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Fixed airflow obstruction relates to eosinophil activation in asthmatics

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Abstract

Background

Some asthmatics develop irreversible chronic airflow obstruction, e.g., fixed airflow obstruction (fixed-AO). This is probably a consequence of airway remodeling, but neither its relation to inflammation, nor which asthma biomarkers can be clinically useful are elucidated. We hypothesized that the presence of type-2 inflammation relates to fixed-AO.

Objectives

Evaluate the presence of four markers for type-2 inflammation in fixed airflow obstruction among asthmatics.

Methods

This was a cross-sectional study of 403 participants with asthma, aged 17-75 years, from three Swedish centers. Fixed airflow obstruction was defined as forced expiratory volume during the first second (FEV₁) over forced vital capacity (FVC) being below the lower limit of normal (LLN). The following type-2 inflammation markers were assessed: exhaled nitric oxide (FeNO), serum periostin, serum eosinophil cationic protein (S-ECP) and urinary eosinophil-derived neurotoxin (U-EDN).

Results

Elevated U-EDN (values in the highest tertile, ≥ 65.95 mg/mol creatinine) was more common in subjects with fixed-AO vs. subjects without fixed-AO: 55% vs. 29%, $p < 0.001$. Elevated U-EDN related to increased likelihood of having fixed-AO in both all subjects and never-smoking subjects, with adjusted (adjusted for sex, age group, use of inhaled corticosteroids last week, atopy, early onset asthma, smoking history and packyears) odds ratios (aOR) of 2.38 (1.28-4.41) and 2.51 (1.04-6.07), respectively. In a separate analysis, having both elevated S-ECP (>20 $\mu\text{g/L}$) and U-EDN was related to having the highest likelihood of fixed-AO (aOR (95% CI) 6.06 (2.32-15.75)). Elevated serum periostin or FeNO did not relate to fixed-AO.

Conclusions and clinical relevance

These findings support that type-2 inflammation, and in particular eosinophil inflammation, is found in asthma with fixed-AO. This could indicate a benefit from eosinophil-directed therapies. Further longitudinal studies are warranted to investigate causality and relation to lung function decline.

Introduction

Asthma is a chronic inflammatory respiratory disease affecting 1-18% of the population worldwide (1). Reversible airflow obstruction and inflammation in the airways are hallmarks of asthma. However, some asthmatics develop irreversible chronic airflow obstruction and decreased lung function as a long-term complication. This is regarded as a consequence of airway remodeling: smooth muscle thickening, basal membrane thickening, goblet cell

hyperplasia and epithelial shedding (2). The mechanisms in this process seem to begin early in the disease progression (3, 4). However, it has been difficult to attribute a clear preventive effect of anti-inflammatory treatment with respect to decline in lung function (5) or histological remodeling changes (3). An explanatory mechanism could be attributed to a low intensity inflammation, leading to histological changes but not necessarily to asthma symptoms. This would eventually lead to inadequately treated asthma, as therapy is mainly guided by symptoms in clinical practice. This could further lead to an inflammation-dependent remodeling of the airways (6-8).

The type of inflammation most commonly seen in asthma is type-2 inflammation (9), dependent on T-helper cells type 2 (Th2), type 2 innate lymphoid cells (ILC-2) and other cell types involved in the production of interleukin (IL-)4, IL-5 and IL-13 (10). Four biomarkers known to reflect type-2 inflammation are: serum periostin (S-periostin), fraction of exhaled NO (FeNO), urinary eosinophil-derived neurotoxin (U-EDN), and serum eosinophil cationic protein (S-ECP). S-periostin is a matricellular protein related to type-2 inflammation (11), IL-4 and IL-13(12, 13). Nitric oxide is produced by the airway epithelium, and is increased by IL-4 and IL-13 in particular (14, 15). Eosinophil levels depend on IL-5 (16, 17). Both eosinophil-derived neurotoxin (EDN), (RNASE2(18)) (previously called eosinophil protein X, EPX (19), note, EPX can also refer to eosinophil peroxidase(18)) and eosinophil cationic protein (RNASE3) (ECP) are basic proteins mainly contained in the granules of eosinophils, but also to a lesser extent in neutrophils, and are released following stimulation (20-22).

We have previously demonstrated that a combination of type-2 biomarkers, FeNO, and S-ECP relates to more asthma attacks the preceding three months(23). It is important to identify if there is a relation between fixed airflow obstruction and type-2 inflammation markers, as these markers of inflammation are regarded as treatable disease traits (24) and therefore might change the course of the disease.

We hypothesized that presence of type-2 inflammation is related to fixed airflow obstruction (fixed-AO) defined as forced expiratory volume the first second (FEV₁) over forced vital capacity (FVC) < lower limit of normal (LLN). The aim of this study was to cross-sectionally investigate the relation between four type-2 inflammatory markers and fixed-AO.

Method

Study group

The study population consisted of participants from three Swedish centers (Umeå, Uppsala and Stockholm) of the Global Asthma and Allergy Excellence Network (GA2LEN) survey (25). In Sweden, 45,000 individuals received a postal survey in 2008. The response rate was 60% (27,866 individuals). From the responders, those with asthma, chronic rhinosinusitis (CRS), both asthma and CRS and controls were selected randomly for a clinical visit between 2008-10. In total, 1174 individuals participated in the clinical visit in Umeå, Uppsala or Stockholm. Asthma was defined as self-reported doctor's diagnosis of asthma in combination with asthma symptoms or asthma treatment during the past 12 months. In all, 403 individuals were included in the study. A total of 82 subjects had concomitant chronic rhinosinusitis (Figure 1). The age range of participants was 17-75 years and the participants were investigated with spirometry, blood and urinary samples and body measures. Adult-onset asthma was defined as onset at 18 years of age or older. The median asthma duration was 19 years, and the group was divided in long and short asthma duration based on this cut-off.

Lung function and definition of fixed airflow obstruction

Spirometry was done using the EasyOne Spirometer (nnd Medizintechnik AG, Zurich, Switzerland). Forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV₁) were registered pre- and 15 min post-bronchodilation (BD) with 200 µg salbutamol. The Global Lung Function Initiative reference values were used (26). Forced exhaled volume in the first second (FEV₁) and forced vital capacity (FVC) were measured pre- and post-BD. Fixed airflow obstruction (AO) was defined as having post-BD FEV₁/FVC < lower limit of normal (LLN) (26).

Questionnaires

Asthma symptoms and attacks were evaluated through structured interviews based on the European Community Respiratory Health Survey (27), performed by experienced interviewers. Use of long-/short-acting beta-adrenoceptor agonists (LABA/SABA), inhaled corticosteroids (ICS) or oral corticosteroids, inhalation of anti-muscarinic drugs and leukotriene receptor antagonists was reported. The current dose ICS used was converted to equivalent dose of Budesonide. The participants were asked about doctor's diagnosis of chronic obstructive lung disease (COPD). A total of 21 subjects reported such a diagnosis. However, half of them had a post-bronchodilation FEV₁/FVC > LLN. We therefore chose not to include self-reported COPD in our analyses, but to adjust for smoking instead (pack-years).

Atopy

Atopy was defined as a positive skin prick test with a wheal at least 3 mm at the widest diameter for any of the investigated aeroallergens. The following aeroallergens were evaluated: timothy grass, mixed grass, *Dermatophagoides pteronyssinus*, cat, dog, birch, cockroach, olive, *Alternaria*, *Artemisia*, *Parietaria* and *Dermatophagoides farina*.

Biomarker analyses

FeNO was assessed using a handheld device with electrochemical sensor in accordance with the European Respiratory Society (ERS)/American Thoracic Society (ATS) guidelines(28), NIOX MINO (NIOX MINO; Aerocrine, Stockholm, Sweden). S-ECP was measured using a fluorescence enzyme immunoassay (ImmunoCAP, Thermo Fisher Scientific, Immunodiagnosics, Uppsala, Sweden). S-periostin was measured using ELISA with two rat anti-human periostin mAbs (clones SS18 and SS17B) (29). U-EDN, (previously called eosinophil protein X, EPX) was measured, in accordance with the manufacturer's instructions, with a sandwich ELISA utilizing a polyclonal EDN antibody as catching antibody and a monoclonal antibody (clone 618) as detecting antibody (Diagnostics Development, Uppsala, Sweden).

Definitions of elevated biomarkers

A FeNO value ≥ 25 ppb was defined as elevated, in accordance with the ATS clinical guidelines (30). An S-ECP level ≥ 20 $\mu\text{g/L}$ was defined as elevated, as previously recommended (31). The cut-offs for S-periostin and U-EDN were defined as the upper tertile, for S-periostin ≥ 74 $\mu\text{g/L}$ and for U-EDN ≥ 65.95 mg/mol creatinine. In the total population consisting of 403 individuals, 372 had S-periostin samples and 32% (118) of them had

elevated S-periostin, 394 had FeNO samples and 32% (126) of them had elevated FeNO, 361 had U-EDN samples and 33% (120) of them had elevated U-EDN, and 341 had S-ECP samples, of which 33% (114) showed elevated S-ECP.

Smoking history

Data on smoking were collected through the structured interviews. The participants were divided into current smokers, previous smokers or never-smokers. A pack year was defined as corresponding to one pack of cigarettes per day during one year's time. Previous and current smokers had smoked for at least one pack year.

Statistical analyses

The statistical analyses were performed using the software Stata/IC 13.1 (Stata Corp, College Station, TX, USA). The differences between the group with fixed-AO and the group without fixed-AO were evaluated regarding population characteristics and symptom history using univariate analyses, Pearson's chi-squared test, t-tests (for normally distributed variables) and Wilcoxon rank-sum test (for variables with skewed distribution). Thereafter, differences in prevalence of elevated inflammatory markers were studied between subjects with fixed-AO and subjects without fixed-AO, using Pearson's chi-squared test. The population group was divided into three age groups, 17-36, 37-53 and 54-75 years old, for sub-analyses. Similarly, the population was divided into three body mass index (BMI) groups, with BMI < 25, 25-30 and ≥ 30 . The correlations between the markers were evaluated, as well as the relation to fixed-AO in the following sub-categories: sex, age groups, atopic status, more or less than 10 pack years' smoking and smoking history. Thereafter, multiple logistic regression analyses were done for fixed-AO as the outcome and elevated inflammatory markers as predictors,

with concomitant adjustment for sex, age group, use of inhaled corticosteroids in the week prior to the clinical visit, atopy, pack years' smoking, early asthma onset and smoking history. These adjusted analyses were also repeated after excluding previous and current smokers.

To assess the validity of the relation between airway obstruction and inflammatory markers, regression analyses were made between log-transformed levels of S-periostin, FeNO, U-EDN, S-ECP and post-bronchodilation FEV1/FVC (expressed as z-score), both with and without adjustments for the potential confounders listed above. For the sub-categories above (sex, age groups, atopic status, more or less than 10 pack years' smoking and smoking history) interaction analyses were done in an adjusted logistic regression model, adjusted for the same covariates above. As a secondary analysis, the eosinophil markers were analyzed in combination in relation to fixed-AO. Missing data was handled with complete cases.

Ethics statement

The study was approved by the Regional Ethical Review Board in Stockholm, Sweden (Dnr. 2008/1100-31/4) for all participating centers, and the collected personal data were processed in accordance with the Swedish Personal Data Act.

Results

Relation between the different type-2 inflammation markers

The investigated type-2 inflammation markers were weakly correlated (Online Table 1), with the highest Pearson's r value of 0.19 ($p < 0.001$) for the correlations between FeNO and U-EDN and between FeNO and S-periostin. The overlap between having elevated S-periostin,

elevated U-EDN, elevated FeNO and elevated S-ECP (in subjects with all markers available, n=302) is shown in Figure 2.

Study population and differences between the groups without and with fixed-AO

Subjects with fixed-AO were older, had been smoking more, were more likely to be men and had atopy to a lower extent than the group without fixed-AO. Moreover subjects with fixed-AO used to a greater extent ICS and LABA than subjects without fixed-AO (Table 1). The daily dose of ICS was also higher in the group with fixed-AO (Table 1). There was however no difference in symptom burden (Online Table 2).

Type-2 biomarkers in relation to fixed airflow obstruction

A higher prevalence of elevated U-EDN was found in subjects with fixed-AO than in those without fixed-AO (Table 2). There were no differences in prevalence of elevated S-periostin, elevated S-ECP or elevated FeNO between subjects with fixed-AO and subjects without fixed-AO (Table 2). U-EDN and S-ECP as continuous variables were increased in subjects with fixed-AO as compared with subjects without fixed-AO (Table 3).

In the multivariate adjusted logistic regressions, both U-EDN and S-ECP were associated with fixed-AO (Table 4). In a multiple logistic regression analysis with all four markers in the same model, adjusted for one another, U-EDN was the only marker still significantly related to fixed-AO (Table 4)

As U-EDN and S-ECP were related to fixed airflow obstruction after adjustments for other variables of interest (Table 4), a sub-analysis was performed with an adjusted logistic

regression with combinations of elevated U-EDN and elevated S-ECP. Having both elevated U-EDN and S-ECP was related to higher odds ratio of having fixed-AO (Table 5).

Sensitivity analyses

Elevated U-EDN was consistently associated with fixed-AO when the same analyses presented in Table 4 were restricted to never-smokers (Online Table 3).

The FEV1/FVC Z-score for the spirometry values post bronchodilation were analyzed in a regression analyses with log-transformed S-periostin, FeNO, U-EDN and S-ECP values. U-EDN ($p=0.003$) and S-ECP ($p=0.016$) significantly associated with lower FEV1/FVC. This was still the case after adjustment for sex, age group, use of inhaled corticosteroids in the week prior to the clinical visit, atopy, pack years' smoking, early asthma onset and smoking history for S-ECP ($p=0.001$)), but not for U-EDN ($p=0.052$). Stratified analyses for sex, age groups, atopic status, more or less than 10 pack years' smoking and smoking history suggested a stronger correlation between male sex and fixed-AO in the group with elevated S-ECP. The relation between elevated U-EDN and fixed-AO was significant in the older age groups (Online Table 4a-e). The relation between S-ECP and fixed-AO was significantly different between men and women, with aOR of having fixed-AO if having elevated S-ECP of 4.1 (95%CI: 1.52-11.1) for men and 1.1 (0.42-3.07) for women, $p_{\text{interaction}}=0.04$, in the adjusted logistic regression interaction analyses.

Discussion

In the present study, elevated levels of an eosinophil activation marker, U-EDN, related to fixed airflow obstruction, both in the whole population and in never-smoking subjects, after adjustments for potential confounders and other type-2 inflammation markers. The association to fixed-AO was even greater in subjects who had a simultaneous elevation of both studied eosinophil markers, S-ECP and U-EDN. S-periostin and FeNO were not related to fixed-AO after adjustments for potential confounders and other investigated type-2 inflammation markers.

All four markers have previously been linked to decreased lung function(32-36), and have to different extent potential to actively contribute to the remodeling process. According to this material, eosinophil activation, and especially EDN, is present in asthmatic subjects with decreased lung function and fixed-AO. While both U-EDN and S-ECP are eosinophil granule proteins with around 70% homology (37), there are differences in function and properties which probably explain the weak correlation between the markers seen in the present study. The release of these granule proteins is also to some extent dependent on different stimuli (22). However, both have properties able to injure the host tissue when released. The fibrogenic properties of ECP have been demonstrated previously, as has its implication in the process of airways remodeling in asthma (38, 39). Higher levels of ECP have been found in smokers and this has been speculated to contribute to accelerated lung function decline in smokers (36). U-EDN is mainly studied in children and a relation to decreased FEV₁ has been found (34, 35, 40). In a longitudinal study by Lugosi *et al.*, U-EDN levels were negatively correlated with FEV₁ and a significant relation to symptomatic asthma, but not to atopy or current medications, was seen (35). Nuijsink *et al.* also found a negative correlation between U-EDN levels and FEV₁, but could not find any relation to other symptoms (34).

We found no correlation to increased FeNO in the group with fixed-AO. Elevated FeNO levels have been related to faster lung function decline in difficult-to-treat asthma (32). In previous studies, periostin has been linked to decreased lung function (33) and eosinophil inflammation (41); however not to severity of disease(42). Since it has a regulatory function in the extracellular matrix (43) (44), it could potentially play a role in the process of airway remodeling in asthma. However, this has not been specifically studied in the aforementioned studies.

Smoking has a negative effect on lung function and it could therefore be argued that the presence of fixed-AO in ever-smoking asthmatics might influence our results. However, when excluding previous and current smokers, the correlation between fixed-AO and elevated U-EDN was still present.

Ageing-related changes in lung function might relate to higher prevalence of fixed-AO in elderly subjects if a fixed FEV₁/FVC ratio is used. However, this was accounted for in the present material by using LLN for defining obstruction(26).

An increase in U-EDN was found with increasing age in our study, where also the oldest participants had a significant correlation between fixed-AO and elevated U-EDN. A longer duration of asthma might result in a higher likelihood of developing fixed-AO. However, the asthma duration did not differ between subjects with fixed-AO and without fixed-AO. Atopic status was less common in the group with fixed-AO, although the correlation between U-EDN and fixed-AO was present also in the atopic group. This could suggest different pathogenesis or location for the decreased lung function seen in asthmatics who develop fixed-AO, with less allergic inflammation, but presence of eosinophil inflammation and less sensitivity to ICS as a common factor. Innate lymphoid cells type-2 (ILC-2) producing IL-5 and IL-13 could be one such factor, driving a type-2 pattern inflammation dependent on IL-5,

with eosinophil predominance and possibly a site of inflammation either systemic or located to the smaller airways less sensitive to inhaled steroids. This in contrast to an inflammation high in IL-4/IL-13 located to the bigger airways or airway mucosa sensitive to ICS, with elevated FeNO and S-periostin levels.

This study encompassed a relatively large amount of adult asthmatic individuals with information about asthma history, medication and life style exposures. Few studies, to our knowledge, have available the type-2 markers studied in the present investigation, together with spirometry pre- and post-bronchodilation. However, the cross-sectional study design makes it difficult both to establish a consistent phenotype of fixed-AO in these subjects, as the subjects were tested at only one time-point, and to evaluate causal relationships between inflammation and fixed airflow obstruction and testing at multiple time points would have done the diagnosis more reliable. Another limitation is the lack of information on blood cell count or markers of neutrophilic inflammation, as these were not measured in the study. Lack of objective evidence for actual histological changes is a further limitation. It cannot be excluded that the decreased lung function might be a result of undertreatment or ongoing exacerbation since the lung function is measured at only one time-point. Nevertheless, the finding of lung function impairment and the association to presence of eosinophil inflammation are still valid; this relation was present also in analyses between FEV1/FVC, used as continuous variable by expressing it as Z-scores, and levels of the eosinophil markers.

It is interesting to speculate if the increased levels eosinophil activation are treatable traits of the disease (24) and might respond to treatment with either inhaled corticosteroids or anti-IL-5. This appears to be supported by studies in both asthma and COPD, where anti-IL-5 treatment seems to result in improvement of lung function (45, 46). In the studied group, a higher use of inhaled corticosteroids was present in the group with fixed-AO, which could be

the result of more severe asthma or a disease less respondent to corticosteroid treatment.

Whether the phenotype of asthma with fixed-airflow obstruction could be more efficiently treated with an eosinophil directed anti-IL 5 therapy is a subject for future studies.

In conclusion, we have demonstrated the presence of type-2 inflammation and in particular eosinophil activation in fixed-AO in asthmatics. This inflammation seems to be most clearly mirrored by release of U-EDN, which might reflect a risk of more pronounced lung function decline and fixed airflow obstruction in asthmatics. This could possibly be targeted using eosinophil-directed therapies. This, as well as the relation to an active remodeling process, should be evaluated in future studies.

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Table 1. Population characteristics both for the total population and for strata based upon presence of fixed-AO.

	Total population n=403	Without fixed-AO n=336	With fixed-AO n=67	p value
Age, mean $\pm$ SD	43 $\pm$ 15	43 $\pm$ 14	50 $\pm$ 14	< 0.001
Atopy	70%	73%	57%	0.009
Female	59%	62%	48%	0.035
BMI, mean $\pm$ SD	26.5 $\pm$ 4.6	26.4 $\pm$ 4.7	26.7 $\pm$ 4.3	0.63
Childhood asthma onset < 18 years	52%	54%	42%	0.064
Age first asthma, median (lower and upper quartile)	15 (7-34)	15 (8-30)	22 (7-40)	0.25
Long asthma duration ($\geq$ 19 years)	56%	54%	64%	0.13
Smoking history				0.026
Never-smoker	56%	57%	45%	
Previous smoker	35%	34%	39%	
Current smoker	9%	7%	16%	
Lifetime smoking history >10 pack years	25%	21%	43%	< 0.001
ICS in last 12 months	47%	45%	60%	0.027
ICS in last week	35%	31%	54%	< 0.001
Oral corticosteroid use in last year	5%	3%	11%	0.009
LABA in last week	30%	27%	43%	0.007
High ICS use (> 400 μ g budesonide equivalents/day)	16%	13%	27%	0.005
Daily dose of ICS (μ g budesonide equivalents), median (lower and upper quartile)	0 (0-250)	0 (0-200)	200 (0-800)	<0.001
Anti-leukotriens in last year	5%	6%	3%	0.38
CRS	20%	20%	22%	0.65

Fixed-AO: fixed airflow obstruction, FEV₁/FVC < LLN post-bronchodilation, ICS: Inhaled corticosteroids, LABA: Long-acting beta-adrenoceptor agonist, CRS: Chronic rhinosinusitis
Evaluated with Pearson's chi-squared test for bivariate variables, t-test or Wilcoxon rank-sum test for continuous variables depending on normal or skewed distribution. Significant results (p <0.05) in bold.

Table 2. Prevalence of elevated biomarker in the group with and without fixed airflow obstruction.

	Without fixed-AO	With fixed- AO	p value
Elevated S-periostin	30%	39%	0.19
Elevated FeNO	35%	31%	0.58
Elevated U-EDN	29%	55%	< 0.001
Elevated S-ECP	32%	43%	0.10

Fixed-AO: fixed airflow obstruction, defined as post-BD FEV₁/FVC < LLN

Elevated levels of biomarkers were used as categorical variables, based on the following cut-offs: ≥ 25 ppb for FeNO, ≥ 20 $\mu\text{g/L}$ for S-ECP level, ≥ 74 $\mu\text{g/L}$ for S-periostin and ≥ 65.95 mg/mol creatinine for U-EDN.

Evaluated with Pearson's chi-squared test. Significant results ($p < 0.05$) in bold.

Table 3. Levels of type-2 biomarkers geometric mean (95%CI) in subjects without and with fixed airflow obstruction, analyzed as log transformed variables.

	Without fixed-AO	With fixed-AO	p value
S-periostin ($\mu\text{g/L}$)	62.0 (59.3-64.9)	67.0 (60.7, 73.8)	0.17
FeNO (ppb)	18.9 (17.7, 20.3)	20.6 (17.2, 24.6)	0.35
U-EDN (mg/mol creatinine)	46.3 (43.2, 49.5)	61.5 (50.4, 75.0)	0.002
S-ECP ($\mu\text{g/L}$)	13.7 (12.6, 14.9)	17.6 (14.0, 22.1)	0.02

Fixed-AO: fixed airflow obstruction, defined as post-BD FEV₁/FVC < LLN

Evaluated with t-tests, significant results ($p < 0.05$) in bold.

Table 4. Adjusted odds ratio (aOR (95%CI)) for asthma with fixed-AO if having elevated levels of different inflammatory markers.

	Asthma with fixed-AO, a*OR	Fixed-AO, model with all markers, a**OR
Elevated S-periostin	1.73 (0.92-3.25)	2.04 (0.99-4.18)
Elevated FeNO	1.15 (0.60-2.20)	0.80 (0.37-1.75)
Elevated U-EDN	2.38 (1.28-4.41)	2.73 (1.33-5.62)
Elevated S-ECP	2.07 (1.07-3.99)	1.98 (0.97-4.05)

*sex, age group, use of ICS in last week, atopy, early onset asthma, smoking history, pack-years

**sex, age group, use of ICS in last week, atopy, early onset asthma, smoking history, pack-years, elevated S-periostin, elevated FeNO, elevated U-EDN, elevated S-ECP

Fixed-AO: fixed airflow obstruction, defined as post-BD FEV₁/FVC < LLN

Elevated levels of biomarkers were used as categorical variables, based on the following cut-offs: ≥ 25 ppb for FeNO, ≥ 20 $\mu\text{g/L}$ for S-ECP level, ≥ 74 $\mu\text{g/L}$ for S-periostin and ≥ 65.95 mg/mol creatinine for U-EDN.

Significant results ($p < 0.05$) in bold.

Table 5. Adjusted odds ratio (aOR (95%CI) for asthma with fixed-AO if having different combinations of elevated ECP and EDN.

	a*OR (95% CI) for fixed-AO	a**OR (95% CI) for fixed-AO
Normal S-ECP	1	1
Normal U-EDN (n=147)		
Elevated ECP	1.52 (0.58-3.99)	1.33 (0.48-3.69)
Normal EDN (n=61)		
Normal ECP	1.76 (0.73-4.26)	2.00 (0.80-4.95)
Elevated EDN (n=64)		
Elevated ECP	6.06 (2.32-15.79)	6.05 (2.24-16.30)
Elevated EDN (n= 34)		

* sex, age group, use of ICS in last week, atopy, early onset asthma, smoking history, pack-years

** sex, age group, use of ICS in last week, atopy, early onset asthma, smoking history, pack-years, elevated S-periostin, elevated FeNO

Fixed-AO: fixed airflow obstruction, defined as post-BD FEV₁/FVC < LLN

Elevated levels of biomarkers were used as categorical variables, based on the following cut-offs: ≥ 25 ppb for FeNO, ≥ 20 μ g/L for S-ECP level, ≥ 74 μ g/L for S-periostin and ≥ 65.95 mg/mol creatinine for U-EDN.

Significant results (p <0.05) in bold.

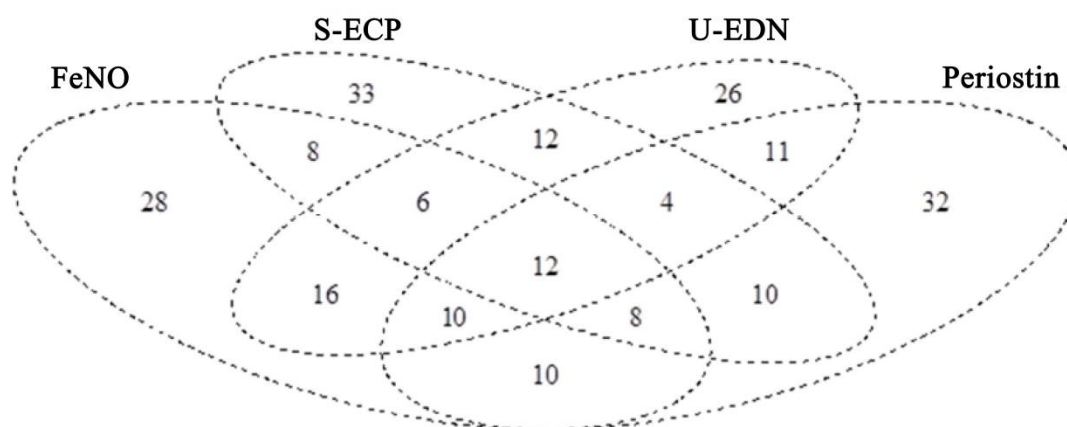
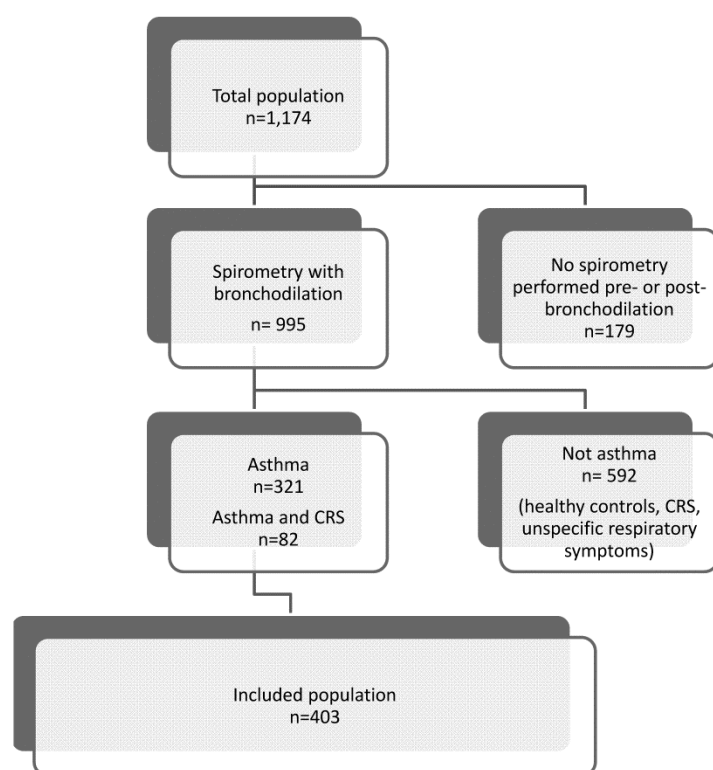
Figure legends

Figure 1. Flowchart of included population.

Figure 2. Overlap of individuals with elevated levels of type 2 inflammation biomarkers.

Only individuals with elevated biomarkers are depicted.

Elevated levels of biomarkers were used as categorical variables, based on the following cut-offs: ≥ 25 ppb for FeNO, ≥ 20 $\mu\text{g/L}$ for S-ECP level, ≥ 74 $\mu\text{g/L}$ (upper tertile) for S-periostin and ≥ 65.95 mg/mol creatinine (upper tertile) for U-EDN.



Online Supplement

Fixed airflow obstruction relates to eosinophil activation in asthmatics

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Online Table 1. Correlation between the four different type-2 markers (Pearson's r value and p value for correlations between log-transformed values).

	FeNO	S-periostin	S-ECP	U-EDN
FeNO	1	0.19 (p < 0.001)	0.17 (p=0.001)	0.19 (p < 0.001)
S-periostin	0.19 (p < 0.001)	1	0.10 (p=0.07)	0.06 (p=0.27)
S-ECP	0.17 (p=0.001)	0.10 (p=0.07)	1	0.14 (p=0.02)
U-EDN	0.19 (p < 0.001)	0.06 (p=0.27)	0.14 (p=0.02)	1

Online Table 2. Symptoms in relation to presence of fixed airway obstruction.

	Without fixed-AO n=336	With fixed-AO n=67	p value
Wheeze	81%	87%	0.28
Wheezing with breathlessness	64%	69%	0.44
Wheezing without cold	62%	61%	0.89
Nocturnal tight chest	38%	46%	0.21
Breathlessness during rest	16%	21%	0.34
Breathlessness during effort	38%	48%	0.13
Nocturnal breathlessness in last 12 months	19%	30%	0.053
Nocturnal breathlessness in last 3 months	10%	18%	0.073
Nocturnal breathlessness in last week	2%	6%	0.12
Winter cough	44%	45%	0.92
Nocturnal cough	51%	40%	0.11
Morning phlegm	30%	40%	0.083
Asthma attack in last 12 months	44%	33%	0.096
Asthma attack in last 3 months	32%	24%	0.22

Fixed-AO: fixed airflow obstruction, FEV₁/FVC < LLN post-bronchodilation

Evaluated with Pearson's chi-squared tests.

Online Table 3. Adjusted odds ratio (aOR (95%CI)) for asthma with fixed-AO in never-smokers (n=227) if having elevated levels of different inflammatory markers.

	Fixed-AO among never-smokers, a*OR	Fixed-AO among never-smokers, a**OR
Elevated S-periostin	1.97 (0.81-4.80)	2.34 (0.83-6.60)
Elevated FeNO	1.66 (0.70-3.92)	1.08 (0.37-3.09)
Elevated U-EDN	2.51 (1.04-6.07)	3.04 (1.11-8.31)
Elevated S-ECP	1.58 (0.66-3.79)	1.27 (0.45-3.55)

*sex, age group, use of ICS in last week, atopy, early onset asthma

**sex, age group, use of ICS in last week, atopy, early onset asthma, elevated S-periostin, elevated FeNO, elevated U-EDN, elevated S-ECP

Fixed-AO: fixed airflow obstruction, defined as post-BD $FEV_1/FVC < LLN$

Elevated levels of biomarkers were used as categorical variables, based on the following cut-offs: ≥ 25 ppb for FeNO, ≥ 20 $\mu\text{g/L}$ for S-ECP level, ≥ 74 $\mu\text{g/L}$ (upper tertile) for S-periostin and ≥ 65.95 mg/mol creatinine (upper tertile) for U-EDN

Significant results ($p < 0.05$) in bold.

Online Table 4. Percentages of elevated markers in the groups without and with fixed-AO with regard to age (panel A), gender (panel B), atopic status (panel C), pack years (panel D), smoking history (panel E).

A	Age groups								
	17-36 years			37-53 years			≥ 54 years		
	No fixed-AO n=125	Fixed-AO n=15	p value	No fixed-AO n=129	Fixed-AO n=20*	p value	No fixed-AO n=82	Fixed-AO n=32	p value
Elevated S-periostin	28%	40%	0.35	33%	26%	0.59	30%	46%	0.12
Elevated FeNO	26%	47%	0.087	30%	30%	0.99	42%	32%	0.32
Elevated U-EDN	22%	38%	0.19	25%	50%	0.032	43%	62%	0.048
Elevated S-ECP	37%	43%	0.65	31%	44%	0.31	24%	42%	0.087

*Fisher's exact test

B	Gender					
	Females			Males		
	No fixed-AO	Fixed-AO	p value	No fixed-AO	Fixed-AO	p value
Elevated S-periostin	31%	38%	0.46	29%	39%	0.26
Elevated FeNO	23%	31%	0.30	45%	38%	0.49
Elevated U-EDN	26%	45%	0.026	34%	65%	0.002
Elevated S-ECP	36%	37%	0.96	24%	48%	0.011

C	Atopic status					
	No atopy			Atopy		
	No fixed-AO	Fixed-AO	p value	No fixed-AO	Fixed-AO	p value
Elevated S-periostin	33%	42%	0.43	29%	40%	0.19
Elevated FeNO	20%	26%	0.51	36%	39%	0.77
Elevated U-EDN	29%	58%	0.009	29%	53%	0.006
Elevated S-ECP	21%	35%	0.19	34%	47%	0.16

D	Pack years					
	< 10 pack years			≥ 10 pack years		
	No fixed-AO n=265	Fixed-AO n=38	p value	No fixed-AO n=71	Fixed-AO n=29	p value
Elevated S-periostin	30%	44%	0.072	33%	31%	0.81
Elevated FeNO	31%	46%	0.066	34%	21%	0.20
Elevated U-EDN	26%	50%	0.003	39%	62%	0.048
Elevated S-ECP	35%	41%	0.47	18%	45%	0.014

E	Smoking history					
	Never-smokers			Previous or current smokers		
	No fixed-AO	Fixed-AO	p value	No fixed-AO	Fixed-AO	p value
Elevated S-periostin	32%	39%	0.41	29%	38%	0.28
Elevated FeNO	31%	48%	0.061	32%	24%	0.35
Elevated U-EDN	25%	50%	0.008	33%	59%	0.007
Elevated S-ECP	36%	46%	0.33	25%	40%	0.093

Fixed-AO: fixed airflow obstruction, FEV₁/FVC < LLN post-bronchodilation

Evaluated with Pearson's chi-squared test. Significant results (p < 0.05) in bold.