Inflammation in asthma: relation to symptomatology, exacerbations and lung function

IDA MOGENSEN
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Abstract

Asthma is an inflammatory disease in the airways. It is characterized by respiratory symptoms such as wheezing, variable airflow obstruction and impaired lung function development. A better understanding of the underlying inflammation is crucial in order to treat and prevent asthma symptoms and lung function deterioration.

We have evaluated six inflammatory markers in relation to asthma symptoms, asthma attacks, and lung function measures (fixed airflow obstruction (FAO) and lung function development over time) in five investigations. The markers (elevated levels) were fraction of exhaled NO (FeNO) (elevated ≥25ppb), serum eosinophil cationic protein (S-ECP) (≥20 µg/L), blood eosinophils (B-Eos) (≥300 cells/µL), urinary eosinophil derived neurotoxin (U-EDN) (≥65.95mg/mol creatinine), serum periostin (S-periostin) (≥74µg/L), and blood neutrophils (B-Neu) (≥5,100 cells/µL). The studied populations consisted of mainly adults (except in Paper II) and included asthmatics from the Swedish part of the Global Allergy and Asthma European Network survey (Papers I and III), the American National Health and Nutrition Examination Survey (Papers II and IV), the Uppsala part of the European Community Respiratory Health Survey I-III, the Vlagtwedde and Vlaardingen study, and the Rotterdam study, the latter two from the Netherlands (Paper V). All study populations were population based, and the asthmatics included had mainly mild to moderately severe asthma.

The main findings are that simultaneously elevated FeNO and S-ECP are associated with more reported asthma symptoms and attacks, and elevated FeNO and B-Eos are associated with lower lung function, suggesting a value in measuring both local (FeNO) and systemic (S-ECP, B-Eos) inflammation in asthma. Eosinophil inflammation (elevated U-EDN and S-periostin) was associated with FAO in asthma, while the other type-2 markers FeNO and S-periostin were not. Elevated B-Eos was further associated to lower lung function measures in a general population, and a faster lung function decline in asthmatics. FeNO was more often elevated in asthmatics, but was difficult to robustly associate to a specific disease characteristic. B-Neu was associated to FAO in participants with current smoking or pronounced smoking history.

In conclusion, asthma with elevated markers for eosinophil inflammation was associated to worse morbidity and lung function development in comparison with asthmatics without elevated markers for eosinophil inflammation. These results indicate ongoing eosinophil inflammation in asthma as closely associated to disease activity and the absence of eosinophil inflammation to less morbidity.

Keywords: Asthma, eosinophil, eosinophil cationic protein, eosinophil derived neurotoxin, periostin, neutrophil, fixed airflow obstruction, lung function, asthma attack, asthma symptom

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There is no affection of the body of which we cannot form a clear and distinct concept.

Baruch Spinoza, *Ethics*
Thanks to Petra Skarmyr for the cover image. The photograph is from "Vilseskogen" https://www.flickr.com/photos/vilseskogen/3836101271, and has been modified.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV Mogensen I, Jacinto T, Alving K, Fonseca J, Janson C, Malinovschi A. Inflammatory patterns in fixed airflow obstruction are dependent on the presence of asthma. Manuscript.


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<tr>
<td>aOR</td>
<td>Adjusted odds ratio</td>
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<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
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<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<td>B-Eos</td>
<td>Blood eosinophils</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>B-Neu</td>
<td>Blood neutrophils</td>
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<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>DAMP</td>
<td>Damage associated molecular pattern</td>
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<td>ECRHS</td>
<td>European Community Respiratory Health Survey</td>
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<tr>
<td>ER</td>
<td>Emergency room</td>
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<td>ERS</td>
<td>European Respiratory Society</td>
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<td>FAO</td>
<td>Fixed airflow obstruction</td>
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<tr>
<td>FeNO</td>
<td>Fraction of exhaled Nitric Oxide</td>
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<tr>
<td>FEV₁</td>
<td>Forced exhaled volume during the first second</td>
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<td>FVC</td>
<td>Forced vital capacity</td>
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<td>GA²LEN</td>
<td>Global Allergy and Asthma European Network</td>
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<td>GINA</td>
<td>Global Initiative for Asthma</td>
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<td>GLI</td>
<td>Global Lung Initiative</td>
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<td>GM</td>
<td>Geometric mean</td>
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<tr>
<td>ICS</td>
<td>Inhaled corticosteroids</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
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<td>INF</td>
<td>Interferon</td>
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<td>LABA</td>
<td>Long-acting β-2 agonist</td>
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<td>LAMA</td>
<td>Long-acting muscarinic antagonist</td>
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<td>LLN</td>
<td>Lower limit of normal</td>
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<td>LTRA</td>
<td>Leukotriene receptor antagonists</td>
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<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<td>NOS</td>
<td>Nitric oxide synthetase</td>
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<tr>
<td>OCS</td>
<td>Oral corticosteroids</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
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<td>SABA</td>
<td>Short acting β-2 agonist</td>
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<td>S-ECP</td>
<td>Serum eosinophil cationic protein</td>
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<td>Sp</td>
<td>Sputum</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
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<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>$T_{\text{reg}}$</td>
<td>Regulatory T-cell</td>
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<td>U-EDN</td>
<td>Urinary eosinophil derived neurotoxin</td>
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<td>V&amp;V</td>
<td>Vlagtwedde and Vlaardingen study</td>
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Introduction

Asthma is a chronic disease in the airways, characterised by variable airflow obstruction. This airflow obstruction is usually caused by inflammation in the airways. The word “asthma” comes from the Greek ἀσθμα, short drawn breath, panting (1). Since antiquity the concept of asthma has evolved considerably, and both the diagnosis and its symptoms encompass a heterogeneous group of diseases. However, most definitions include the traits covered by the definition given by the Global Initiative For Asthma (GINA) (2), which claims asthma to be characterised by variable symptoms of wheeze, shortness of breath, chest tightness, and/or cough, and by fluctuating expiratory airflow limitation. These symptoms are triggered by different stimuli such as allergens, inhaled substances, exercise, or infections leading to hyper-reactive airways. Chronic airway inflammation is usually present, and the airway inflammation in asthma is believed to underlie the disease severity with symptom burden, low physical ability, and long-term complications such as persistently reduced lung function.

Concepts and investigation

As suggested above, the term asthma is currently a general concept gathering several disease mechanisms all resulting in obstructive breathing and hyper-reactive airways sensitive to certain stimuli, rather than a homogeneous diagnosis. A way to characterise asthma further is to divide and classify into endotypes and phenotypes. One definition of this is to refer endotype to the underlying biological mechanism and pathogenesis, while the phenotype is a specific clinical characteristic (3). Thus, asthma can be associated to IgE sensitisation (endotype), and can be eosinophilic and steroid responsive (phenotype) in a certain person. This categorisation is related to the emerging concept of treatable traits (4), where underlying endotypic or phenotypic characteristics, rather than the umbrella diagnosis, are supposed to guide treatment.

The method used here to better understand traits and phenotypes in asthma will be epidemiological. The investigations are explanatory and mainly cross-sectional, while Paper V also has a longitudinal part. The aim is to associate the biomarkers for inflammation with phenotypic characteristics such as asthma symptoms, attacks, and lung function. The goal is a better understanding of aspects of the inflammation and related clinical presentation.
Here, I will present the underlying inflammation and characteristics defining some asthmatic endotypes and phenotypes. Thereafter, I will provide an overview of some diagnosis and treatment aspects and present a specific phenotypic trait, fixed airflow obstruction. The second part of the introduction will discuss the concept of biomarkers and the biomarkers evaluated in this thesis.

Inflammation in asthma

One of the most common phenotypic classifications is based on the cellular (mainly granulocytic) profile in sputum, blood or bronchoalveolar lavage (BAL) (5). This is largely due to new treatment possibilities related to inflammatory characteristics. The cellular profile can result from various underlying inflammatory mechanisms (endotypes) leading to the recruitment of different dominating cell types. This is related to the cytokine pattern dominating in the inflammation, which depends on several factors. The T-helper (Th) cell differentiation is dependent on the cytokines produced by the antigen-presenting cells and the cytokine environment at activation, which in turn are dependent on the type of antigen presented (6). This results in a certain cytokine pattern promoting specific differentiation of naïve CD4+ T-cells (Th) cells at activation (7). This will skew the inflammation in a certain direction. Intracellular bacteria and viruses able to replicate inside macrophages give rise to high levels of interferon (INF)-γ and interleukin (IL)-12, produced by among others natural killer (NK) cells, macrophages, and dendritic cells. This leads to Th1 differentiation, promotes class switch to immunoglobulin (Ig) G antibodies, and also further IFN-γ production in a positive feedback loop (6, 7). If the dominating cytokine is IL-4, a differentiation towards a Th2 subset will occur instead. IL-4 activates GATA3 in naïve CD4+ T-cells, which activates IL-4 and IL-13 encoding genes, and thus also works through a positive feedback loop. IL-4 promotes antibody switch to IgE. IL-6 further enhances the Th2 proliferation through a suppression of the Th1 pathway (6). On the other hand, IL-6, together with transforming growth factor (TGF)-β, promote Th17 differentiation and neutrophil recruitment, a process also requiring IL-21 and IL-23 for amplification and stabilisation (6). IL-6 is produced as a response to extracellular bacteria and fungi (7). In the absence of IL-6, TGF-β instead induces induced regulatory T-cells (T_{reg}), modulating and resolving immune responses. The Th subsets mutually suppress one another, leading to a cytokine milieu skewed in a certain direction (6). A common way to classify inflammation in asthma is into “type-2 inflammation” when the levels of Th2 associated cytokines are elevated, in contrast to “non-type-2 inflammation” low in Th2 associated cytokines.

The differentiated Th cells maintain the inflammatory environment and through further cytokine production and recruitment of other cells promote a certain immunological response. Innate lymphoid cells (ILC), numbered 1-3,
reside in the tissue and produce cytokines corresponding to their Th cell counterparts (Th17 corresponds to ILC3). These cytokines are able to enhance the inflammation (7). Some of the most important activating pathways are presented in Figure 1 (4, 6-9).

The immune reactions occurring in the tissue are dependent on further stimuli from invading viruses or bacteria, leading to recruitment and activation of macrophages and granulocytes, among other cells. The airway epithelium is important in the immune response. In type-1 (Th1 associated) and type-3 (Th17 associated) reactions, infected and dying cells and pathogens release pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP), resulting in activation of cytotoxic T cells, NK cells, and ILC1 or ILC3, and subsequent Th1 or Th17 differentiation and proliferation. The release of IL-17 and IL-22 by Th17 and ILC3 further recruits neutrophils to the site of inflammation (8). In tissue injury, the innate immune response to DAMP can lead to neutrophil recruitment also in the absence of bacteria (9). In type-2 inflammation, the immediate response to helminths, venoms, or allergens is performed by mast cells, releasing histamine and cytokines such as tumour necrosis factor (TNF), IL-4, IL-25 and IL-13. This reaction promotes Th2 activation through IL-4 release. Epithelial cells have crucial functions in the immune response in addition to their barrier function. As a response to cell damage caused by helminths or toxins, the epithelium promotes a type-2 response, where ILC-2 cells probably play an important role. ILC2 are believed to be less dependent on allergic sensitisation (10), but produce both IL-13 and IL-5, maintaining type-2 inflammation and promoting eosinophil recruitment. IL-5 is the most important cytokine for eosinophil recruitment and differentiation in the bone marrow (7).

These inflammatory pathways lead to domination of differing granulocytes in the airway lumen and tissues and systemically in the blood. Eosinophilic asthma, neutrophilic asthma, mixed eosinophilic and neutrophilic asthma, and “paucigranulocytic asthma”, where neither eosinophils nor neutrophils are found, are the dominating cellular phenotypes (5). Eosinophilic asthma can be allergic, often presenting high in symptoms, but responding well to inhaled corticosteroids (ICS), or with a later onset, commonly with more severe disease, less steroid responsive, and less prevalent allergic sensitisation (11). Neutrophilic asthma is often steroid-insensitive (12), has a correlation to smoke exposure, and is often found to be associated with low lung function (12). Paucigranulocytic asthma is hypothesised by some scholars to be a result of “burnt out” inflammation, with severe tissue destruction and changes (13), but could also indicate a less active or a well-treated inflammation.
Figure 1. Inflammatory pathways leading to granulocyte recruitment in asthmatic airway inflammation.
Compartment of inflammation

In addition to the type of inflammation, the location of the inflammation is also important for the phenotype. In spirometric testing, the large airways are assessed, while the small airways are probably important in both asthma and chronic obstructive pulmonary disease (COPD). In asthma, 50–60% of the patients are estimated to have small airway engagement (14). Small airway engagement has been found to be more common in asthma with fixed airflow obstruction (FAO) (15). Long-term complications are probably also dependent on the main site of inflammation in the airways. In some asthmatics, the systemic engagement is prominent with levels of inflammatory cells affected, and other markers also present in the systemic circulation.

Endotypic mechanisms affecting inflammation and phenotype

Several factors are associated with the clinical presentation of the disease and affect the airway inflammation.

Allergic sensitisation

The presence of allergic sensitisation or atopy, which is allergic sensitisation with concomitant allergic symptoms, is common in asthma, especially in individuals with an early disease onset (11). This means that the individual has IgE antibodies with a specificity for allergens, i.e., is sensitised. The allergic reaction can trigger an asthmatic reaction. The airborne allergens are (most often) inhaled, and bind to IgE, leading to mast cell degranulation. This in turn leads to a type-1 hypersensitivity reaction with immediate tissue swelling and bronchial constriction. Thereafter, a late phase reaction ensues, with cellular influx in the bronchi and initiation of a Th2-driven inflammation (8). Atopy has been linked to steroid-responsive eosinophilic inflammation (16).

Overweight and obesity

Overweight and obesity affect the respiratory system in several ways, but the mechanical effects on the respiratory work due to obesity are probably separate from the effects due to asthma (17). The lung volume is decreased, seen as a reduction in functional residual capacity and to some extent the total lung capacity (18). A reduced compliance of the respiratory system is further associated with obesity (19) and the bronchoconstriction after methacholine provocation seems to be differently distributed in obese and non-obese individuals (20). Higher body mass index (BMI) is correlated with worse asthma control, and obesity is more prevalent among asthmatics (21, 22). Higher weight has
been associated with increased asthma incidence (23) and weight loss with significant improvement in asthma control (24-26). Fat tissue is both endocrine and immunologically active. Eosinophil inflammation has been found to associate to higher BMI in asthma (27) as well as neutrophilic inflammation (28, 29) or simply less inflammation (30). There are also alterations in the hormonal expression related to obesity, with increased leptin and decreased adiponectin levels in obese asthmatics, which is believed to affect the disease (31, 32).

Smoking

The lungs are constructed to transport oxygen from the air to the blood. However, the large surface and large flow of air continuously passing the lung tissues make them vulnerable to pollutants and toxic exposures. Cigarette smoke is a well-known toxic exposure affecting the lung tissue in several ways. The additive component in cigarette smoke is nicotine, a substance with both pro- and anti-inflammatory properties (33). In studies on rheumatoid arthritis, nicotine has been found to induce neutrophil extracellular trap release through stimulation of the 7α subunit of the nicotinic acetylcholine receptor (34). The neutrophil extracellular trap is composed of chromatine (DNA) from the neutrophil and expulsed from it to catch and incapacitate invading organisms, mainly bacteria (35), but it can also cause tissue injury. However, nicotine can also have anti-inflammatory properties, decreasing TNF levels, downregulating inducible nitric oxide synthetase (NOS), upregulating Treg response (36) and decreasing IL-2, IL6, CXC motif ligand (CXCL) 8, and IL17A (33). Cigarette smoking, leading to more exposures than solely to nicotine, is associated with increased sputum CXCL8 (37), mucosal IL-17A expressing cells (38), as well as IL-17A in BAL (39), and is related to both increased systemic leukocyte levels (40) and sputum neutrophilia (37). However, in one study, cells expressing IL-17A and IL-17F were not more common in the submucosa of smokers without COPD than in healthy controls, while this was seen in both (non-smoking) COPD (IL-17A) and asthma (IL-17A and F) (41). Asthma and allergy in childhood have been associated with nicotine exposure in utero, however this association seem to be less pronounced when the exposure is after birth or later in life (23, 42, 43). One interpretation could be that nicotine per se (through placenta or breast milk) shifts the inflammation towards a more Th2-driven type, but that cigarette smoke induces other responses. In conclusion, an impact of cigarette smoke exposure on lung health and asthma is beyond doubt, as well as its effect on the immune system. However, the mode of impact is complex and nicotine has both pro- and anti-inflammatory properties altering the immune response.
Sex, age, and age of onset

There are sex-specific differences in asthma morbidity and differences in how the disease manifests at different ages (44). Older asthmatics have been found to have more symptoms and higher levels of both sputum neutrophils (12) and eosinophils (45). The age of asthma onset also correlates with certain phenotypes. Allergic asthma usually has onset during childhood, while non-atopic eosinophilic asthma often has later onset (11). Risk factors for disease seem to be different among men and women. Female sex hormones can affect asthma morbidity (46). Females – especially obese, non-atopic females with a history of smoking – seem to be at higher risk for developing adult onset asthma (47, 48). The immune system, anatomy, and exposure change over life could be a reason for the different phenotype occurrence patterns seen at certain ages.

Spirometric measures for lung function

Dynamic spirometry is one of the most important tools for assessing lung function and breathing disorders. In spirometry, the main measures are forced exhaled volume during the first second (FEV1), forced vital capacity (FEV), and their ratio FEV1/FVC (49). The participant takes a deep breath and exhales as fast as possible into a mouthpiece. The exhaled volume during the first second and the total exhaled volume are measured, as well as the airflow velocity. FEV1 measures a decreased ability to exhale quickly, possibly due to an increased resistance in the (large) airways, but can also be a consequence of small lung volumes. The ratio FEV1/FVC is used to define airflow obstruction, taking the vital capacity into account in relation to the FEV1. A ratio under 0.7 is usually used as a cut-off for obstructive impairment. Reference values are often used, taking height, sex, age, and ethnicity into account, to compensate for constitutional factors (50-53). Thus, a predicted value or a lower limit of normal (LLN) for a certain individual can be calculated. Reversibility of bronchial obstruction can be tested with administration of a bronchodilator and comparison of pre- and post-bronchodilatory results. An increase in FEV1 of at least 200 mL and > 12% of the pre-bronchodilatory value is an indicator of asthma (2).

Impaired lung function trajectories

Low lung function can be attributed to several causes. Under normal conditions, the lungs develop until the age of around 25 years, plateauing for around 5 years and then slowly declining through life (54). Several conditions, such as smoke exposure both during life and in utero, preterm birth with affected
lung maturation (55), and childhood respiratory infections can affect the lung function at birth and in early life. After that, lung function tends to follow a trajectory at approximately the same percentage of the expected level (56). Early events can therefore affect the maximally attained lung function (57, 58), though there is some evidence of a catching up in preterm-born children exposed to beneficial conditions during the first years of life (59). Exposures such as smoking and air pollution, and respiratory diseases like asthma, can however contribute to impaired lung growth and faster decline. A low maximally attained lung function as well as a fast decline can contribute substantially to low lung function at higher age (54, 60-62). Factors affecting maximally attained lung function are shown in Figure 2.

Figure 2. Trajectories of lung function development through life and associated risk factors for impaired lung function at higher age.
Fixed airflow obstruction, airway remodelling and lung function decline in asthma

A long-term complication in asthma is FAO (Figure 3), usually defined as post-bronchodilation FEV1/FVC < LLN or 0.7. This can be a consequence of impaired lung development, and is seen as connected to the development of airway remodelling and/or faster lung function decline. Airway remodelling is a condition commonly seen in asthma with basement membrane thickening, myocyte hyperplasia, epithelium thickening and loss of integrity, subepithelial fibrosis, and goblet cell hyperplasia (63, 64), eventually leading to an inability to regain a normal lung function between asthma deteriorations. Its symptomatology has some similarities with COPD, also presenting with non-reversible lung-function decline and chronic inflammation. However, development of COPD is usually caused by tobacco smoke or exposures to noxious gases leading to the airway abnormalities (65).

The cause of airway remodelling is believed to be dependent on chronic inflammation. However, it has been difficult to link any specific marker or type of inflammation to airway remodelling changes. Furthermore, the changes associated with airway remodelling can be seen early in asthmatic individuals. Already in childhood morphological alterations have been found in individuals with severe asthma with reduced lung function compared to individuals with reversible obstructions (66). In preschool children with wheezing, more eosinophilic inflammation and airway remodelling changes were found compared with in healthy controls (67).

Higher blood and sputum eosinophils are associated with lower lung function in asthmatics (12). In some studies, eosinophilic inflammation has also been associated with faster lung function decline among asthmatics (68, 69), while others have not been able to find this (70, 71). In COPD, higher neutrophil levels in sputum have been associated with a faster FEV1 decline in smokers (72). Neutrophil airway inflammation in asthma has been associated with
lower FEV$_1$ in cross-sectional studies of adults (12, 73, 74), however, in asthmatic children, intra-epithelial neutrophils in the airways were associated with a better lung function in one study (75).

**Medications in asthma**

A range of bronchodilating and anti-inflammatory medications is used in asthma. The most important anti-inflammatory group is that of ICS. ICS are now recommended as first-line therapy already in mild asthma (2). Oral corticosteroids (OCS) are also used. ICS are locally administered in the airways and have a predominantly local effect, while OCS have systemic effects. Unfortunately, both ICS and OCS have steroid-related side effects, and not all patients respond to treatment. Other oral medications include leukotriene receptor antagonists (LTRA). For bronchodilation, short-acting β-2 agonists (SABA), long-acting β-2 agonists (LABA) and long-acting muscarinic antagonists (LAMA) are used. New treatments include monoclonal antibodies inhibiting specific receptors or cytokines involved in the inflammatory process such as IL-5 antagonists, IL-4 / IL-13 antagonists, and IgE antagonists (2). A novel therapy is monoclonal antibodies binding to TSLP, inhibiting the inflammation early in the process (76). Development of novel biomarkers is to a large extent focused on predicting and assessing response to treatment and identifying novel targets for therapy.

**Biomarkers in asthma**

The definition of “biomarker” is often given as “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention” (77). Several different areas of use are established, and a biomarker in a specific context can be classified as: diagnostic, monitoring, pharmacodynamic/response, predictive, prognostic, surrogate endpoint, or safety and susceptibility/risk (78). In asthma research and clinically, there are biomarkers available for several of these purposes. When phenotyping asthma based on the dominating inflammatory pattern, biomarkers are the main tool for assessing the inflammation, and are thus used as diagnostic tools. However, the same markers are often also used for predictive, pharmacodynamic, and monitoring purposes, or prognostically for assessing risk for exacerbations or long-term outcomes. The close relation between phenotypes and biomarkers used in other contexts creates certain pitfalls in the interpretation of the association between biomarker level and outcome. The relevance of a low level of eosinophil markers is probably minor in an individual whose asthma is dependent on neutrophilic inflammation,
while it, in an asthmatic person with predominantly eosinophilic inflammation, can be a result of appropriate treatment (as the phenotype is affected by the treatment).

To better cluster endotypic or phenotypic properties present in a specific individual, the concept of “treatable traits” (79) has been developed (80). A treatable trait is defined as “a therapeutic target identified by phenotypes or endotypes through a validated biomarker” (81), and moves the focus from the diagnosis to an individual trait of the airway disease in a particular patient. Such traits can be, for example, eosinophilic asthma, frequent exacerbations, FAO, or current smoking. The biomarker (which can be a molecule, but also another measure such as a radiographic sign or a comorbidity) is then used as a trait identification marker (81). In this context, the biomarker is seen as a marker for a treatable property, coexisting with other treatable properties, and not primarily related to a diagnostic label such as “asthma” or “COPD”.

Inflammation and disease clusters

Inflammation is not necessarily correlated with symptom burden. Some asthmatics have a significant inflammation with few symptoms, while the level of inflammation and symptoms correlate fairly well in others. In non-type-2 asthma, with many cases being less responsive to corticosteroid treatment, symptom-based treatment can lead to the overuse of a non-efficient treatment. A cluster analysis by Haldar et al. on asthma phenotypes (82) pinpointed the dissociation between inflammation and symptom intensity, which is also mentioned in the GINA characterisation above. They found that the correlation between inflammation and symptom intensity was very strong in some clusters, while there was a large discrepancy in others. Distinguishing patients with a treatable inflammation, and the corresponding beneficial treatment, is a crucial task in a personalised treatment approach. In this context, a better characterisation of the associations of inflammatory biomarkers with phenotypic clusters, specific outcomes, and other biomarkers would be of value.

Biomarkers evaluated in this thesis

The inflammatory biomarkers evaluated in this thesis are: eosinophil count measured in blood (B-Eos), eosinophil-derived neurotoxin measured in urine (U-EDN), eosinophil cationic protein measured in serum (S-ECP), fraction of exhaled nitric oxide (FeNO) measured in exhaled air, periostin measured in serum (S-periostin), and neutrophil count measured in blood (B-Neu). All these markers, except B-Neu, are associated with type-2 inflammation. A summary of the evaluated biomarkers is presented in Table 1.
Blood eosinophils (B-Eos)

B-Eos is measured in peripheral venous blood, and is one of the most studied eosinophil markers due to its accessibility and usefulness in assessing eosinophil airway inflammation. B-Eos is fairly well correlated with sputum eosinophils (sp-Eos) (83, 84), at least among asthmatics (85). However, B-Eos has been found to be less affected by ICS than sp-Eos (86-88). B-Eos is affected by OCS (89) and biologic treatment with anti-IL-5 monoclonal antibodies (90, 91), indicating local versus systemic engagement of the asthmatic inflammation. Systemic inflammation does not exclude a simultaneous local inflammation, and is probably often a result of this in lung disease. However, elevated B-Eos levels have been observed to associate with a faster lung function decline over time also in respiratory healthy general populations, when compared to low levels (68, 92). B-Eos is increased by smoking (40, 93) and increasing age (94). When eosinophils are activated, one mode of action is to release the contents of their granulae: EDN, ECP, eosinophil peroxidase, and major basic proteins 1 and 2 (95). These proteins are possible to measure, and could be used as a measure of a more specific eosinophil activation, even if they are also produced in smaller amounts by other cells, such as liver, placenta, and other granulocytes (95, 96).

Urinary eosinophil derived neurotoxin (U-EDN)

EDN (RNASE2 (97)), previously called eosinophil protein X (98), is a basic protein contained in the eosinophil granulae. EDN has neurotoxic properties, but is less toxic for mammalian and helminthic cells than ECP, for example. It has anti-viral properties through RNAse activity against single-stranded RNA (95, 99, 100). EDN is also measurable in serum, and has then been linked to severe asthma in adults (101, 102) and wheezing in infants (103). U-EDN is mainly evaluated in paediatric research, where the non-invasive measurement is attractive. In children, it has been associated mainly with lower FEV₁ (100, 104, 105), but also with asthma and respiratory symptoms (106, 107); it seems to be less related to allergic sensitisation than ECP (108).

Serum eosinophil cationic protein (S-ECP)

ECP (RNASE3 (97)) is a basic protein, contained in eosinophil granulae, like EDN. It is toxic for helminths, bacteria, hemoflagellates and to some extent single-stranded RNA. It is cell-toxic and tissue-destructive, also for the host, and is probably important in the defence against parasites (95, 109). It has also been shown to have pro-fibrotic properties of potential importance for airway remodelling (110, 111). ECP seems to be more affected than EDN by allergic sensitisation and exposure (108, 112, 113). It is to some extent decreased by ICS treatment (114). ECP has been fairly well-studied in asthma, but has not
been proven to be a better marker than eosinophils with regard to asthma outcomes (87, 115). ECP is modified during release and exists in two main subgroups, of which one is cytotoxic (116). We do not have information on the proportions of the respective subgroups in our studies.

Fraction of exhaled Nitric Oxide (FeNO)
The majority of the nitric oxide (NO) measured in exhaled air is produced in the airway epithelium. FeNO reflects airway inflammation and is a predictor of sp-Eos (83). There is a constitutive production dependent on nitric oxide synthetase (NOS), where subtype 1 is expressed in neurons, subtype 3 in endothelial cells, and subtype 2, inducible NOS, is expressed by several cell types, such as macrophages and epithelial cells (117, 118). However, the amounts produced by NOS-1 and -3 are minor in relation to the amount produced by inducible NOS. Some NO is formed in the oral cavity, independent of NOS, from nitrate in the saliva, which is reduced to nitrite by bacteria, and then is further reduced to NO. The levels formed in the pharynx and mouth depend on the diet, and nitrate-rich food can affect the FeNO levels (117, 119). However, the main production in response to inflammatory activity is dependent on inducible NOS. FeNO levels are affected by blocking of IL-4 and IL-13 (120-122), and this is believed to be the main pathway of formation (117). Raised FeNO levels are a sign of steroid-responsive disease (123), and levels are decreased by steroid treatment (86, 124). Under normal physiological circumstances, FeNO is produced both in the small airways and alveoli and the bronchi. Using different exhalation flow-rates the amounts produced in the alveolar part of the lungs can be assessed (125). In the work presented here, a constant flow-rate of 50 mL/second has been used, which does not assess the alveolar production specifically, and is the flow-rate recommended in clinical practice (126). The FeNO level is positively correlated with height and age (94), as well as atopy, also in the absence of asthma (127, 128). Males have higher NO values than females partly because of greater height and larger lungs. There also seems to be a sex-specific difference (129). Smoking decreases FeNO values (127, 130).

Serum periostin (S-periostin)
Periostin is an extracellular matrix protein and is elevated due to bone growth. It has been evaluated as a marker for cancer-related activity in some types of cancers (131) and also as a marker for type-2 inflammation increased by IL-4 and IL-13 (132, 133). It has been associated with airway remodelling and fixed airflow obstruction and reduced lung function (88, 134-136) and is decreased as a response to ICS (88). However, asthma has not been found to relate to higher levels of periostin per se (134). Further, S-periostin is decreased by smoking and higher BMI (137). This, together with the effect from bone
growth and other medical conditions, can make the level difficult to interpret in many patient groups.

Blood neutrophils (B-Neu)

Neutrophils are one of the most important cells in the innate response to extracellular bacteria and invading pathogens (7), and are also recruited as a response to tissue injury (138). Elevated B-Neu is a sign of non-type-2 inflammation. The level in blood is not well-correlated with levels in BAL, sputum and lung tissue (84, 139), but could reflect a systemic inflammation skewed towards a non-type-2 pattern. Low B-Neu has been found to be a predictor of steroid-responsive disease independent of eosinophil levels (140), and higher B-Neu is associated with a worse prognosis (141), severe disease (142), and steroid resistance (16). Smoking increases neutrophil levels (40), and is shown to cause neutrophil necrosis leading to DAMP activation and neutrophil recruitment (143), probably as a result of both increased risk of infections in smoking-injured lungs and immunological mechanisms (38). Ongoing neutrophilic inflammation in the lungs may cause further injury due to release of the neutrophil granulae contents (such as neutrophil elastase) and formation of neutrophil extracellular traps (144, 145). Corticosteroids affect neutrophil function in several ways, such as accumulation in the blood, impaired migration (146) and delayed apoptosis (147, 148), leading to both pro- and anti-inflammatory effects.

Table 1. Summary of the biomarkers evaluated in this work.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Compartiment</th>
<th>Local/ systemic</th>
<th>Affected by ICS/OCS</th>
<th>Responding to biological treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeNO</td>
<td>Airways</td>
<td>Local</td>
<td>Decreases</td>
<td>Anti-IL4/IL-13</td>
</tr>
<tr>
<td>S-ECP</td>
<td>Serum</td>
<td>Systemic</td>
<td>Decreases</td>
<td>?</td>
</tr>
<tr>
<td>U-EDN</td>
<td>Urine</td>
<td>Systemic</td>
<td>Decreases</td>
<td>?</td>
</tr>
<tr>
<td>S-periostin</td>
<td>Serum</td>
<td>Systemic</td>
<td>Decreases</td>
<td>Probably anti-IL-4/IL-13</td>
</tr>
<tr>
<td>B-Eos</td>
<td>Blood</td>
<td>Systemic (and local)</td>
<td>Decreased by OCS</td>
<td>Anti-IL-5</td>
</tr>
<tr>
<td>B-Neu</td>
<td>Blood</td>
<td>Systemic, often not local</td>
<td>No</td>
<td>-</td>
</tr>
</tbody>
</table>

The biomarkers and the aspects of asthma they are intended to evaluate are summarised in Table 1 and Figure 4:
Figure 4. The biomarker matrix (green bubble) and the studied outcomes in relation to these biomarkers.
General aim of the thesis

The main aim of this thesis was to evaluate how inflammatory biomarkers reflecting different inflammatory pathways and locations associated with asthma symptoms, asthma attacks, and lung function. The rationale for this was to better characterise aspects of inflammation in asthma and their association with phenotypes of the disease.

In Papers I–II, the impact of simultaneously elevated markers for local and systemic inflammation was assessed in relation to reported burden of asthma symptoms, asthma attacks, and lung function. In Papers III–V, inflammatory markers were assessed in relation to fixed airflow obstruction and lung function.

Specific aims

I  To investigate the significance of simultaneously elevated levels of FeNO, a marker for local inflammation in the airways, and S-ECP, a marker for systemic inflammation, in relation to asthma symptoms and asthma attacks in a population-based group of asthmatics.

II To investigate the significance of simultaneously elevated levels of FeNO and B-Eos in relation to asthma symptoms, asthma attacks, and lung function in a population-based group of asthmatics.

III To investigate four markers for type-2 inflammation, FeNO, U-EDN, S-ECP, and S-periostin, and their association to fixed airflow obstruction in asthma.

IV To investigate three inflammatory markers, FeNO, B-Eos, and B-Neu, and their associations to fixed airflow obstruction in a general population with or without asthma.

V To investigate the association between B-Eos levels and lung function and lung function development over time in a general population and among asthmatics.
Materials and Methods

Study populations

Global Allergy and Asthma European Network Survey (Paper I and Paper III)
The Swedish part of the Global Allergy and Asthma European Network (GA²LEN) Survey was initiated in 2008. The first part consisted of a postal survey where a total of 45,000 individuals was contacted, 10,000 from Stockholm, Uppsala, and Umeå, respectively, and 15,000 from Gothenburg. The response rate was about 62% (27,866 individuals). In the second step, individuals who had answered the postal survey were invited to a clinical visit. The participants were randomly selected within four strata (asthma (1), chronic rhinosinusitis (2), asthma and chronic rhinosinusitis (3), and a control group (4)) with this pre-stratification made based on the answers in the initial survey (149, 150). The aim was to include up to 120 participants in strata 1, 2, and 4, and 40 in stratum 3, from each study centre. In total, 1,329 were examined (83%) The asthma diagnosis was reaffirmed at the clinical visit, with 604 participants classified as asthmatics. The diagnosis was defined as present when a participant had a self-reported doctor’s diagnosis of asthma, had used asthma medication during the previous year and/or had asthma symptoms during the previous year (wheezing, attack of shortness of breath, awakening in the night with breathlessness). The participants from Uppsala, Stockholm, and Umeå provided extra samples for measurements of serum eosinophil cationic protein (S-ECP), serum periostin (S-periostin), and urinary eosinophil-derived neurotoxin (U-EDN), in addition to fractional exhaled NO (FeNO). Therefore, the participants from Gothenburg were excluded from the related analyses.

In Paper I, all participants from Uppsala, Stockholm, and Umeå with present asthma, FeNO and S-ECP measurements were included. This resulted in a study population with 339 participants. In Paper III, the participants from Uppsala, Stockholm and Umeå with present asthma were included, resulting in 403 participants. The participants were 17–75 years old.
The National Health and Nutrition Examination Survey (Paper II and Paper IV)

The National Health and Nutrition Examination Survey (NHANES) is an American survey with the overall aim to assess the health and nutritional status of the non-institutionalised population in the United States of America (USA). It is conducted by the National Center for Health Statistics of the Centers for Disease Control and Prevention. Every year, around 7,000 individuals are invited to participate, and the data are released in two-year cycles. The participants are selected through a complex stratified multistage cluster sampling scheme, to represent the population of the USA. The cycles 2007–08, 2009–10 and 2011–12 were used as FeNO measurements were performed only during these cycles. The data collection is performed at three levels. First, a household screening is performed to find eligible participants, assessed using a pre-determined set of selection probabilities for the demographic subdomain desired. The eligible participant is then interviewed and undergoes a physical examination (151). The unweighted response rate was 70–79% in these cycles (152). In the statistical analysis, non-response is taken into account through weighting. FeNO was measured during these cycles, as were blood eosinophils (B-Eos) and blood neutrophils (B-Neu). In Paper II, all participants over 6 years of age with a self-reported health care professional’s diagnosis of asthma and B-Eos and FeNO measurements were included. In total, 1,419 participants were included. In Paper IV, all participants 20 years of age or older with spirometry measurements fulfilling the American Thoracic Society/European Respiratory Society (ATS/ERS) eligibility criteria were included, in total 11,579 participants.

The European Community Respiratory Health Survey, Vlagtwedde and Vlaardingen study and the Rotterdam study (Paper V)

The European Community Respiratory Health Survey

The European Community Respiratory Health Survey (ECRHS) is a European survey performed in several European countries. In Paper V, participants from Uppsala were investigated. In total, 3,600 randomly selected participants from the entire Uppsala population, in the age range of 20–44 years, were chosen and sent a questionnaire in 1990; the response rate was 87%. Among them, a random sample of 800 individuals, as well as a set of non-randomly selected responders (in total 216 additional individuals) reporting asthma symptoms in the initial questionnaire (153) (response rate 83%), was invited for a clinical examination. This was performed in 1991–92 (response rate 68%). In total, 836 subjects were examined and, of them, 676 individuals performed spirometry and had B-Eos measured at baseline. These were used as inclusion criteria
for Paper V. Of them, 487 subjects participated in at least one of the two follow-ups with clinical visits in 1999–2000 and 2011–12. In the ECRHS, current asthma was defined as self-reported doctor’s asthma diagnosis and current asthma medication and/or respiratory symptoms during the previous year.

**The Vlagtwedde and Vlaardingen study**

Vlagtwedde and Vlaardingen are two communities in the Netherlands. The Vlagtwedde and Vlaardingen study was initiated in 1965 and included the populations from these two communities, with > 8,000 participants (154-156). The participants have been followed approximately every third year with spirometry, among other examinations. In total, 6,684 participants were included in Paper V, and 3,642 were followed for up to 25 years, including subjects who had at least two spirometry measurements over a period of at least 5 years (with at most 8 follow-ups) and a B-Eos measurement. Asthma was considered as present if a positive answer to the question “Have you ever had attacks of shortness of breath with wheezing at rest?” was recorded.

**The Rotterdam study**

The Rotterdam study is a cohort in the Netherlands from the Ommord area close to Rotterdam. It was initiated in 1989. It is a population-based cohort study and includes 20,000 participants aged ≥ 45 years. Its aim is to assess risk factors and occurrence of chronic diseases among elderly people (157). The population has been enriched with new cohorts approximately every tenth year (158). The population included in Paper V participated in the follow-up between 2014 and 2016 (RS-I-6 and RS-II-4), which encompassed 2,857 participants. The data were used cross-sectionally. To be included in Paper V, a participant had to have an interpretable spirometry and a measured B-Eos. In total, 1,737 individuals were eligible. In the Rotterdam study, current asthma was defined as a self-reported doctor’s diagnosis of asthma, use of asthma medication and/or asthma symptoms in the preceding year (159).

**Questionnaire-defined variables**

**Symptoms and asthma attacks**

The questionnaires in the GA²LEN survey were based on the ECRHS questionnaire (160). The interviews were performed by experienced personnel in a structured way. The symptoms reported and included in the analysis were: wheezing – and, if yes, during exercise and/or in absence of a current cold – nocturnal chest tightness, breathlessness at rest, after physical exercise, and/or nocturnal attacks of breathlessness, all during the preceding 12 months. Asthma attacks in the preceding 12 and 3 months were also assessed.
The NHANES interviews were performed by experienced interviewers, in a structured way; the questions are available at www.nhanes.com (161). The symptoms should have been present during the preceding 12 months, and were: wheezing and, if yes, effect on sleep, daily activities, exercise, and occurrence of nightly cough in absence of respiratory infection. Moreover a composite variable referred to as “two asthma symptoms”, was defined as at least two of the aforementioned symptoms. Further symptoms were health care visits due to asthma, days of missed school/work due to wheezing, asthma attacks, and asthma-related emergency room (ER) visits during the preceding year.

The term “asthma exacerbations” will in the text refer to asthma attacks and ER visits due to asthma.

Medication
In GA²LEN, use of SABA, LABA, LAMA, and ICS was reported for the preceding year and the preceding week. OCS and LTRA use was reported for the preceding year. The ICS dose was converted to equivalent budesonide dose and use of > 400 µg budesonide per day was defined as high use (Paper III).

In NHANES, medications prescribed for wheezing were reported for the preceding year, and asthma medication for the preceding 3 months. Information on the use of ICS, LABA, OCS, and LTRA was given for the preceding month, while information on OCS or ICS use was given for the two days before FeNO measurements.

In the ECRHS, ICS and OCS use was reported for the preceding 12 months. Cumulative use (months used) was calculated for the longitudinal analysis. During the time of the Vlagtwedde and Vlaardingen study, ICS was not used; no data were available regarding OCS prescription. In the Rotterdam study, ICS and OCS prescription data were obtained through automated pharmacy records, for a period of 90 days prior to the blood analysis.

Smoking (Papers I–V)
Smoking was self-reported and the number of pack-years calculated. The population was divided into never, previous, and current smokers. In Papers II and IV, S-cotinine was used to assess smoking exposure.

Other self-reported variables
In NHANES, self-reported symptoms of hay fever in the preceding year were used as a proxy for allergy.
Spirometry

In GA²LEN, spirometry was performed in accordance with the ATS standards. An EasyOne Spirometer (nnd Medizintechnik AG, Switzerland) was used. All subjects without contraindications performed both a pre- and post-bronchodilation examination, and bronchodilation was done with 200 μg salbutamol. For Paper I, the Hedenström (51, 52) reference values were used as predicted values of FEV₁ and FVC, and Hankinson (53) was used for the LLN of FEV₁/FVC. In Paper III the Global Lung Initiative (GLI) reference values (50) were used.

In NHANES, spirometry was done in accordance with the ATS standards. An Ohio 822/827 dry-rolling seal volume spirometer (Ohio Medical, Gurnee, IL, USA) was used. Bronchodilation was done with two puffs of 108 μg albuterol in participants with a FEV₁/FVC ratio < 0.7 or LLN. In Papers II and IV, the Hankinson reference values were used, corrected for ethnicity (53, 162).

In ECRHS, lung function was measured using a dry-rolling seal spirometer system (Sensor Medics 2130, Sensor Medics, Anaheim, California, USA). The GLI reference values (50) were used.

In the Vlagtwedde and Vlaardingen study, a water-sealed spirometer (Lode Spirograph D52, Lode, Groningen, the Netherlands) was used. The GLI reference values (50) were used.

In Rotterdam, an electronic spirometer with pneumotachograph (Jaeger Master Screen PFT Cardinal Health, Hoechberg, Germany) was used. The GLI reference values (50) were used for the spirometry measurements.

Fixed airflow obstruction

FAO was, in both the GA²LEN survey and NHANES, defined as a post-bronchodilation FEV₁/FVC < LLN.

Laboratory measures

Fraction of exhaled Nitric Oxide

In both the GA²LEN survey and NHANES, FeNO was measured in accordance with the ATS/ERS (163) recommendations using a handheld device with an electrochemical sensor, NIOX MINO (NIOX MINO, Aerocrine, Stockholm, Sweden) at the exhalation flow rate of 50 mL/second.
Blood eosinophils
In NHANES, B-Eos was analysed using a quantitative haematologic analyser and leukocyte differential cell counter: Beckman Coulter HMX (Beckman Coulter, Fullerton, CA, USA). B-Eos was measured with a precision of 100 cells/μL.

In the ECRHS, B-Eos was measured with a Hemalog 2R (Tecknicon Chemicals Company, Tournai, Belgium) in 5 mL blood supplemented with EDTA (0.34 mol/L) (153); the precision was 10 cells/μL.

In the Vlagtwedde and Vlaardingen study, B-Eos was measured in a 1:11 dilution with a Bürker counting chamber (164).

In the Rotterdam study, B-Eos counts were measured with a Sysmex XS-800 Hematology analyser in blood supplemented with EDTA.

Urinary eosinophil derived neurotoxin
In GA²LEN, U-EDN was measured using a sandwich ELISA utilising a polyclonal EDN antibody as the catching antibody and a monoclonal antibody (clone 618) as the detecting antibody, in accordance with the manufacturer’s instructions (Diagnostics Development, Uppsala, Sweden).

Serum eosinophil cationic protein
In GA²LEN, S-ECP was measured with a fluorescence enzyme immunoassay (ImmunoCAP, Thermo Fisher Scientific, Immunodiagnostics, Uppsala, Sweden).

Serum periostin
In GA²LEN, S-periostin was measured using ELISA with two rat anti-human periostin monoclonal antibodies (clones SS18 and SS17B) (134).

Blood neutrophils
In NHANES, B-Neu was measured with the quantitative haematologic analyser and leukocyte differential cell counter, the Beckman Coulter HMX (Beckman Coulter, Fullerton, CA, USA). B-Neu was measured with a precision of 100 cells/μL.

Serum cotinine
In NHANES, S-cotinine was used as a marker for smoke exposure. A level ≥ 3 ng/mL was defined as indicating exposure (165).
Body mass index
BMI was calculated as weight in kilograms divided with height in meter squared. BMI groups were set to < 25 (normal weight), 25–30 (overweight), and > 30 (obese). In Paper II, the BMI group for the participants between 6 and 20 years of age was calculated and defined using the WHO references (166).

Skin prick test and allergy assessment
Skin prick tests were performed in the GA²LEN survey, used in Paper I and Paper III. It was considered positive with wheal ≥ 3 mm assessed for timothy grass, mixed grass, *Dermatophagoides pteronyssinus*, cat, dog, birch, cockroach, olive, *Alternaria*, *Artemisia*, *Parietaria*, and *Dermatophagoides farina*.

Study designs and statistical analyses
A summary of the included studies and key concepts of the Papers I–V are presented in Table 2.

Paper I
In Paper I, the main aim was to evaluate singly and simultaneously elevated levels of FeNO and S-ECP in relation to asthma symptoms and exacerbations. The hypothesis was that both local inflammation (measured through FeNO) and systemic inflammation (measured through S-ECP) were associated with more severe asthma morbidity. The group with 339 asthmatics was divided in four groups: with normal S-ECP (defined as < 20 μg/L) and normal FeNO (< 25 ppb); with elevated S-ECP (≥ 20 μg/L) and normal FeNO; with elevated FeNO (≥ 25 ppb) and normal S-ECP, and with both S-ECP and FeNO elevated. The groups were compared using univariate analyses (chi-squared test) with the group with both S-ECP and FeNO at normal levels as reference, for the outcome asthma symptoms and exacerbations. Thereafter, the groups were compared in a multivariate logistic regression, adjusted for age, sex, BMI, smoking history, study centre, allergic sensitisation, and use of medication in the previous week (LABA, ICS) and year (LTRA and OCS), with the outcome asthma symptoms and exacerbations. The multivariate analysis was done for the symptoms with significant associations to S-ECP and FeNO in the univariate analysis. The study design in presented in Figure 5.
Paper II

In Paper II, the main aim was to evaluate the association between elevated FeNO (local inflammation) and B-Eos levels (systemic inflammation), and asthma symptomatology and lung function. The hypothesis was that both systemic and local inflammation is associated with worse asthma morbidity. Further, different cut-offs for the inflammatory markers were investigated, as were differences between age groups. An additional outcome was asthma symptoms and exacerbations and obstructive breathing as measured through spirometry. First, different cut-offs were evaluated for FeNO (20/25 ppb, 30/35 ppb, and 35/50 ppb, with the lower values for participants under 12 years of age based on the ATS clinical guidelines (126)) and for B-Eos (300 cells/μL, 400 cells/μL and 500 cells/μL) in relation to symptoms and lung function, analysed with univariate logistic regressions. Thereafter, a comparison was performed between four groups: a reference group with normal FeNO levels (< 25 ppb for adults and < 20 ppb for children under 12 years of age) and normal B-Eos levels (< 300 cells/μL); a group with elevated FeNO levels (≥ 25 ppb and ≥ 20 ppb, respectively) and normal B-Eos levels; a group with elevated B-Eos levels (> 300 cells/μL) and normal FeNO levels; and a group with both FeNO and B-Eos elevated. Comparisons were univariate with chi-squared tests, and then performed using a multivariate logistic regression adjusted for sex, ethnicity, age, age at asthma onset, hay fever, BMI, S-cotinine, smoking status, use of anti-inflammatory medication, use of corticosteroids in the two days prior to FeNO measurement, and survey year. A sub-analysis was later done with the same adjusted logistic analysis stratified for age 6–17, 18–44 and > 44 years old. The study design in presented in Figure 5.

Figure 5. Study design and statistical analyses in Paper I and Paper II.
Paper III

In Paper III, four type-2 inflammatory markers were investigated in relation to fixed airflow obstruction among asthmatics in the GA²LEN survey. The hypothesis was that a more active type-2 inflammation was related to FAO in asthma. The markers evaluated were: FeNO, U-EDN, S-ECP, and S-periostin. They were divided into normal and elevated: FeNO elevated ≥ 25 ppb, U-EDN ≥ 65.95 mg/mol creatinine, S-ECP ≥ 20μg/L, and S-periostin ≥ 74μg/L. The group was divided in one sub-group without FAO and one sub-group with FAO. The sub-groups were compared regarding prevalence of elevated levels of the inflammatory markers, with the inflammatory markers as continuous variables. Thereafter, logistic regressions were performed, adjusted for sex, age, use of ICS last week, allergic sensitisation, early-onset asthma, smoking history, and pack-years, with the elevated markers as predictors and FAO as outcome (Figure 6). Stratified univariate analyses were done for age, allergic sensitisation, sex, never or ever smoking, and more or less than 10 pack-years of smoking.

Figure 6. Study design and statistical analyses in Paper III.

Paper IV

In Paper IV, the general population ≥ 20 years of age from NHANES was investigated with the aim to distinguish inflammatory patterns in asthma and FAO among adults. FAO was defined as FEV₁/FVC < LLN post bronchodilation. The markers evaluated were FeNO (elevated ≥ 25 ppb), B-Eos (elevated ≥ 300 cells/μL), and B-Neu (elevated ≥ 5,100 cells/μL). The population was divided into a reference group without asthma and without FAO (the control group), a group with asthma but no FAO, a group with asthma and FAO,
and a group with FAO but no asthma (the case groups). The groups were compared regarding the level and prevalence of elevated markers with an unpaired t-test and chi-squared tests, and, thereafter, with adjusted logistic regressions with elevated level of the biomarker as outcome and adjusted for sex, BMI, ethnicity, age, steroid use in the two days preceding FeNO measurements, study year, S-cotinine level, smoking status, and use of anti-inflammatory medication (Figure 7). Stratified analyses were done for smoking status and pack-years.

![Figure 7. Study design and statistical analyses in Paper IV.](image)

**Paper V**

In Paper V, the association between B-Eos levels at baseline and lung function development over time was assessed among adults > 18 years old. The hypothesis was that a higher level of B-Eos was related to faster lung function decline over time. Four cohorts were evaluated cross-sectionally: two young (ECRHS and Vlagwedde and Vlaardingen < 45 years of age) and two old (Vlagwedde and Vlaardingen ≥ 45 years old) and the Rotterdam cohort; with B-Eos categorised as normal (< 300 cells/µL) or elevated (≥ 300 cells/µL) as predictor and FEV₁, VC, and FEV₁/VC as outcome. In the ECRHS and Vlagtwedde and Vlaardingen cohorts, the association between baseline B-Eos (normal/elevated) was also analysed over 20 years’ time with the outcome being change in FEV₁, VC, and FEV₁/VC. The groups were cross-sectionally analysed with linear regression, both unadjusted and adjusted for sex, height, age, smoking, and pack-years. A meta-analysis was thereafter done with the two
younger cohorts and the two older cohorts, respectively. The longitudinal analysis was done with a mixed model for each cohort, adjusted for the same covariates as above. Thereafter, a meta-analysis between the younger cohorts was performed, Figure 8. Stratification for current asthma was done, with stratified analyses performed in the same manner as described above for the cohorts divided into asthmatics and non-asthmatics.

Sensitivity analyses were done with adjustments for ICS and OCS use in the cohorts from ECRHS and Rotterdam.

Figure 8. Study design for Paper V.

Ethical permissions

Ethical permission was given for the GA²LEN survey by the Regional Ethical Review Board in Stockholm, Sweden (Diary number 2008/1100-31/4) for all participating centres. The personal data collected were processed in accordance with the Swedish Personal Data Act.

Ethical permission for NHANES was given for the protocols (Ethics Review Board protocol numbers #2006-2007 and #2011-2017) by The National Center for Health Statistics Research Ethics Review Board; the participants provided written informed consent.
Ethical permission for ECRHS Uppsala was given by the Regional Ethical Review Board in Uppsala, Sweden (Diary number 1991/33; 1999/313; 2010/068); all participants signed an informed consent form.

The Vlagtwedde and Vlaardingen study protocol was approved by the Medical Ethics Committee of the University Medical Centre Groningen, Groningen, the Netherlands. All participants provided written informed consent.

The Rotterdam study was approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Centre and by the review board of The Netherlands Ministry of Health, Welfare and Sports (Diary number MEC 02.115).
Table 2. Studies included in Papers I–V, and definitions of key concepts.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Survey</th>
<th>Assessed biomarker(s)</th>
<th>Aim</th>
<th>Outcome variables</th>
<th>Study design</th>
<th>Population</th>
<th>Asthma definition</th>
<th>Main analysis model</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>GA³LEN</td>
<td>FeNO, ECP</td>
<td>Assess local and systemic inflammation</td>
<td>Asthma symptoms, Exacerbations</td>
<td>Cross-sectional</td>
<td>Asthmatics, Population-based</td>
<td>Doctor’s diagnosis and medication and/or symptoms in preceding year</td>
<td>Adjusted logistic regression</td>
</tr>
<tr>
<td>II</td>
<td>NHANES</td>
<td>FeNO, B-Eos</td>
<td>Assess local and systemic inflammation</td>
<td>Asthma symptoms, Exacerbations, Lung function</td>
<td>Cross-sectional</td>
<td>Asthmatics, Population-based</td>
<td>Self-reported diagnosis, set by health care professional</td>
<td>Adjusted logistic regression</td>
</tr>
<tr>
<td>III</td>
<td>GA³LEN</td>
<td>S-ECP, U-EDN, FeNO, S-periostin</td>
<td>Assess association with FAO</td>
<td>Prevalence of elevated level of assessed biomarker in asthmatics with FAO</td>
<td>Cross-sectional</td>
<td>Asthmatics, Population-based</td>
<td>Doctor’s diagnosis and medication and/or symptoms in preceding year</td>
<td>Adjusted logistic regression</td>
</tr>
<tr>
<td>IV</td>
<td>NHANES</td>
<td>FeNO, B-Eos, B-Neu</td>
<td>Assess association with lung function</td>
<td>Prevalence of elevated level of assessed biomarker in asthmatics and non-asthmatics with FAO compared with controls</td>
<td>Cross-sectional</td>
<td>General population, divided into asthmatics and non-asthmatics</td>
<td>Self-reported diagnosis, set by health care professional</td>
<td>Adjusted logistic regression</td>
</tr>
<tr>
<td>Paper</td>
<td>Survey</td>
<td>Assessed biomarker(s)</td>
<td>Aim</td>
<td>Outcome variables</td>
<td>Study design</td>
<td>Population</td>
<td>Asthma definition</td>
<td>Main analysis model</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------</td>
<td>-----------------------</td>
<td>------------------------------------------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
<td>-------------------------------------------------</td>
<td>---------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>V</td>
<td>ECRHS</td>
<td>B-Eos</td>
<td>Assess association with lung function</td>
<td>Lung function impairment and decline</td>
<td>Cross-sectional and longitudinal</td>
<td>General population, stratified for asthma</td>
<td>Doctor’s diagnosis and medication and/or symptoms in preceding year</td>
<td>Adjusted linear regression Adjusted mixed model</td>
</tr>
<tr>
<td>V</td>
<td>Vlagtwedde Vlaardingen</td>
<td>B-Eos</td>
<td>Assess association with lung function</td>
<td>Lung function impairment and decline</td>
<td>Cross-sectional and longitudinal</td>
<td>General population, stratified for asthma</td>
<td>Self-reported respiratory symptoms</td>
<td>Adjusted linear regression Adjusted mixed model</td>
</tr>
<tr>
<td>V</td>
<td>Rotterdam</td>
<td>B-Eos</td>
<td>Assess association with lung function</td>
<td>Lung function impairment</td>
<td>Cross-sectional</td>
<td>General population, stratified for asthma</td>
<td>Doctor’s diagnosis and medication and/or symptoms in preceding year</td>
<td>Adjusted linear regression</td>
</tr>
</tbody>
</table>


Results

Local and systemic inflammation, Papers I–II

The hypothesis in Papers I and II was that a simultaneous local and systemic inflammation in asthma would relate to more severe asthma morbidity, when retrospectively assessed. Furthermore, it was hypothesised that FeNO and the eosinophil marker represented different aspects of asthmatic inflammation and would have differing symptomatology. Local inflammation in the airways was measured through FeNO and systemic inflammation through S-ECP (Paper I) or B-Eos (Paper II). The two investigations were quite different regarding the investigated populations, presented in Table 3. The population in Paper I was over 18 years of age, had lower BMI, and had a high degree of allergic sensitisation as well as few current smokers compared with that in Paper II.

Table 3. Characteristics of included study populations in Paper I and Paper II

<table>
<thead>
<tr>
<th>Paper I</th>
<th>Number (percent)</th>
<th>Paper II</th>
<th>Absolute number (weighted percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 339</td>
<td></td>
<td>N = 1,419</td>
<td></td>
</tr>
<tr>
<td>Normal FeNO, Normal S-ECP</td>
<td>164 (48%)</td>
<td>Normal FeNO, Normal B-Eos</td>
<td>680 (48%)</td>
</tr>
<tr>
<td>Elevated FeNO</td>
<td>63 (19%)</td>
<td>Elevated FeNO</td>
<td>147 (10%)</td>
</tr>
<tr>
<td>Elevated S-ECP</td>
<td>63 (20%)</td>
<td>Elevated B-Eos</td>
<td>296 (21%)</td>
</tr>
<tr>
<td>Elevated FeNO, Elevated S-ECP</td>
<td>45 (13%)</td>
<td>Elevated FeNO, Elevated B-Eos</td>
<td>296 (21%)</td>
</tr>
<tr>
<td>Female sex</td>
<td>200 (59%)</td>
<td>Female sex</td>
<td>784 (58%)</td>
</tr>
<tr>
<td>Allergic sensitisation</td>
<td>246 (76%)</td>
<td>Hay fever</td>
<td>392 (34%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Median (range) 40 (17–75) years</td>
<td>Median (range) 23 (6–79) years</td>
<td></td>
</tr>
<tr>
<td>&lt; 18</td>
<td>562 (27%)</td>
<td>562 (27%)</td>
<td></td>
</tr>
<tr>
<td>17–40</td>
<td>159 (47%)</td>
<td>18–44</td>
<td>489 (42%)</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>180 (53%)</td>
<td>&gt; 44</td>
<td>368 (31%)</td>
</tr>
<tr>
<td>BMI:</td>
<td></td>
<td>BMI:</td>
<td></td>
</tr>
<tr>
<td>&lt; 18.5</td>
<td>5 (1.5%)</td>
<td>&lt; 25</td>
<td>464 (33%)</td>
</tr>
<tr>
<td>18.5–25</td>
<td>153 (45%)</td>
<td>&lt; 25</td>
<td>464 (33%)</td>
</tr>
<tr>
<td></td>
<td>Paper I</td>
<td>Paper II</td>
<td>Absolute number</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td>N = 339</td>
<td>N = 1,419</td>
<td>(weighted percent)</td>
</tr>
<tr>
<td>25–30</td>
<td>112 (33%)</td>
<td>25–30</td>
<td>362 (28%)</td>
</tr>
<tr>
<td>≥ 30</td>
<td>69 (20%)</td>
<td>≥ 30</td>
<td>586 (39%)</td>
</tr>
<tr>
<td>Asthma onset ≥ 18 years old</td>
<td>149 (44%)</td>
<td>Asthma onset ≥ 18 years old</td>
<td>408 (35%)</td>
</tr>
<tr>
<td>Never smoker</td>
<td>206 (61%)</td>
<td>Never smoker*</td>
<td>404 (52%)</td>
</tr>
<tr>
<td>Previous smoker</td>
<td>111 (33%)</td>
<td>Previous smoker*</td>
<td>184 (25%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>22 (6.5%)</td>
<td>Current smoker*</td>
<td>199 (23%)</td>
</tr>
</tbody>
</table>

θ In the population ≥18 years old, 47% of the population had an asthma onset at age 18 or later. *Among participants ≥ 20 years old;

**Fraction of exhaled Nitric Oxide**

Elevated FeNO was, in both populations, associated with male sex and allergic sensitisation/hay fever, compared with the group with normal FeNO and S-ECP/B-Eos. In Paper II, it was also associated with lower BMI and shorter smoking history.

In Paper I, elevated FeNO showed some association with wheezing in the absence of cold and breathlessness after physical exercise in the adjusted analysis, also independent of elevated S-ECP. However, FeNO was not associated with a higher prevalence of reduced lung function (FEV₁ < 80% of predicted or FEV₁/FVC < LLN) or with asthma attacks. In Paper II, the positive association between elevated FeNO and asthma symptoms was absent; in contrast, elevated FeNO was associated with fewer asthma-related emergency room visits both in the unadjusted and adjusted analysis, as well as independently of B-Eos level. However, elevated FeNO was associated with a FEV₁/FVC < LLN pre-bronchodilation.

**Serum eosinophil cationic protein and blood eosinophils**

Singly elevated S-ECP was associated with a greater number of asthma attacks in the preceding three months, also independent of FeNO. It had no association with more asthma symptoms or reduced lung function. In Paper II, elevated B-Eos was associated with asthma symptoms and exacerbations (asthma attacks and ER visits), both unadjusted and independent of FeNO. However, singly elevated S-ECP with concomitant normal FeNO was not associated with any of the outcomes in the unadjusted model and only with more ER visits in the adjusted model.
Simultaneous local and systemic inflammation

In Paper I, when both FeNO and S-ECP were elevated, there was a stronger association with both wheezing without a cold, breathlessness after exercise, and asthma attacks, after adjustment (Figure 9). In Paper II, simultaneously elevated FeNO and B-Eos were, in the adjusted model, associated with wheezing (Figure 4), wheezing disturbing the sleep (adjusted odds ratio (OR): 1.88 (95% confidence interval (CI): 1.27–2.78)), and FEV$_1$ < 80% of predicted and FEV$_1$/FVC < LLN, both pre-bronchodilation and post-bronchodilation (Figure 9). There was an interaction in the association between simultaneously elevated FeNO and B-Eos and a FEV$_1$ < 80% of predicted, depending on BMI, with a stronger association in the group with BMI ≤ 25 than in the group with BMI > 30 (odds ratio (OR) 6.2 (CI: 1.9–20.1) vs. OR: 1.7 (CI: 0.74–3.9)), $P_{interaction}$ 0.018.

Summary, Papers I and II

In conclusion, elevated FeNO was associated with increased symptom burden in Paper I. In Paper II, this was not found, but a negative association between elevated FeNO and asthma morbidity could be noted. Elevated S-ECP was associated with more asthma exacerbations in Paper I, and a similar pattern was found for elevated B-Eos in Paper II. Having both elevated FeNO and elevated S-ECP was associated with both more asthma symptoms and more asthma attacks in Paper I. In Paper II, there was some association between simultaneously elevated FeNO and B-Eos and wheezing symptoms, but the main finding was an association with lower lung function. This was not found in Paper I, although it was only analysed in an unadjusted model.
Figure 9. Adjusted odds ratio (on a logarithmic scale) for having had an asthma attack, asthma symptoms, and affected lung function, in the groups with one or two of the markers elevated, compared with the groups without any elevated markers.

In Paper I (GA<sup>2</sup>LEN), adjustments were made for: sex, age, BMI, study centre, smoking habits, allergic sensitisation, OCS use in the preceding year, LABA use in the preceding week, ICS use in the preceding week, and LTRA use in the preceding week.

In Paper II (NHANES), adjustments were made for: sex, ethnicity, age, age at asthma onset, hay fever, BMI group, S-cotinine, smoking habits, corticosteroid use in the two days preceding examination, study year, and use of anti-inflammatory medication.
Fixed airflow obstruction

FAO in asthmatics and its association with inflammatory markers was investigated in Paper III. In Paper IV, FAO in both asthmatics and non-asthmatics was investigated in relation to inflammatory markers.

In both papers, FAO was associated with higher age, male sex, being a former or current smoker, and having more pack-years of smoking history. In Paper IV, higher BMI was found in the asthma group without FAO and lower BMI in the group with FAO without asthma compared with among controls. In Paper III, absence of allergic sensitisation was associated with FAO; no difference in hay fever prevalence was found between the asthma groups in Paper IV. Among asthmatics, age of asthma onset was not associated with FAO. However, in Paper IV, asthmatics with FAO had more asthma symptoms reported and longer asthma duration. This was not seen in Paper III. In both papers, the group with asthma and FAO used more ICS and OCS than the group with asthma without FAO.

Paper III

Among the investigated markers in Paper III (FeNO, S-ECP, U-EDN, S-periostin), only elevated U-EDN was significantly more prevalent in the group with FAO (55%) versus in the group without FAO (29%), \( p < 0.001 \) (Figure 10). When analysed as continuous variables, both U-EDN (FAO: geometric mean (GM): 61.5 mg/mol creatinine (95% CI: 50.4–75.0) vs. no FAO: GM 46.3 mg/mol creatinine (95% CI: 43.2–49.5) \( p = 0.002 \)) and S-ECP (FAO: GM = 17.6 µg/L (95% CI: 14.0–22.1) no FAO: GM = 13.7 µg/L (95% CI: 12.6–14.9), \( p = 0.02 \)), were found to be higher in the group with FAO than in the group without FAO.

![Figure 10](image.png)

*Figure 10.* Percent with elevated marker (FeNO ≥ 25 ppb; U-EDN ≥ 65.95 mg/mol creatinine; S-ECP ≥ 20 µg/L and S-periostin ≥ 74 µg/L) in the group without FAO compared with the group with FAO.
In the adjusted logistic regression, adjusted for sex, age group, use of ICS in the preceding week, allergic sensitisation, early onset asthma, smoking history, and pack-years, both having elevated U-EDN and having elevated S-ECP yielded higher OR for having FAO. This was consistent for U-EDN also when adjusted for the other markers in the same model (Table 4). However, simultaneously elevated U-EDN and S-ECP had an aOR at 6.04 (CI: 2.32–15.8) adjusted for the same covariates; however, the group was small (n = 34).

Table 4. Adjusted odds ratios for having FAO when a certain marker is elevated (FeNO ≥ 25 ppb; U-EDN ≥ 65.95 mg/mol creatinine; S-ECP ≥ 20 μg/L and S-periostin ≥ 74 μg/L)

<table>
<thead>
<tr>
<th>Inflammatory marker</th>
<th>Asthma with FAO adjusted* odds ratio (95% CI)</th>
<th>Asthma with FAO adjusted** odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeNO</td>
<td>$1.15 \ (0.60–2.20)$</td>
<td>$0.80 \ (0.37–1.75)$</td>
</tr>
<tr>
<td>U-EDN</td>
<td>$2.38 \ (1.28–4.41)$</td>
<td>$2.73 \ (1.33–5.62)$</td>
</tr>
<tr>
<td>S-ECP</td>
<td>$2.07 \ (1.07–3.99)$</td>
<td>$1.98 \ (0.97–4.05)$</td>
</tr>
<tr>
<td>S-periostin</td>
<td>$1.73 \ (0.92–3.25)$</td>
<td>$2.04 \ (0.99–4.18)$</td>
</tr>
</tbody>
</table>

*sex, age group, use of ICS in the preceding week, allergic sensitisation, early onset asthma, smoking history, pack-years.

**sex, age group, use of ICS in the preceding week, allergic sensitisation, early onset asthma, smoking history, pack-years, elevated FeNO, elevated U-EDN, elevated S-ECP, elevated S-periostin.

**Smoking, allergic sensitisation, sex differences, and age differences**

The association between FAO and elevated U-EDN was not significant in the youngest age group (17–36 years old), but was significant in the older age groups. The association was still present, independent of smoking and smoking history (pack-years), sex, and allergic sensitisation. In contrast, FAO only associated with elevated S-ECP among participants with more than 10 years of smoking history and in males (Tables 5–9).
Tables 5-9. Percent with elevated markers in the groups without and with FAO, stratified for age, sex, allergic sensitisation, pack-years smoking, and never or ever smoking.

Table 5. Stratified analysis in relation to age group.

<table>
<thead>
<tr>
<th>Age group</th>
<th>17–36 years</th>
<th>37–53 years</th>
<th>≥ 54 years</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No FAO n = 125</td>
<td>FAO n = 15</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>FAO n = 129</td>
<td>n = 20</td>
<td></td>
</tr>
<tr>
<td>Elevated FeNO</td>
<td>26%</td>
<td>47%</td>
<td>0.087</td>
</tr>
<tr>
<td>Elevated U-EDN</td>
<td>22%</td>
<td>38%</td>
<td>0.19</td>
</tr>
<tr>
<td>Elevated S-ECP</td>
<td>37%</td>
<td>43%</td>
<td>0.65</td>
</tr>
<tr>
<td>Elevated S-periostin</td>
<td>28%</td>
<td>40%</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Table 6. Stratified analysis in relation to sex.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No FAO n = 170</td>
<td>FAO n = 27</td>
</tr>
<tr>
<td></td>
<td>No FAO n = 115</td>
<td>FAO n = 29</td>
</tr>
<tr>
<td>Elevated FeNO</td>
<td>23%</td>
<td>31%</td>
</tr>
<tr>
<td>Elevated U-EDN</td>
<td>26%</td>
<td>45%</td>
</tr>
<tr>
<td>Elevated S-ECP</td>
<td>36%</td>
<td>37%</td>
</tr>
<tr>
<td>Elevated S-periostin</td>
<td>31%</td>
<td>38%</td>
</tr>
</tbody>
</table>
Table 7. Stratified analysis in relation to allergic sensitisation.

<table>
<thead>
<tr>
<th>Allergic sensitisation</th>
<th>No allergic sensitisation</th>
<th>Allergic sensitisation</th>
<th>p value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No FAO n = 66</td>
<td>FAO n = 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated FeNO</td>
<td>20%</td>
<td>26%</td>
<td>0.51</td>
<td>36%</td>
</tr>
<tr>
<td>Elevated U-EDN</td>
<td>29%</td>
<td>58%</td>
<td>0.009</td>
<td>29%</td>
</tr>
<tr>
<td>Elevated S-ECP</td>
<td>21%</td>
<td>35%</td>
<td>0.19</td>
<td>34%</td>
</tr>
<tr>
<td>Elevated S-periostin</td>
<td>33%</td>
<td>42%</td>
<td>0.43</td>
<td>29%</td>
</tr>
</tbody>
</table>

Table 8. Stratified analysis in relation to pack-years smoking.

<table>
<thead>
<tr>
<th>Pack-years</th>
<th>&lt; 10 pack-years</th>
<th>≥ 10 pack-years</th>
<th>p value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No FAO n = 265</td>
<td>FAO n = 38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated FeNO</td>
<td>31%</td>
<td>46%</td>
<td>0.066</td>
<td>34%</td>
</tr>
<tr>
<td>Elevated U-EDN</td>
<td>26%</td>
<td>50%</td>
<td>0.003</td>
<td>39%</td>
</tr>
<tr>
<td>Elevated S-ECP</td>
<td>35%</td>
<td>41%</td>
<td>0.47</td>
<td>18%</td>
</tr>
<tr>
<td>Elevated S-periostin</td>
<td>30%</td>
<td>44%</td>
<td>0.072</td>
<td>33%</td>
</tr>
</tbody>
</table>
Table 9. Stratified analysis in relation to smoking habits.

<table>
<thead>
<tr>
<th>Smoking history</th>
<th>Never smokers</th>
<th>Previous and current smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No FAO</td>
<td>FAO</td>
</tr>
<tr>
<td>Elev. FeNO</td>
<td>n = 171</td>
<td>n = 26</td>
</tr>
<tr>
<td>31%</td>
<td>48%</td>
<td>0.061</td>
</tr>
<tr>
<td>Elev. U-EDN</td>
<td>25%</td>
<td>50%</td>
</tr>
<tr>
<td>Elev. S-ECP</td>
<td>36%</td>
<td>46%</td>
</tr>
<tr>
<td>Elev. S-periostin</td>
<td>32%</td>
<td>39%</td>
</tr>
</tbody>
</table>
Paper IV

In Paper IV, the associations between FAO and elevated FeNO, B-Eos, and B-Neu were investigated in asthmatics and non-asthmatics. In univariate analyses performed with chi-squared tests, asthma both with and without FAO was associated with elevated FeNO levels. All groups – asthma without FAO, asthma with FAO, and FAO without asthma – had higher prevalence of both elevated B-Eos and elevated B-Neu than the control group. Asthma with FAO had a higher prevalence of elevated B-Eos than both the asthma group without FAO and the FAO group without asthma (Figure 11).

Figure 11. Comparison of the prevalence of elevated markers in the different asthma/FAO groups. From the left, the panels show the prevalence of elevated levels of FeNO (≥ 25 ppb), B-Eos (≥ 300 cells/µL), and B-Neu (≥ 5,100 cells/µL), respectively. The arrows indicate significant differences in prevalence, analysed with chi-squared test: * p < 0.05; **p < 0.001.

In the adjusted logistic regression (adjusted for sex, ethnicity, age, ICS/OCS use during the two days preceding FeNO measurement, study year, cotinine level, use of anti-inflammatory medication, and smoking history), asthma (regardless of FAO) was still associated with both elevated FeNO and B-Eos. However, the group with both asthma and FAO had the highest odds ratios for both markers. FAO without asthma was the only group associated with higher B-Neu after adjustment (Table 10).

Table 10. Adjusted odds ratios for having elevated levels of the respective markers in the case groups compared with the control group.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Asthma without FAO aOR</th>
<th>Asthma with FAO aOR</th>
<th>FAO without asthma aOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeNO</td>
<td>2.61 (2.02–3.37)</td>
<td>3.82 (2.09–6.97)</td>
<td>1.13 (0.86–1.48)</td>
</tr>
<tr>
<td>B-Eos</td>
<td>1.38 (1.13–1.67)</td>
<td>2.46 (1.43–4.22)</td>
<td>1.25 (0.95–1.65)</td>
</tr>
<tr>
<td>B-Neu</td>
<td>1.05 (0.82–1.35)</td>
<td>1.23 (0.74–2.04)</td>
<td>1.41 (1.09–1.86)</td>
</tr>
</tbody>
</table>

*Adjusted for: sex, ethnicity, age, ICS/OCS use during the two days preceding FeNO measurement, study year, cotinine level, use of anti-inflammatory medication, and smoking history.
Smoking history and pack-years smoking
Stratified analyses for never smokers, previous smokers and current smokers as well as for less or more than 10 pack-years of smoking history, showed that elevated FeNO levels were more prevalent in all case groups compared with controls among never-smokers. In asthmatics, regardless of FAO, elevated FeNO was more prevalent among ex-smokers and among participants with less than 10 pack-years of smoking history. Among smokers, elevated FeNO was only associated with asthma without FAO, and not with any group among participants with more than 10 pack-years of smoking history.

Elevated B-Eos was more prevalent among asthmatics without FAO than among controls, regardless of smoking history. However, this difference was not seen among participants with more than 10 years of smoking history. In asthmatics with FAO, a higher prevalence of elevated B-Eos was seen when they were stratified for smoking history compared with controls, as well as when stratified for 10 pack-years of smoking history. This was not a significant difference among never or current smokers. No significant differences between controls and the group with FAO without asthma remained in the smoking-stratified analyses.

Among current smokers and participants with more than 10 pack-years of smoking history, elevated B-Neu was more prevalent in the group with asthma without FAO and the group with FAO without asthma than in the control group. The group with asthma and FAO had similar prevalence rates as the two other case groups. However, the difference was not significant.

Summary of results, Paper III and Paper IV
A summary of the findings of Paper III and Paper IV is presented in Table 11. In both papers, no differences regarding elevated FeNO prevalence were found between the asthmatic groups, regardless of presence of FAO. B-Eos and the eosinophil markers S-ECP and U-EDN were associated with FAO in asthmatics, and also in non-asthmatics. U-EDN was found to be robustly associated with FAO, independently of allergic sensitisation, smoking, and sex. There was an association between FAO and elevated B-Neu, but only among ex- and current smokers. In the adjusted analysis, a higher odds ratio for elevated B-Neu was not found in the groups with asthma, but in the group with FAO without asthma compared with controls. FAO in asthma was not found to associate with elevated S-periostin.
Table 11. Summary of the inflammatory markers evaluated in Paper III and Paper IV.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Association with FAO</th>
<th>Association with asthma</th>
<th>Association with smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeNO</td>
<td>No</td>
<td>Yes</td>
<td>Yes, lower</td>
</tr>
<tr>
<td>U-EDN</td>
<td>Yes</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>S-ECP</td>
<td>Some</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>B-Eos</td>
<td>Yes</td>
<td>Yes</td>
<td>Some</td>
</tr>
<tr>
<td>Eosinophilic inflammation</td>
<td>Yes</td>
<td>Yes</td>
<td>Some</td>
</tr>
<tr>
<td>S-periostin</td>
<td>No</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>B-Neu</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Eosinophilic inflammation and lung function

Paper V

In Paper V, the association between eosinophilic inflammation, measured in blood through B-Eos, and lung function was investigated in different populations, both cross-sectionally and longitudinally. Both baseline characteristics (Table 12) and the associations between B-Eos and lung function were found to differ between the studies.

Table 12. Baseline characteristics of the populations included in Paper V

<table>
<thead>
<tr>
<th>Variable</th>
<th>V&amp;V 18–44 years of age</th>
<th>ECRHS 20–45 years of age</th>
<th>V&amp;V 45–74 years of age</th>
<th>Rotterdam 70–95 years of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>4,881</td>
<td>676</td>
<td>1,803</td>
<td>1,737</td>
</tr>
<tr>
<td>Female n (%)</td>
<td>2,348 (48%)</td>
<td>341 (50%)</td>
<td>987 (55%)</td>
<td>952 (55%)</td>
</tr>
<tr>
<td>Age mean ± SD</td>
<td>30.3 ± 8.0</td>
<td>33 ± 7.3</td>
<td>53 ± 5.9</td>
<td>78 ± 5.1</td>
</tr>
<tr>
<td>Never smokers n (%)</td>
<td>1,559 (32%)</td>
<td>338 (50%)</td>
<td>896 (50%)</td>
<td>589 (34%)</td>
</tr>
<tr>
<td>Previous smokers n (%)</td>
<td>490 (10%)</td>
<td>154 (23%)</td>
<td>146 (8%)</td>
<td>1,036 (60%)</td>
</tr>
<tr>
<td>Current smokers n (%)</td>
<td>2,832 (58%)</td>
<td>182 (27%)</td>
<td>761 (42%)</td>
<td>111 (6%)</td>
</tr>
<tr>
<td>Pack-years median (IQR)†</td>
<td>4.2 (0.9–11.3)</td>
<td>8.5 (3.2–17.0)</td>
<td>23.1 (12.9–36.0)</td>
<td>18.1 (6.8–35.0)</td>
</tr>
<tr>
<td>Asthma n (%)</td>
<td>176 (3%)</td>
<td>65 (3.6%)</td>
<td>127 (7.5%)</td>
<td>-</td>
</tr>
<tr>
<td>Current asthma n (%)</td>
<td>90 (13%)</td>
<td>38 (6%)</td>
<td>-</td>
<td>155 (9%)</td>
</tr>
<tr>
<td>ICS use n (%)</td>
<td>-</td>
<td>38 (6%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Cross-sectional results

Cross-sectionally, the two younger cohorts (18–45 years old) consistently had a negative association between elevated B-Eos levels and lower lung function (FEV₁, VC, and FEV₁/VC), analysed in a multiple regression model, adjusted for previous or current smoking, pack-year smoking, age, sex, and height. In the older groups (> 44 years of age), an association was seen only with FEV₁ and FEV₁/VC, analysed in the same model.

Both the younger Vlagtwedde and Vlaardingen cohort and the ECRHS cohort, when stratified for asthma, showed a significant association between elevated B-Eos and lower FEV₁ and VC (but not FEV₁/VC ratio) among non-asthmatics. In the Vlagtwedde and Vlaardingen cohort, this negative association between elevated B-Eos and all lung function measurements was present and showed larger differences in lung function among the asthmatics. On the other hand, the ECRHS cohort showed no significant association between elevated B-Eos and lung function measures among asthmatics. In the older subgroups in the Vlagtwedde and Vlaardingen cohort there was an association between elevated B-Eos and lower VC among asthmatics, and between B-Eos and lower FEV₁/VC ratio among the non-asthmatics. In the Rotterdam cohort, no significant associations between elevated B-Eos and lung function were found when stratified for asthma.

Adjustment for ICS or OCS use in the analyses lead to minor changes in significance associations.

Longitudinal results

In the longitudinal analysis, done with a mixed model adjusted for age, height, sex, and pack-years, all in interaction with time, elevated B-Eos at baseline associated with a faster FEV₁ and FVC decline only in the ECRHS cohort.
In the analysis stratified for asthma, an elevated B-Eos level was associated with a faster VC decline among asthmatics from the younger Vlagtwedde and Vlaardingen cohort and with a faster FEV\textsubscript{1} decline among asthmatics from the ECRHS cohort, compared with asthmatics with a normal B-Eos level in the respective cohorts. The group of asthmatics from the older Vlagtwedde and Vlaardingen cohort was too small to analyse. Additional adjustment for cumulative ICS use in the asthma stratified analysis (the ECRHS cohort) did not change the results.

**Meta results**
The results from the age stratified meta-analyses are presented in Figure 12, both asthma stratified and for the whole populations.

*Figure 12.* Differences in lung function measures between the group with B-Eos levels ≥ 300 cells/µL in comparison with the group with < 300 cells/µL (reference), analysed stratified for asthma and in the whole group. The results are presented as meta-analyses for the separate age strata (grey background for cohorts < 45 years of age at baseline).
Discussion

The asthma morbidities discussed here were asthma symptoms, asthma attacks (exacerbations), FAO, obstructive pattern of spirometry, low lung function, and lung function decline in asthma and, to some extent, in non-asthmatics. The aim was to evaluate proposed biomarkers for inflammation in asthma for a better understanding of the morbidities associated with these inflammatory patterns.

Symptoms

Asthma symptoms and related biomarkers were assessed mainly in Papers I and II. We found a weak association between FAO and asthma symptoms in Paper IV, but no such association in Paper III.

In Paper I, elevated FeNO had some association with wheezing in the absence of a cold (unadjusted) and breathlessness after exercise (adjusted), but no significant association with any of the other evaluated symptoms. The association was stronger when S-ECP was also elevated. However, singly elevated S-ECP without elevated FeNO did not associate with more asthma symptoms compared to the group with low levels. In Paper II, singly elevated FeNO was not found to associate with more asthma symptoms, and there was a weak association when B-Eos was also elevated. This was similar to the association between elevated B-Eos and symptom burden, and there was no clear added value from FeNO.

Regarding singly elevated FeNO, the findings in Papers I and II are contradictory. The differences between the study groups could be an explanation for the differing associations found, and these are discussed in the next paragraph.

Differences between the populations: age, allergic sensitisation, smoking, and BMI

The NHANES study had participants aged between 6 and 18 years, and this group was overrepresented among the participants with simultaneously elevated FeNO and B-Eos. However, in the age-stratified analysis, the weak association between elevated FeNO and symptoms was present also in the youngest age group (see Paper II, supplementary material).

In the GA2LEN population, the prevalence of allergic sensitisation was high, 76%. The proxy for atopy used in the NHANES – hay fever – affected
only 34% of the population, which was surprising. This is probably partly a
sign of self-reported hay fever being an unreliable proxy for atopy. The
younger age group would be expected to contribute to a higher prevalence of
allergic asthma. However, a true difference in prevalence of allergic sensiti-
sation could indicate a larger value from measuring FeNO in individuals with
allergic sensitisation and a Th2-driven airway inflammation.

Smoking was more prevalent among the participants in Paper II. Only in-
dividuals older than 20 years were interviewed about smoking, but in this
group 23% were current smokers and 25% were ex-smokers, compared with
6.5% current smokers and 33% ex-smokers in Paper I. Smoke exposure meas-
ured with cotinine levels in serum in Paper II indicated 23% to be regularly
exposed to cigarette smoke (in the entire population), indicating that a sub-
stantial proportion of the younger individuals either were smokers or were
exposed to passive smoking. Since both current and previous smoking affect
FeNO levels (167) and the inflammation in asthma, this could probably con-
tribute to the different associations found between FeNO and symptom burden
in the respective papers.

The impact of obesity could also affect the associations between inflamma-
tory markers and symptoms, being more common in the population in Paper
II. As described above, obesity affects asthma morbidity in several ways with
altered eosinophil metabolism and neutrophil recruitment (28). However, no
clear indications of a large effect on FeNO has been found (168, 169).

In conclusion, the investigations presented in this work did not find a reli-
able biomarker associated with asthma symptoms. Rather, the results highlight
the importance of interpreting FeNO with caution, and taking into account
factors affecting the FeNO level, and suggest that FeNO is of value only in
specific patient populations.

Asthma attacks and asthma requiring emergency room
visits

Biomarkers in association with asthma attacks or asthma in need of urgent
care were also investigated mainly in Paper I and Paper II. In Paper III and
Paper IV, no associations between FAO and asthma attacks were found, al-
though this was not the main research question.

In Paper I, elevated S-ECP associated with having had an asthma attack in
the preceding three months. This was more pronounced if FeNO was also el-
evated. In Paper II, elevated B-Eos also associated with both asthma attacks
and ER visits due to asthma in the preceding year. However, in this study,
simultaneously elevated FeNO and B-Eos had a weaker association with
asthma attacks in the preceding year, and elevated FeNO was negatively as-
associated with ER visits.
In conclusion, elevated S-ECP, especially in combination with elevated FeNO, was associated with more asthma attacks. Elevated B-Eos associated with more asthma attacks and ER visits on its own. The associations with FeNO are probably affected by the same constitutional differences between the participants in Paper I and Paper II discussed in the previous section. However, the eosinophil markers seems to have been less affected by these factors.

Impaired lung function

Lung function was assessed in all papers; in Paper III and Paper IV specifically as FAO and in the others more generally. In Paper V, lung function over time was also evaluated.

Cross-sectional analyses

In Paper I, reduced FEV₁ pre-bronchodilation (FEV₁ < 80% of predicted) was not found to associate with either elevated FeNO, elevated S-ECP, or both markers elevated. In Paper II, FEV₁ < 80% of predicted was associated with elevated B-Eos, as well as having a higher prevalence in participants with both FeNO and B-Eos elevated. This association between having both FeNO and B-Eos elevated was also found to a lower FEV₁/FVC < LLN both pre-and post-bronchodilation. In Paper V, lower FEV₁ and lower FEV₁/FVC < LLN pre-bronchodilation was associated with elevated levels of B-Eos; in the younger groups also to a lower VC. Stratified for asthma, the asthma group from the ECRHS showed a tendency for higher lung function in contrast to the asthma group from Vlagtwedde and Vlaardingen.

Fixed airflow obstruction

FAO was found to associate with eosinophilic inflammation. In Paper III, FAO was evaluated in asthmatics, and found to associate most robustly with the marker U-EDN. S-ECP was also found to be more prevalently elevated among asthmatics with FAO than those without FAO, but was more affected by smoking history and sex than U-EDN. In Paper IV, FAO in asthma was associated with a higher prevalence of elevated B-Eos compared with among healthy controls and also compared with among asthmatics without FAO (univariately assessed). However, in Paper II, with a somewhat overlapping population, having both FeNO and B-Eos elevated was associated with FAO in the adjusted analysis, but not in the univariate analysis, and also not when FeNO and B-Eos were analysed separately at different cut-offs.

The increased prevalence of elevated level of B-Neu in non-asthmatics with FAO, and before adjustment also among asthmatics, was in smoking-stratified analyses seen only in current smokers and in individuals with more than 10 years of smoking history. This suggests an association between B-Neu and the
condition of the smoking exposed lung, even though B-Neu levels are weakly correlated with sputum neutrophils under asthmatic conditions (84).

**Longitudinal analyses**
In contrast to what was seen in the cross-sectional analyses, elevated B-Eos at baseline was associated with faster lung function decline (FEV₁ and FVC) among the asthmatics, but not among the non-asthmatics, in the longitudinal analysis performed in Paper V. An interesting aspect of the results from Vlagtwedde and Vlaardingen is the association between B-Eos and lung function decline in asthmatics, without ICS treatment, consistent with the findings from ECRHS, where ICS treatment was used in one third of the asthmatics.

**Age, allergic sensitisation and smoking, in relation to lung function**

**Age**
Lung function is highly dependent on age, as discussed in the Introduction. Using values in percent of predicted or related to LLN compensates for this effect. However, in young individuals, a low lung function can be suspected to be a sign of uncontrolled inflammation, rather than of structural changes in the airway tissue (airway remodelling). In Paper III, in the age-stratified analysis, the prevalence of elevated FeNO was higher among the participants with FAO (47%) than without FAO (26%) in the youngest age group, although not significantly, p = 0.085. In the oldest age group, the condition tended to be reversed (FAO: 31%, no FAO: 42%, p = 0.32). This in contrast to the results for U-EDN, where the higher prevalence of elevated U-SEND in the group with FAO compared with in the group without FAO was significant only in the higher age strata.

In Paper V, there was an association between elevated B-Eos and lower FEV₁ and VC both among asthmatics and non-asthmatics in the younger cohorts (meta-analyses). In the older cohorts, an association was found between elevated B-Eos and lower FEV₁ and a lower FEV₁/VC ratio. Hence, in the old cohorts, the association was mainly between elevated B-Eos and obstructive lung function, while in the young cohorts it was between B-Eos and smaller lung volumes. Elevated B-Eos in younger individuals could be related to a lower peak lung function, while in older individuals it might be more closely related to a more pronounced decrease in FEV₁. The associations between elevated B-Eos and lower lung function were more pronounced in the young cohorts, suggesting active inflammation to be a more important factor at young age, in line with the results from Paper III.

The investigation on lung function decline in Paper V supported this hypothesis to some extent, where the association between elevated B-Eos levels and a faster lung function decline (FEV₁ and FVC) was found only in the
young cohorts, restricted to the asthmatics. However, a weakness was the small number of asthmatics in the older cohort from Vlagtwedde and Vlaarding, permitting interpretation only of the strata with non-asthmatics.

**Allergic sensitisation**
In Paper III, allergic sensitisation was more prevalent in the group without FAO than in the group with FAO. In Paper IV, no difference was seen between the two asthma groups (with or without FAO) in self-reported episodes of hay fever during the preceding year. The stratification for allergic sensitisation in Paper III did not reveal any interactions in relation to the assessed biomarkers. Elevated FeNO and S-ECP was more common in the group with allergic sensitisation, but did not differ in relation to FAO. Interestingly, the prevalence of elevated U-EDN was similar in the sub-groups with and without allergic sensitisation, but was significantly higher among participants with FAO in both strata. The association between asthma without allergic sensitisation and FAO, with increased levels of a proposed type-2 marker, raises the question whether U-EDN better reflects a non-Th2 associated type-2 pathway, such as ILC-2-driven eosinophilic asthma.

**Smoking**
Smoking was associated with FAO in both Paper III and Paper IV. Both in the asthma group with FAO and in the group with FAO but without asthma in Paper IV, three fourth of the participants had a smoking history, indicating smoking to be an important factor for FAO development irrespective of asthma. The never smoking groups in the stratified analysis in Paper IV also had a different inflammatory pattern, with significantly higher prevalence of elevated FeNO in all case groups, but no difference in the prevalence of elevated B-Neu. The results possibly suggest some degree of undiagnosed asthmatics in the group with FAO without asthma. In Paper III, the association between elevated FeNO and FAO showed an interaction regarding smoking, with a lower prevalence associated with FAO compared with no FAO among former or current smokers. A lower prevalence was also seen in participants with $\geq 10$ pack-years of smoking history, but a higher prevalence associated with FAO compared to no FAO in never smokers and participants with $< 10$ pack-years, although not significantly.

**Effect of medication, symptoms and lung function**
In these investigations, Paper IV revealed a slightly higher symptom burden in asthmatics with FAO compared with asthmatics without FAO. This was not seen in Paper III. However, in Papers I and II, the eosinophil markers associated with increased burden of exacerbations, and in Paper II also with lower lung function. The relation between asthma exacerbations and faster lung
function decline has been studied and established previously (170-174). It is difficult to determine if the effect on lung function is dependent on the exacerbations, where inflammatory bursts associated with asthma exacerbations could lead to irreversible tissue damage, or if it is an effect of a poorly controlled inflammation leading to exacerbations.

Anti-inflammatory medication, referring mainly to (inhaled) corticosteroids, is an important disease-modifying factor. It is also one of the most important reasons to identify inflammatory biomarkers in asthma. In these investigations, steroid dose was found to be higher in asthmatics with FAO (Paper III), anti-inflammatory medication was more common in asthmatics with FAO than in asthmatics without FAO in Paper IV. The higher use of corticosteroids, despite a similar symptom burden in the groups, is intriguing. It could be interpreted as an adequate treatment regimen in more severe asthma. However, it could also be a sign of treatment failure, where the treatment does not have the intended effect and is increased in an unresponsive disease.

Earlier studies investigating corticosteroid use and its effect on lung function development have come to contradictory conclusions. In a randomised control trial comparing placebo, nedocromil, and budesonide in children, budesonide treatment was not found to be protective against fast FEV\textsubscript{1} decline. The participants with a faster FEV\textsubscript{1} decline were less atopic and of male sex (175). However, in a study by O’Byrne et al., budesonide protected against exacerbations (176) and, when an exacerbation did occur, decreased the excess lung function decline in the group experiencing exacerbations. This was also found in a study on children, where budesonide protected against both exacerbations and lung function decline (177). Other studies have also found beneficial effects on lung function development after introduction of ICS (178). The time from asthma onset to intervention seems to be of importance. Also anti-IL-5 treatment has been found to improve FEV\textsubscript{1} in severe asthma, but only among participants without any exacerbations; this protective effect was not seen among participants with exacerbations (173). Interestingly, in a study by Matsunaga et al., a greater decline in FEV\textsubscript{1} correlated with a reduction in reversibility (174), linking the lung function decline to development of FAO.

In Paper V, the population from Vlagtwedde and Vlaardingen did not have ICS treatment, while the ECRHS and Rotterdam populations had. Interestingly, the asthmatics from ECRHS did not show any difference in lung function cross-sectionally between the group with elevated B-Eos and normal B-Eos levels; but a tendency to better lung function in the group with elevated levels. This was the opposite of what was seen for the asthmatics from Vlagtwedde and Vlaardingen, where the asthma group with elevated B-Eos had a worse lung function than the asthma group with normal B-Eos. One explanation could be a protective effect from ICS treatment in the ECRHS group. However, this was not seen in the longitudinal investigation, where the asthma groups with elevated levels had a worse decline in both the ECRHS and the
Vlagtwedde and Vlaardingen cohorts. An interpretation of this could be that a steroid sensitive eosinophil inflammation is more closely associated to acute broncho-constriction, while steroid insensitive eosinophil inflammation is associated to airway remodelling changes and long term lung function decline.

In conclusion, eosinophilic inflammation was found to be associated with both FAO and lower lung function, and is likely to be involved in the presence and development of reduced lung function to some extent. In these investigations, although only evaluated in one material, U-EDN seemed to best reflect post-bronchodilatory reduced FEV$_1$/FVC and FAO in asthma. The analyses done in general populations (Paper IV and Paper V) highlight the risk of analysing an effect of asthma rather than an effect of a specific inflammatory pathway.

Summary of evaluated biomarkers and traits related to certain outcomes

The biomarkers evaluated here have added some knowledge on inflammation-associated outcomes in asthma. The main findings are visualized in Figure 13 and summarized in Table 13.

FeNO had some association with asthma symptoms, as well as a negative association with asthma exacerbations. It was found to associate mainly with asthma, and lower lung function, to a certain extent, together with elevated B-Eos in Paper II and in non-asthmatics with FAO but no smoking history in Paper IV. An interpretation of this could be that FeNO, at least in the absence
of simultaneous eosinophilia, is a sign of a benign type-2-associated asthma, mainly representing mucosal engagement. Previous research into the association of FeNO with ICS sensitivity (123), allergic sensitisation and IL-4- and IL-13-dependent pathways (121) further strengthens this interpretation.

U-EDN was here found to be associated with FAO in asthma. This association was robust, also when taking allergic sensitisation, sex, and smoking into account. EDN has anti-viral properties and a release related to viral infections would be plausible. Earlier studies have found a correlation between U-EDN and FEV1, and decreased levels of U-EDN with increased lung function (100). Further studies are needed to better understand the physiological background, and whether U-EDN could be a useful marker for predicting or monitoring lung function development, or if there are beneficial effects of increased corticosteroid treatment. The non-invasive measurement has an added benefit in comparison with other eosinophil markers.

S-ECP was found to associate with asthma exacerbations in Paper I, especially with simultaneous FeNO elevation. However, it was not associated with lung function. In Paper III, there was some association with FAO, but this was mainly seen among men and smokers. The findings here suggested U-EDN to be a better marker of lung function-associated morbidity. Still, S-ECP, especially in combination with FeNO, appeared to be associated with increased symptom and exacerbation burden, reflecting eosinophilic inflammation. However, the results here do not indicate that it is a better marker of eosinophilic inflammation than B-Eos.

B-Eos was found to associate with both increased symptoms and exacerbation burden, and FAO in asthmatics. B-Eos is an extensively studied marker in asthma, and the main additive findings in these investigations are the stronger association with low lung function, with simultaneously elevated FeNO, and worse lung function development assessed over time in asthma. Furthermore, we could not see any evidence of a protective effect from ICS on excessive lung function decline in asthma in our material, although not the main analysis.

S-periostin was only assessed in relation to FAO, and we could not find any convincing association in this study. In this investigation, only cross-sectional associations were evaluated, and it is not possible to draw conclusions on the potential usefulness of periostin in a longitudinal setting. However, the sensitivity to other factors affecting the S-periostin level makes it problematic as a reliable marker for inflammation in asthma.

B-Neu was found to be higher in smokers with FAO than non-asthmatic smokers without FAO and in both asthmatics and non-asthmatics as compared with in non-asthmatic smokers stratified for more than 10 pack-years. The poor correlation with sputum neutrophils makes B-Neu difficult to interpret in regard to airway inflammation. However, our findings indicate an association with FAO in smokers, with lower prevalence of elevated B-Neu in participants.
without FAO and asthma. This association may be a sign of increased susceptibility to infections, leading to neutrophil recruitment in individuals with an airway pathology. Further studies are needed to evaluate more specific causes of these association and the signification of B-Neu in lung disease.

Table 13. Summary of the evaluated inflammatory biomarkers and the associated outcomes.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Implications from Papers I–V</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeNO</td>
<td>Together with elevated S-ECP (symptoms, exacerbations) or elevated B-Eos (lung function) sign of more severe disease. If elevated without simultaneously elevated eosinophil marker, related to more benign disease.</td>
</tr>
<tr>
<td>U-EDN</td>
<td>More prevalently elevated in asthmatics with FAO, independent of allergic sensitisation, sex, and smoking.</td>
</tr>
<tr>
<td>S-ECP</td>
<td>Associated with exacerbations, especially with simultaneously elevated FeNO levels. Some association with FAO, especially in presence of elevated U-EDN.</td>
</tr>
<tr>
<td>B-Eos</td>
<td>Associated with asthma symptoms and exacerbations; associated with FAO and accelerated lung function decline in asthmatics.</td>
</tr>
<tr>
<td>S-periostin</td>
<td>Not found to be associated with FAO.</td>
</tr>
<tr>
<td>B-Neu</td>
<td>Associated with FAO in smokers.</td>
</tr>
</tbody>
</table>

Methodological aspects

In epidemiology, the selection of data on which the investigations are based is essential to both the internal and the external validity of the results. Further, the strategy chosen for analysis of the data and handling of covariates other than the main variables of interest can both avoid and introduce pitfalls in the interpretation. A systematic error is affecting the whole population or analysis. A systematic error is commonly called a bias. Common causes include a wrongly calibrated instrument, a very low response rate from a certain subgroup of the population investigated, leading to a selection bias, or confounding depending on a common root of both cause and effect that might explain the proposed causality (179). A random error is the variability in the data without a given explanation (chance), or the deviation from the true value in a response (179, 180); it will be smaller when a larger population is investigated.

Systematic errors

**Study populations and selection of participants**

In an investigation, a subset of a population is studied and the conclusions drawn are dependent on whether the sampled subset is representative. Selection bias can occur when certain subpopulations respond more or less frequent than others and this leads to a selection of participants with certain characteristics.
The response rate in GA\textsuperscript{2}LEN in the first round was approximately 60%, which is acceptable. In the second round, there was an 83% response rate and the participants were selected randomly, depending on the respiratory symptoms reported in the first questionnaire, to fill up certain quota (asthma, asthma and chronic rhinosinusitis, chronic rhinosinusitis, and controls) (149, 150). If the responses to the first questionnaire selected a certain subgroup of asthmatics, this could lead to a selection bias. In the second round, with the clinical examination, the response rate was good.

In the NHANES, there was a crude response rate of 70–79% (152). However, this was adjusted for by the complex multistage sampling procedure and the weighted analysis (151). These are statistical methods where answers are weighted to adjust for underrepresented subpopulations based on register variables (characteristics), to compensate for the differing response rates in the studied sample. The results from the NHANES can be assumed to represent the American population well. Thus, this should be the case for the subgroup included in this investigation.

In the ECRHS I, there was a high response rate of 87% in the first round, and an 83% response rate in the second round (clinical visit) (153). The population participating in the ECRHS II and/or ECRHS III follow-up was 72% of the entire group, and its baseline characteristics were similar to those of the whole population.

In the Vlagtwedde and Vlaardingen study, the total population receiving an invitation to the study consisted of approximately 10,000 individuals (156). In Paper V, due to the inclusion criteria, 6,684 individuals were included and 54% from the cross-sectional group continued to the follow-up. Here too, the baseline characteristics were similar among the participants in the cross-sectional and longitudinal parts.

In the Rotterdam study, the data used in the present analysis were taken from part of the population from their sixth visit (RS-I-6) and from another part on their fourth visit (RS-II-4). The first cohort were included in 1990, and the baseline response rate was 78%; the second cohort was included in 2000 and had a 67% response rate (158). Only data from the last follow-up were used. At this examination, 61% of the invited participants were eligible to be included in the present analysis and this group was representative of the group included at baseline (181).

**Asthma diagnosis**

In GA\textsuperscript{2}LEN, ECRHS, and Rotterdam, the diagnosis of asthma was defined as self-reported doctor’s diagnosis of asthma, together with symptoms and/or current medication for asthma in the preceding year. The definition in the NHANES was a self-reported doctor’s diagnosis, together with self-reported current asthma, which could lead to a different selection of the asthmatic subgroup, possibly accounting for some of the differences found between Paper I
and Paper II. In Paper V, the Vlagtwedde and Vlaardingen study had a different definition than the other cohorts, with self-reported wheezing in absence of a cold used to define asthma that might lead to heterogeneity in the asthma group.

**Confounding, interactions, and collider effects**

In the analyses, efforts were made to take confounding into account through stratification and adjusted analyses. Smoking and smoking history affect all aspects of respiratory health and inflammation and are thus important potential confounders. Therefore, adjustments were made in all papers for both current smoking status and cumulative exposure (pack-years). Age, age of asthma onset, allergic sensitisation, anti-inflammatory medication, and BMI are also potential confounders. The reported use of medication can be affected by difficulties to remember medication intake, reporting of a prescribed dose rather than a used dose, and can further vary significantly over time. Further, it can be hypothesised to have a complex association with symptom burden. This is a weakness and could influence the results. Both adjusted and unadjusted calculations were presented in the main analyses.

Stratification further enables identification of interactions between effects in sub-groups of the population. Such stratification was performed and presented to better explain the associations.

A risk with adjusted analyses and stratifications is the introduction of a selection bias through adjustment for a collider, dependent on a common effect from both exposure and effect. This introduces a selection bias in the analysis and can lead to incorrect conclusions.

**Random error**

In Papers I and II, where asthma symptoms and asthma attacks are discussed, only retrospective data on asthma morbidity were available. This is a limitation, where difficulties to remember asthma symptoms and attacks can introduce both less and more reported morbidity than the true level. We assumed this to be randomly distributed. The questionnaire used in GA²LEN was based on the ECRHS questionnaire, which is a widely used respiratory questionnaire. Experienced research personnel did the examinations and performed the interviews in a standardised way. In the NHANES, the examinations and interviews were also performed by experienced personnel in a standardised way.

The biomarkers assessed show variability over time (182). Retrospective reporting of asthma symptoms could attenuate the association and, crucially, makes predictive properties impossible to evaluate. An asthma worsening would likely lead to intensified treatment with corticosteroids affecting FeNO (86, 124), S-ECP (114), and B-Eos levels (89). This could lead to a systematic error, however, it would most likely attenuate the association between the markers and the outcomes. In both the GA²LEN and the NHANES materials,
steroid use was reported. Additionally, in the NHANES, steroid use during the two days preceding examination was reported and adjusted for, and this might be a more appropriate way to adjust for effects on biomarkers prone to promptly respond to steroids, such as FeNO.

The spirometry measures evaluated in Papers I–IV were performed at the same time point as the other examinations, assessing an association not affected by changes in medication in the meantime or recall bias.

In Paper V, the three populations were analysed with the same statistical model, but investigated separately. The definition of asthma is not identical between the studies, as mentioned above, leading to difficulties in the interpretation. The different time frames during which the cohorts were analysed, as well as differences in environmental exposures related to location, can probably affect the results. This can be seen as a strength for the generalisability, but also leads to difficulties in the interpretation of associations.
Conclusions

General conclusion
Asthma with elevated markers for eosinophilic inflammation was associated with worse lung function and asthma morbidity than asthma without elevated markers for eosinophilic inflammation. This indicates eosinophilic inflammation to be highly important for asthma morbidity, and absence of eosinophilic inflammation to be associated with less morbidity in comparison.

Specific conclusions

I–II Simultaneously elevated levels of FeNO and S-ECP was associated with more reported asthma symptoms and asthma attacks, while simultaneously elevated FeNO and B-Eos were not. However, elevated FeNO and B-Eos did associate with lower lung function. Concomitant local and systemic inflammation is concluded to be associated with worse morbidity, but the implications of elevated FeNO should be interpreted with caution.

III Eosinophilic inflammation was concluded to associate with fixed airflow obstruction, and U-EDN was seen to be a more robust marker than S-ECP. S-periostin and FeNO did not associate with fixed airflow obstruction in asthma.

IV Elevated B-Eos was associated with fixed airflow obstruction in asthma. FeNO was associated with asthma, but not with fixed airflow obstruction. Elevated B-Neu was associated with fixed airflow obstruction and asthma among smokers and participants with a smoking history.

V Elevated B-Eos was associated with lower lung function cross-sectionally in a general population, and with faster lung function decline, restricted to asthmatics.
Clinical implications
In this investigation, eosinophilic inflammation was found to be associated with worse outcomes in asthma. Treating eosinophilia in asthmatics with signs of eosinophilic inflammation is found to improve morbidity in terms of symptoms and attacks. It may also be important with regard to preserved lung function.

Future perspectives
Despite improved treatment possibilities, not all asthmatics have good asthma control. Furthermore, asthma is associated with both worse peak lung function and faster decline. Gaining better understanding of the mechanisms behind this impaired lung function development, and finding treatments to prevent it, will remain important tasks for the future. In this work, biomarkers for inflammation will probably play an important role, both in the selection of the right patients for treatment, and also in the finding of new potential treatment targets.

In the investigations presented here, eosinophils and eosinophil markers were found to be associated with both asthma symptoms and attacks, as well as with worse lung function measures. Corticosteroid treatment has so far not shown any clear protective effect (175-177). Whether a more efficient suppression of the eosinophils with monoclonal antibodies can improve long-term lung function outcomes is still not known; neither are other long-term consequences of eosinophil suppression.

Biological treatments with monoclonal anti IL-5 antibodies have been found to be effective in reducing steroid dependence, and to keep asthma under control in severe eosinophilic asthma (90, 91). Neutrophil inflammation has been shown to imply a less good response to corticosteroids, and may be an effect modifier or a cause of persistent symptoms. The combination of several biomarkers may give a better prediction of treatment response to certain treatments.

Other knowledge gaps are treatment options in non-type-2 asthma, which remain sparse, and when to abstain from corticosteroid treatment, which may in some cases do more harm than good.

In conclusion, biomarkers will probably be important for future research, in finding novel treatment targets, in predicting treatment response to existing medications, and to endotype and phenotype individual patients. The overarching aim is to be able to keep asthma under control and prevent excessive lung function loss.
Sammanfattning på svenska


Aasma är en vanlig sjukdom (med en prevalens mellan 1−18% i olika delar av världen (2)), och trots förbättrade behandlingsmetoder är aasma fortfarande en potentiellt livshotande sjukdom; det är många som inte når bra astmakontroll och många drabbas av en progredierande lungfunktionsnedsättning.

De främsta framstegen under senare år i omhändertagande av astmapatien ter är en bättre kunskap om den underliggande kroniska luftvägsinflammation som de flesta astmatiker har och som är en viktig orsak till den morbiditet som aasmajukdomen innebär. Det har lett till mer skrådarsyst behandling av inflammationen där specifika inflammatoriska mekanismer hämmas. För att veta vilka patienter som kan ha nytta av vilka behandlingar och för att hitta behandlingsbara aspekter av sjukdomen är idag en stor del av aasmaforsk ningen inriktad på biomarker. En biomarker kan vara en molekyl eller ett protein som mäts i kroppen, eller andra undersökningsparametrar (som t.ex. röntgenfynd), som man försöker koppla till olika aspekter av en sjukdom. Dessa kan sedan användas för att förutspå behandlingsvar, förstå sjukdomsmekanismer eller för att hitta angreppmekanismer för nya läkemedel.

Ofta delas aasma in i undergrupper, fenotyper, beroende på vilka typer av inflammatoriska celler som förekommer i luftvägarna och blodet. En vanlig fenotyp är eosinofil aasma, där en typ av vita blodkroppar, eosinofiler, dominerar inflammationen. Eosinofil inflammation triggas ofta av allergier, men kan också ha andra orsaker. En annan typ av vit blodkropp, neutrofilen, kan också vara framträdande vid astmatisk inflammation. Neutrofiler är viktiga i det direkta försvaret mot infektioner, och förhöjda nivåer av neutrofiler kan spegla en infektion men kan också vara ett svar på annan akut vävnadsskada. Dessa celler är också viktiga biomarker för vilken typ av inflammation som är dominerande hos en specifik individ. Den indelningen kan breddas ytterligare till en indelning i aasma med ”typ-2” inflammation, baserat på ett underliggande inflammatoriskt mönster som ofta avspeglas i förhöjda eosinofilin- nivåer, men som också kan mätas med flera andra biomarker (bland andra
fraktion utandad kväveoxid (FeNO) som återkommer nedan); och ”icke-typ-2” inflammation där förhöjda neutrofilnivåer är vanligare.

I den här avhandlingen har sex olika biomarkörer undersökt i relation till astmasjuklighet (symptom, exacerbationer) och lungfunktion. Lungfunktion har mätts med dynamisk spirometri, både över tid och i tvärsnitt. I arbete III och IV har framför allt astma med fixerad obstruktion undersökt. Fixerad obstruktion är en beteckning på en kronisk lungfunktionsnedsättning där kvoten mellan den forcerade utandade volymen den första sekunden (FEV₁) och den forcerade vitalkapaciteten (FVC) inte normaliseras av bronkvidgande behandling. Kvoten FEV₁/FVC är således även efter bronkdilatation <0,7 eller under den nedre normalgränsen (beräknad utifrån kön, ålder och längd), och är ett mått på en obstruktiv lungfunktionsnedsättning.

I arbete I undersöktes FeNO i utandningsluft och eosinofil katjonprotein i serum (S-ECP) som utsöndras av eosinofiler vid aktivering, i relation till rapporterade astmasymtom och astmaattacker det senaste året. Undersökningssgruppen, som bestod av astmatiker från en allmän population, delades sedan in i fyra grupper, en med normala nivåer av FeNO och S-ECP, en med förhöjt FeNO (≥25 ppb), en med förhöjt S-ECP (≥20 µg/L) och en med förhöjda nivåer av bägge markörerna. Dessa grupper jämfördes sedan avseende rapporterade astmasymtom och attacker. Vad vi kunde se var att förhöjt FeNO kunde kopplas i någon mån till mer symptom, förhöjt S-ECP till mer astmaattacker, men att bägge förhöjda hade den starkaste kopplingen till mer astmasymtom och astmaattacker.

I arbete II undersöktes en större grupp astmatiker också från en (amerikansk) allmän population. Den gruppen delades in fyra grupper även den: en grupp med normalt FeNO (<20 eller 25 ppb beroende på ålder), och normala blodeosinofilnivåer (<0,3x10⁹ celler/L); en grupp med förhöjt FeNO, en grupp med förhöjda blodeosinofilnivåer och en grupp med bägge markörerna förhöjda. Här kunde vi inte se någon koppling mellan förhöjt FeNO och mer symptom, men dock till färre astmaattacker det senaste året. Förhöjda blodeosinofilnivåer var dock kopplats till mer astmaattacker. Här undersöktes även lungfunktion, och att ha både förhöjt FeNO och blodeosinofilnivå var kopplat till sämre lungfunktion.

I arbete III undersöktes astmatiker med och utan fixerad obstruktion och jämfördes avseende nivåer av fyra biomarkörer för inflammation: FeNO (definierat som förhöjt ≥25 ppb), eosinofil nervtoxin i urin (U-EDN) (förhöjt: ≥65,95mg/mol creatinine), S-ECP (förhöjt: ≥20 µg/L) och periostin mätt i serum (S-periostin), förhöjt: ≥74µg/L. EDN är liksom ECP ett eosinofil granulaeprotein som utsöndras vid aktivering av de eosinofila cellerna. Periostin är ett protein som förekommer i extracellulärmatrix, och som har associerats med typ-2 inflammation, men som också påverkas av till exempel bennybildning. U-EDN var den markör som tydligast var kopplat till en fixerad obstruktion i
den här undersökningen, medan S-ECP var det i viss mån. FeNO var inte associerat till fixerad obstruktion. Sammantaget var det den eosinofila inflammationen som var tydligast kopplad till fixerad obstruktion.

I arbete IV inkluderades både personer med och utan astma från en allmän population. De delades in i fyra grupper: en grupp med personer utan astma och utan fixerad obstruktion (kontrollgrupp), en grupp där deltagarna hade astma men inte fixerad obstruktion, en grupp med astma och fixerad obstruktion och en grupp utan astma men med fixerad obstruktion. Dessa grupper jämfördes avseende prevalens av förhöjda nivåer FeNO (≥25 ppb), blodeosinofiler (≥0,3x10⁹ celler/L) och blodneutrofiler (≥5,1x10⁹ celler/L). Resultaten visade att förhöjt FeNO var vanligare i astmagrupperna, men inte kopplat till fixerad obstruktion, blodeosinofiler var oftare förhöjda i alla grupperna jämfört med kontrollerna men med högs prevalens i astmagruppen med fixerad obstruktion. Blodneutrofiler var oftare förhöjda i gruppen utan astma men med fixerad obstruktion. Förhöjda nivåer av blodneutrofiler var dock nära kopplat till rökning och rökexponering.

I arbete V undersöcktes huruvida förhöjda blodeosinofilnivåer (≥0,3x10⁹ celler/L) var associerat till lägre lungfunktion i en allmän befolkning och hos astmatiker. Därefter studerades också om lungfunktionen snabbare försämrades överbtid (upp till 20 år) hos personer med förhöjda blodeosinofilnivåer vid baslinjen. Tre kohorter undersöks, en från Uppsala, Sverige (European Community Respiratory Health Survey I-III, ”ECRHS”, med deltagare <45 år) och två från Nederländerna (deltagare från Vlagtwedde och Vlaardingestudien och Rotterdamstudien). Rotterdamstudien analyserades endast i tvärsnitt. Vlagtwedde och Vlaardingepopulationen delades i två grupper, en yngre <45 år (som jämfördes med ECRHS-populationen) och en äldre ≥ 45 år (som jämfördes med gruppen från Rotterdamstudien där deltagarna var 70−95 år gamla). Resultaten visade en lägre lungfunktion i tvärsnitt kopplat till förhöjda blodeosinofilnivåer. Gruppen som hade förhöjda blodeosinofiler vid baslinjen hade en snabbare lungfunktionsförlust övertid. Vid en uppdelning i astmatiker och ickeastmatiker kvarstod skillnaden bara i astmagruppen, följaktligen, att ha astma och höga blodeosinofilnivåer var kopplat till en snabbare lungfunktionsförlust jämfört med att ha astma men normala blodeosinofiler. I gruppen utan astma syntes ingen skillnad i lungfunktionsförkust kopplat till blodeosinofilnivå.

Slutsats

Resultaten i de här studierna tyder på att hos personer med astma är framför allt eosinofil inflammation kopplat till en sämre lungfunktion och svårare astmasjuklighet än hos personer med astma utan tecken till eosinofil inflammation. Det talar för att aktiv eosinofil inflammation är ett allvarligt tecken vid astma, även vid mildare former av sjukdomen.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)