previously eosinophil protein X) may reflect eosinophil activation in patients with asthma (26, 27), and higher levels are associated with eosinophilic asthma in children (28). Somewhat surprisingly, we found no association between serum S-ECP and periostin.

The current observation that higher periostin levels are associated with poorer lung function in patients with asthma is of particular interest and confirms previous observations (20). It has been reported that higher circulating periostin levels are associated with a greater decline in FEV₁ over a period of four years (29). Matsusaka *et al* also observe that asthmatic patients with higher periostin levels have a lower FEV₁/FVC ratio (23). This may relate to the involvement of periostin in tissue repair and matrix organization. In addition to periostin being up-regulated by IL-13, a key cytokine involved in subepithelial fibrosis and

smooth muscle hyperplasia (4), periostin is also known to interact with collagen, fibronectin and integrins within the extracellular matrix (30), and to increase TGF β production by epithelial cells, a growth factor also involved in fibrotic mechanisms e.g. via its effects on fibroblast collagen production (2). Fittingly, Hoshino and colleagues recently demonstrated an association between serum periostin levels and markers of airway remodeling such as airway wall area (31). Periostin levels may therefore reflect both processes of eosinophilic inflammation and airway remodeling.

In epithelial cells, the POSTN gene has been shown to be down-regulated by corticosteroid treatment (4), suggesting that corticosteroids could in theory reduce circulating periostin levels. Accordingly, small reductions in baseline serum periostin levels have been observed following 12 or 16 weeks of ICS treatment (20, 32). Potentially, serum periostin could therefore be higher in asthmatics, but reduced due to medication use. However, grouping asthmatic patients according to ICS or OCS use has not revealed significant differences in periostin levels (20, 21, 22), and periostin levels have even been reported to be higher in patients taking high doses of ICS compared to those taking lower doses (29). In this cross-sectional study of predominantly mild asthmatics, about one third of patients reported ICS use in the past week, and therefore the current cohort may not be able to adequately reveal potential effects of corticosteroid use, or be of relevance to severe asthma.

In order to address the fact that many of the studies discussed use different assays to measure periostin and report different levels, we have performed a comparison between two of the most published and characterized methods; the assay developed by Izuhara and colleagues (14) and the Elecsys[®] periostin assay developed by Roche Diagnostics (15). There was a strong correlation between results obtained using the two techniques, although the ShinoTest assay consistently detected higher levels than the Roche Elecsys[®] assay. Biological information obtained using these well-characterized assays is therefore

likely similar, even though individual values may not be directly compared. Furthermore, it is important to note that the values obtained from the two assays differ more as periostin concentration increases. Ideally, it would be equally important to compare and characterize other commercially available periostin assays which all use different antibodies with differing affinities, sensitivities, and different reagents and standards for calibration. Furthermore, eight different periostin isoforms have been identified to date (33), and these may be detected to a different extent by different assays.

In the present study, certain constitutional and lifestyle factors were found to affect periostin levels which may complicate its use as a biomarker in asthma. Periostin was reduced in current smokers compared to never smokers or previous smokers. This effect was apparent in subjects both with and without asthma, and confirms previous observations (19, 22). Interestingly, periostin levels were also higher in older subjects, whereas lower levels were associated with higher BMI, both in those with and without airway disease. The negative association between periostin and BMI has been reported (19, 20). One hypothesis may be that circulating periostin also reflects periosteum cell activity in an ongoing effort to maintain bone quality (1). Risk of osteoporosis increases with age, and a higher BMI is protective for the development of osteoporosis (34), possibly implicating normal bone metabolism as a confounding factor of relevance to circulating periostin levels.

The present study is based on patients with asthma from a sample of the general population. An advantage of this is that the results have a high external validity but a drawback is that the majority of patients in this kind of sample have relatively mild asthma. The definition of asthma in this study was based on a combination of self-reported diagnosis and asthma symptoms or current asthma medication. This kind of diagnostic criteria has been shown to have a high sensitivity when compared to clinical diagnosing (35), but there is naturally always a risk of misclassification.

The present findings support the notion that circulating periostin levels are associated with “type-2” or eosinophilic inflammation at a group level in asthma, although on the whole the associations are relatively weak. Furthermore, the reported effects of smoking, age and BMI on periostin levels also need to be considered, and may complicate the clinical usefulness of periostin as a subtype-specific biomarker of airway inflammation.

Acknowledgements

The study was carried out as part of the GA(2)LEN survey and follow-up, and was supported by the EU FP6 project GA(2)LEN (EU contract number FOOD-CT-2004-506378), The Centre for Allergy Research at Karolinska Institutet, The Swedish Heart Lung Foundation, the Medical Research Council, the Vårdal Foundation, Stockholm County Council (ALF), the Swedish Asthma and Allergy Association, the Swedish Foundation for Strategic Research, Konsul Th C Berghs Foundation, the Karolinska Institutet SciLifeLab Collaborations on Translational Medicine (ChAMP) project and Karolinska Institutet.

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Table 1. Univariate correlations between periostin and disease-related variables.

	No asthma (n=633)	p-value	Asthma (n=460)	p-value
Age (10 years)	2.2 (1.0,3.4)	<0.0004	2.7 (1.0-4.4)	0.002
BMI (5 units)	-4.3 (-6.3,-2.2)	<0.0001	-5.2 (-7.8, -2.7)	0.0001
Pack years	-0.08 (-0.22, 0.05)	0.23	0.02 (-0.16, 0.21)	0.79
FEV ₁ (10% units)	-0.2 (-1.5,1.1)	0.77	-2.8 (-4.2, -1.3)	<0.0001
FVC	-0.5 (-1.8, 0.8)	0.46	-2.4 (-3.9, -0.8)	0.003
FEV ₁ /FVC	-0.03 (-0.30, 0.24)	0.82	-0.26 (-0.52, 0.01)	0.06
AQLQ total	-	-	-1.0 (-3.8, 1.8)	0.46
EQ5D index	9.9 (-0.2, 19.9)	0.054	-2.9 (-14.4, 8.6)	0.62
Total IgE (log)	1.4 (0.04, 2.8)	<0.04	3.7 (2.1, 5.4)	<0.0001
S-ECP	-5.1 (-11.1, 0.9)	0.09	6.9(-1.4, 15.1)	0.10
FeNO (log)	10.5 (8.6, 18.0)	0.007	19.4 (10.7, 28.0)	<0.0001
U-EDN (log)	5.1 (-2.0, 12.2)	0.16	13.5 (4.4-22.5)	0.004
Methacholine PD ₂₀ (log)*	-	-	-3.1 (-12.0-5.6)	0.48

Results of univariate regression analyses are shown as beta (95% CI) with p-value.

*Methacholine challenge was performed in a subset of 55 asthmatic subjects only.

Table 2. Multiple regression including variables that were statistically significantly associated with periostin in the univariate analyses.

	No asthma	p-value	Asthma	p-value
Age 16-30	1		1	
>30-45	-2.2 (-8.0, 3.7)	0.47	4.3 (-3.1, 11.7)	0.25
>45-60	2.0 (-4.0, 8.0)	0.52	7.9 (-0.22, 16.0)	0.06
>60-75	14.3 (7.7, 20.8)	<0.0001	14.9 (4.9, 24.8)	0.004
Never-smoker	1		1	
Ex-smokers	-1.9 (-6.5, 2.7)	0.42	-0.57 (-6.8, 5.6)	0.86
Current smokers	-8.5 (-14.5, -2.6)	0.005	-10.9 (-21.4, -0.52)	0.04
BMI 5 units	-5.1 (-7.4, -2.8)	<0.0001	-6.3 (-9.3, -3.4)	<0.0001
FEV1 10% units	0.56 (-0.85, 2.0)	0.44	-2.4 (-4.1, -0.77)	0.004
Total IgE (log)	1.5 (0.08, 3.0)	0.04	3.1 (1.1, 5.1)	0.002
U-EDN and FeNO low*	1		1	
U-EDN high FeNO low*	-0.42 (-5.7, 4.9)	0.88	-4.1 (-12.0, 3.9)	0.32
U-EDN low FeNO high*	-3.1 (-8.7, 2.5)	0.27	-0.03 (-7.2, 7.1)	0.99
U-EDN and FeNO high*	10.7 (0.82, 20.5)	0.03	10.0 (1.1, 18.9)	0.03

Results of multivariate regression analyses are shown as beta (95% CI) with p-value.

*U-EDN and FeNO grouped in relation to having U-EDN and FeNO high (≥ 73 $\mu\text{g}/\text{mmol}$ creatinine and 25 ppb, respectively) or U-EDN and FeNO low (<73 $\mu\text{g}/\text{mmol}$ creatinine and <25 ppb, respectively).

Figure Legends

Figure 1. Median serum periostin levels in participants with or without asthma and chronic rhinosinusitis (CRS). Groups were compared by Kruskal-Wallis test with Dunn's correction for multiple comparisons. No significant differences were found among any of the subject groups.

Figure 2. Effects of constitutional factors on circulating periostin levels. Median periostin levels are shown in participants with or without asthma (without asthma ●, with asthma ●) in relation to (A) gender, (B) age (15- year intervals), (C) smoking status or (D) BMI (kg/m^2). P values reflect results of Mann-Whitney comparison (gender), or Kruskal-Wallis 3- or 4-group comparison (age, smoking status, BMI). *Comparisons where $p < 0.05$ following Dunn's correction for multiple tests.

Figure 3. Periostin levels in relation to Type-2 biomarkers. Median periostin levels are shown in participants with or without asthma (without asthma ●, with asthma ●), grouped in relation to having high levels of U-EDN and FeNO (≥ 73 $\mu\text{g}/\text{mmol}$ creatinine and 25 ppb, respectively) compared to U-EDN and FeNO low (< 73 $\mu\text{g}/\text{mmol}$ creatinine and < 25 ppb, respectively). Groups were compared by Kruskal-Wallis test (4 groups), and p-values are shown for comparisons significant following Dunn's correction for multiple tests.





