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– microRNA, nasal nitric oxide and proteome analysis

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DOCTORAL DISSERTATION

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CONTENTS

ABSTRACT

The prevalence of chronic rhinitis and asthma in the adult population is high worldwide. These diseases often coexist; the vast majority of asthmatics have rhinitis and many patients with rhinitis also have asthma. The upper and lower airway inflammation in allergic rhinitis and asthma have several common characteristics. Thus, rhinitis and asthma can be seen as manifestations of one disease with a common underlying inflammatory process.

The aim of this thesis was to investigate airway inflammation biomarkers in asthma and rhinitis in the context of rhinitis and asthma as one disease. We assessed markers of inflammation in the upper and lower airways of patients with allergic and nonallergic rhinitis and asthma.

A total of 336 men and women aged between 31 and 49 years from separate study populations were examined. Study population A was divided into four groups on the basis of their medical history and clinical examination: allergic rhinitis, allergic rhinitis with concomitant asthma, nonallergic rhinitis and healthy controls. Similarly, population B was divided into three groups: persistent and non-persistent long term asthma, and healthy controls. We assessed inflammatory cells, cytokines and microRNA levels in the nasal biopsies, and exhaled and nasal nitric oxide levels in both populations. In addition, in Population A, nasal computed tomography (CT) scans as well as sputum and nasal lavage fluid proteomics were analysed.

We found only modest differences between the nasal inflammatory cells and cytokine levels in the nasal biopsies of the patients with allergic rhinitis and asthma and the controls. In population A, we found up-regulations of microRNAs miR-155, miR-205 and miR-498 in the nasal biopsies of the subjects with current allergic rhinitis, and a

down-regulation of let-7e in asthmatic patients without current rhinitis symptoms compared to healthy controls. In population B, miR-498, miR-187, miR-874, miR-143 and miR-886-3 were up-regulated in the nasal biopsies of the patients with long-term asthma, and miR-18a, miR-126, let 7e, miR-155 and miR-224 were down-regulated. Four of these microRNAs were also down-regulated in the asthmatic patients without allergic rhinitis. We only detected trends of differences between the microRNA expression of the non-persistent and persistent asthma groups.

Compared to the controls, the level of nasal nitric oxide was slightly elevated in the subjects with allergic rhinitis in the study population A, but not in those with nonallergic rhinitis. A positive correlation between the nasal and exhaled nitric oxide levels ($r=0.38$, $p<0.01$) and an inverse correlation between the nasal nitric oxide level and sinus ostia obstruction ($r=-0.27$, $p=0.013$) was detected. When we evaluated the allergic rhinitis patients without marked sinus ostial obstruction, the nasal nitric oxide level correlated positively with the sinus opacification score ($r=0.25$, $p=0.033$) as well as with the nasal eosinophil count ($r=0.29$, $p=0.030$).

In the subgroup of Population A, we identified 31 different proteins in the sputum proteome analysis, most of which were also found in nasal lavage fluid. An increased abundance of fatty acid binding protein 5 (FABP5) was found in the sputum of the asthmatics. In the immunological validation of Population A, we found increased levels of FABP5 protein both in the sputum and in the nasal lavage fluid of the asthmatics. Positive correlations between the FABP5 and vascular endothelial growth factor (VEGF) levels ($r=0.66$, $p<0.01$), as well as between the FABP and cysteinyl leukotriene levels ($r=0.54$, $p<0.01$) were detected in the nasal lavage fluid, suggesting that FABP5 may contribute to airway inflammation and remodeling.

Conclusions: We found differentially expressed microRNAs in nasal mucosa in allergic rhinitis and asthma. Among the asthmatics, differences in the microRNAs were also detected when no significant changes in the inflammatory cells and cytokines were found. In the future, microRNAs arrays might be useful as a sensitive method for assessing airway inflammation. The nasal nitric oxide level reflects eosinophilic inflammation in nasal mucosa in allergic rhinitis. However, the level is dependent on sinus ostia obstruction, reducing its feasibility in monitoring allergic inflammation. The marker of asthmatic inflammation that we found

in sputum, was also detected in the nasal lavage fluid of the asthmatics. Samples from upper airways are easy to obtain, and our findings suggest that they might be useful in investigating lower airway inflammation in asthma.

TIIVISTELMÄ

Kroonista nuhaa ja astmaa esiintyy yleisesti aikuisikäisissä väestöissä ympäri maailman. Suurimmalla osalla astmaatikoista on myös krooninen nuha ja monilla nuhapotilailla todetaan astma. Astmaan ja allergiseen nuhaan liittyvässä hengitystietulehduksessa todetaan samankaltaisia piirteitä. Näin ollen astma ja nuha voidaan nähdä saman sairauden eri ilmentyminä, joihin liittyy samanlainen hengitysteiden tulehdusprosessi.

Tavoitteenamme oli tutkia astmaan ja nuhaan liittyvää hengitystietulehdusta perustuen ajatukseen, että astma ja nuha kuuluvat samaan tautikokonaisuuteen. Tutkimme ylä- ja alahengitysteiden tulehdusmerkkiaineita allergista ja ei-allergista nuhaa ja astmaa sairastavilla henkilöillä.

Yhteensä 336 iältään 31–49-vuotiasta henkilöä osallistui tutkimuksiin muodostaen kaksi tutkimusaineistoa. Tutkimusaineistoon A kuuluvat henkilöt jaettiin neljään ryhmään sairaushistorian ja kliinisten tutkimusten perusteella. Ryhmät olivat allergista nuhaa, allergista nuhaa ja astmaa, ei-allergista nuhaa sairastavat sekä terveet verrokit. Tutkimusaineistoon B kuuluvat pitkään astmaa sairastaneet ja terveet henkilöt jaettiin kolmeen ryhmään: oireettomat tai ajoittaisesti oireilevaa astmaa sairastavat, jatkuvasti oireilevaa astmaa sairastavat ja terveet verrokit. Analysoimme tulehdussoluja, sytokiini- ja microRNA-tasoja nenäbiopsianäytteistä sekä uloshengitysilman ja nenän typpioksidipitoisuutta molemmissa tutkimusaineistossa. Aineistossa A tutkimme lisäksi nenän tietokonekuvia sekä indusoituja ysköksiä ja nenähuuhtelunäytteitä käyttäen proteomiikkamenetelmää.

Nenän limakalvonäytteiden tulehdussoluissa ja sytokiinitasoissa todettiin vain vähäisiä muutoksia astmaa ja nuhaa sairastavilla terveisiin verrokkeihin verrattuna. Tutkimusaineistossa A tutkittavilla, joilla oli tutkimushetkellä allergisen nuhan oireita, todettiin micro-RNA-tasojen

miR-155, miR-205 ja miR-498 olevan koholla. Astmaa ja allergista nuhaa sairastavilla henkilöillä, joilla ei ollut tutkimushetkellä nuhaoireita, todettiin let-7e-tason madaltuminen. Tutkimusaineistossa B pitkään astmaa sairastaneilla henkilöillä todettiin miR-498-, miR-187-, miR-874-, miR-143- ja miR-886-3-tasojen lisääntyneen ja miR-18a-, miR-126-, let 7e-, miR-155- ja miR-224-tasojen vähentyneen nenän limakalvolla. Näistä neljän microRNA:n ilmentymisen todettiin vähentyneen myös niillä astmaatikoilla, joilla ei todettu allergista nuhaa. MicroRNA tasoissa todettiin vain viitteellisiä eroja oireettomia tai ajoittaisesti oireilevaa astmaa sairastavia ja jatkuvasti oireilevaa astmaa sairastavia verrattaessa.

Aineistossa A nenän typpioksidipitoisuus oli lievästi koholla allergista nuhaa sairastavilla, sen sijaan ei-allergista nuhaa sairastavien taso ei poikennut kontrolliryhmästä. Nenän ja uloshengitysilman typpioksidipitoisuuden välillä todettiin positiivinen korrelaatio ($r = 0.38$, $p < 0.01$). Lisäksi nenän typpioksidin ja sivuonteloiden aukkojen eli ostiumien ahtauman välillä todettiin negatiivinen korrelaatio ($r = -0.27$, $p = 0.013$). Kun tutkimme allergista nuhaa sairastavia henkilöitä, joiden sivuonteloiden ostiumit eivät olleet merkittävästi ahtautuneet, totesimme nenän typpioksidipitoisuuden korreloivan positiivisesti sivuonteloiden radiologisen samentuman tason ($r = 0.25$, $p = 0.033$) kanssa sekä nenäbiopsian eosinofiilien määrän kanssa ($r = 0.29$, $p = 0.030$).

Tunnistimme 31 eri proteiinia ysköksen proteomianalyysissä, joka suoritettiin osalle aineiston A näytteistä. Suurin osa proteiineista tunnistettiin myös nenähuuhtelunesteestä. Fatty acid binding protein 5 (FABP5) -pitoisuus oli lisääntynyt astmaatikkojen ysköksissä. Immunologisessa validoinnissa aineistossa A totesimme FABP5-tason olevan koholla astmaa sairastavilla sekä ysköksessä että nenähuuhtelunesteessä. FABP5-taso korreloi positiivisesti vascular endothelial growth factor (VEGF) -tason ($r = 0.66$, $p < 0.01$) ja kysteinyyli leukotrieenitason ($r = 0.54$, $p < 0.01$) kanssa nenähuuhtelunesteessä viitaten FABP5-proteiinin osallistuvan tulehdukseen ja tyvikalvon paksuuntumiseen johtavaan prosessiin.

Johtopäätökset: Totesimme microRNA-tasoissa muutoksia nenän limakalvolla allergista nuhaa ja astmaa sairastavilla henkilöillä. Muutoksia todettiin astmaatikoilla myös silloin, kun merkittäviä muutoksia tulehdussoluissa tai sytokiineisssa ei todettu. Tulevaisuudessa microRNA-analyysistä voi kehittyä herkkä menetelmä hengitystietulehduksen

arviointiin. Nenän typpioksidipitoisuus kuvastaa eosinofiilista tulehdusta nenän limakalvolla. Nenän sivuonteloiden ostiumien ahtauma kuitenkin vaikuttaa typpioksiditasoon vähentäen mittauksen käyttökelpoisuutta allergisen tulehduksen seurannassa. Löysimme astmaattiseen tulehdukseen liittyvän merkkiaineen ysköksestä, ja sen taso oli koholla myös nenähuuhtelunesteessä astmaa sairastavilla. Ylähengitysteistä on alahengitysteitä helpompi ottaa näytteitä, ja jatkossa ylähengitysteistä otettavat näytteet voivatkin olla hyödyllisiä myös astmaattisen tulehduksen mittaamisessa.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals:

I Suojalehto H, Toskala E, Kilpeläinen M, Majuri ML, Mitts C, Lindström I, Puustinen A, Plosila T, Sipilä J, Wolff H, Alenius H. MicroRNA profiles in nasal mucosa of patients with allergic and nonallergic rhinitis and asthma. Int Forum Allergy Rhinol. 2013;3(8):612–20

II Suojalehto H, Lindström I, Majuri ML, Mitts C, Karjalainen J, Wolff H, Alenius H. Altered microRNA expression of nasal mucosa in long-term asthma and allergic rhinitis. Int Arch Allergy Immunol. 2014;163(3):168–78

III Suojalehto H, Vehmas T, Lindström I, Kennedy DW, Kilpeläinen M, Plosila T, Savukoski S, Sipilä J, Varpula M, Wolff H, Alenius H, Toskala E. Nasal nitric oxide is dependent on sinus obstruction in allergic rhinitis. Laryngoscope. 2014 Jun;124(6):E213–8

IV Suojalehto H*, Kinaret P*, Kilpeläinen M, Toskala E, Ahonen N, Wolff H, Alenius H, Puustinen A. Level of Fatty Acid Binding Protein 5 (FABP5) is increased in sputum of allergic asthmatics and links to airway remodeling and inflammation. Submitted June 30, 2014. *Shared first authorship

ABBREVIATIONS

2D	Two-dimensional
ARIA	Allergic Rhinitis and its Impact on Asthma
ATS	American Thoracic society
BMI	Body mass index
CAH6	Carbonic anhydrase 6
cDNA	complementary DNA
CH3L2	Chinatinase-3-like-protein 2
CRISP3	Cysteine-rich secretory protein 3
CT	Computed tomography
CysLT	Cysteinyl leukotriene
DTT	Dithiothreitol
DIGE	Differential gel electrophoresis
ECP	Eosinophil cationic protein
ECRHS	European Community Respiratory Health Survey
EPO	Eosinophil peroxidase
EPX	Eosinophil protein X
ERS	European Respiratory Society
FABP5	Fatty acid binding protein 5
FEV_1	Forced expiratory flow in one second
FVC	Forced vital capacity
GO	Gene Ontology
GINA	Global Initiate for Asthma
GM-CFS	Granulocyte-macrophage colony stimulating factor
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible nitric oxide synthase

LC	Liquid chromatography
LT	Leukotriene
MBP	Major basic protein
MHC	Major histocompatibility complex
miRNA	MicroRNA
mRNA	Messenger RNA
MS	Mass spectrometry
NOS	Nitric oxide synthase
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PG	Prostaglandin
Q	Quartile
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPT	Skin prick test
TGF	Transforming growth factor
Th	T helper
TSLP	Thymic stromal lymphopoietin
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

Asthma and chronic rhinitis are both common chronic diseases all over the world (Zacharasiewicz et al. 2003; Bahadori et al. 2009). The prevalence of asthma and allergic rhinitis has increased rapidly in the latter half of the last century, however in some developed countries with a high prevalence, a levelling off in this prevalence has been detected in recent years (Eder et al. 2006; Bousquet et al. 2008b). In Finland, no signs of plateauing have been detected. In the recent report, the prevalence of phycisian-diagnosed asthma was 10.0% and the prevalence of allergic rhinoconjunctivitis was 44.4% in the adult Finnish population (Kainu et al. 2013). It has been indicated that asthma and rhinitis often coexist. In a Swedish study, 63.9% of asthmatics had concomitant allergic rhinitis and 39.8% had chronic rhinitis, and 19.8% of subjects with allergic rhinitis also had asthma (Eriksson et al. 2011).

Upper and lower airways form a single entity. Dividing respiratory diseases into two categories based on medical specialities (ear, nose and throat or lung diseases) sometimes seems to blur this fact. The main physiological function of the nose is to condition the inhaled air before it reaches the lower airways. Nasal cavities have a good capacity to humidify and filter the air, and nasal mucus and mucociliary clearance are essential in the filtering of inhaled particles and gaseous materials. In addition, nitric oxide produced in upper airways has a protective role in the entire respiratory track, as it has antiviral, bacteriostatic and bronchodilatory effects and it improves oxygenation (Lundberg et al. 1999). Nasal epithelium has also an important role in immunity, it is constantly engaged in immunomodulation between the host and the environment. The nasal and bronchial mucosa have histological similarities and allergic inflammation in the nasal mucosa and in the bronchus displays several common characteristics including Immunoglobulin E (IgE) dependent

mast cell activation, eosinophilic infiltration and an increase of T helper 2 (Th2) type lymphocytes and cytokines. Moreover, not only has a similar inflammation in the upper and lower airways has been found, but nasal inflammation has been shown to have effects on the lower airways and vice versa (Togias 2003; Braunstahl et al. 2006). Allergic asthma and rhinitis can be seen as manifestations of one disease, in the concept of "one airway one disease" or "united airway disease".

In recent years the methods to analyse biological samples have developed rapidly, providing good opportunity to investigate mechanisms of allergic inflammation and to identify new biomarkers. The objective of the present series of studies was to assess airway inflammation in rhinitis and asthma, and to identify potential biomarkers in asthma and rhinitis in the upper and lower airways in the "one airway one disease" context. We evaluated microRNA (miRNA) expressions in the nasal mucosa as well as the nasal nitric oxide levels of subjects with rhinitis and asthma. Furthermore, we conducted proteomic analysis of the induced sputum to reveal differences in the protein abundances.

2 LITERATURE REVIEW

2.1 Asthma

2.1.1 Definition and diagnosis of asthma

The pathogenesis of asthma is not completely understood, so much of its definition is descriptive. The current Global Initiative for Asthma (GINA) (2014) guideline defines asthma as "a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheezing, shortness of breath, chest tightness and coughing that vary over time and in intensity, together with variable expiratory airflow limitation".

Although airway inflammation has been found to be essential in the pathogenesis of asthma, based on the current international and Finnish guidelines the diagnosis is based on typical symptoms of asthma and changes in pulmonary function. In the current GINA guidelines, the diagnostic criteria are documented airflow limitation, at least once during the diagnostic process forced expiratory flow in one second (FEV_1)/ Forced vital capacity (FVC), and documented excessive variation in lung function including one of the following: 1) the increase of FEV_1 ≥12% and 200ml in response to bronchodilator in spirometry, 2) average diurnal daily variation of peak expiratory flow (PEF) >10% in twice-daily PEF over two weeks, 3) the increase of FEV_1 ≥12% and 200ml (or PEF by >20%) from baseline after four weeks of anti-inflammatory treatment, 4) fall in FEV_1 >10% and 200ml from baseline in the exercise test, 5) positive bronchial challenge test, or 6) variation of FEV_1 ≥12% and 200ml between visits outside respiratory infections (GINA 2014). The Finnish criteria include one of the following findings 1) the increase of FEV_1 or FVC ≥12% and 200ml in response to bronchodilator in spirometry, 2)

the ≥15% increase of PEF and 60 l/min in response to bronchodilator or diurnal variation of ≥20% and 60 l/min in PEF at least three times during two weeks of monitoring, 3) an increase in FEV_1 of ≥15% or an average PEF if at least 20% after anti-inflammatory treatment, 4) moderate or severe hyperresponsiveness in the histamine or methacoline test or 5) a ≥15% fall in FEV_1 in the exercise test (Asthma: Current Care Guidelines Abstract, 2012).

2.1.2 Phenotypes of asthma

Almost 70 years ago, Rackeman (1947) introduced the concept of extrinsic (allergic) and intrinsic (nonallergic) subtypes of asthma based on the clinical manifestation of the disease. Extrinsic asthma had an early onset, was associated to atopy (IgE detected to specific allergens), allergic triggers could be identified, and other allergic diseases or family history of allergic diseases were also detected. Intrinsic asthma had onset in adulthood, was not related to allergic sensitisation, and exacerbation related to aspirin intake could be detected in some cases.

In recent years, asthma heterogeneity has been better understood and several studies utilizing cluster analyses have increased the knowledge of phenotypes i.e. combinations of clinical characteristics and their link to biology (Weatherall et al. 2009; Siroux et al. 2011; Anto et al. 2012). However, more information is still needed to form a full picture of true asthma phenotypes. In addition, many environmental factors such as smoking, infection and occupational exposures can influence the underlying inflammatory processes.

Haldar and colleagues (2008) divided asthma patients into five clinical phenotypes: 1) *Early symptom predominant*, having early onset, normal body mass index (BMI) and high symptom expression, 2) *obese non-eosinophilic* with late onset, female preponderance and high symptom expression, 3) *mixed middle-aged* cohort with well-controlled symptoms, inflammation and benign prognosis, 4) *early onset atopic asthma* having concordant symptoms, inflammation and airway dysfunction, and 5) *inflammation predominant* with late onset, mostly men, few daily symptoms, but active eosinophilic inflammation.

More recently, Wenzel and colleagues (2012) categorised adult asthma into five phenotypes. 1) *Early-onset asthma* phenotype usually originat-

ing in early childhood, with an atopic and allergic component and the severity of asthma varying from mild to severe. It typically coexists with other atopic diseases, allergic rhinitis and atopic dermatitis, and the level of total and allergen-specific IgE is often high. Th2-type immune process is usually associated with this phenotype. 2) In *late-onset eosinophilic asthma,* elevated numbers of eosinophils can be found in sputum, in bronchoscopic samples or in blood. However, allergy is seldom detected. The onset of asthma is in adulthood, it is often severe from the beginning, and associated with sinusitis, nasal polyps and is sometimes aspirin-exacerbated. Th2-type inflammation is also associated with this phenotype. 3) Subjects with *exercise-induced asthma* usually have mild asthma and experience bronchoconstriction in response to exercise. It is associated with mast cells and their mediators and Th2-type immunity. 4) *Obesity-related asthma* originates in adulthood mostly in women; these asthmatics are often very symptomatic, but airway hyperresponsiveness is seldom detected. This phenotype is not associated with Th2-type inflammation. 5) In the *neutophilic asthma* phenotype, neutrophilia is detected in sputum. This asthma phenotype is associated with clinical features of low FEV_1 and air trapping. Th2-type inflammation is not detected. Instead, neutrophilia is linked with Th17 inflammation. It is estimated that 50% of people with asthma belong to the Th2-associated phenotypes.

Moreover, the term endotype has been used to distinguish subtypes of asthma. Endotype is defined as the subtype of a condition that is defined by distinct functional or pathophysiological mechanism (Anderson 2008; Lötvall et al. 2011). Phenotypic characteristics represent observations of the clinical dimensions of asthma, whereas an asthma endotype represents a mechanistically coherent disease entity. Each endotype may include several phenotypes, or some phenotypes may be present in more than one endotype. Lötvall and colleagues (2011) chose seven parameters to differentiate categories including clinical characteristics, biomarkers (eosionophilia, exhaled nitric oxide, skin prick tests (SPT), IgE, lung physiology, genetics, histopathology, epidemiology) and treatment response. They present six endotypes. 1) *Aspirin-sensitive asthma,* where the disease mechanism is likely eicosanoid related. 2) *Allergic bronchopulmonary mycosis* being a hypersensitive reaction to the colonisation of the airways (usually Aspergillus fumigatus). 3) *Adult allergic asthma* and

4) *Children with asthma-predictive indices* are driven by a Th2 driven inflammatory process. 5) *The severe late-onset hypereosinophilic asthma* group includes about 20% of asthma patients; these patients are non-atopic and the disease mechanisms are still mainly unknown. 6) *Cross-country skiers' asthma* is clinically defined as asthma symptoms associated with strenuous skiing-related exercise. It is seldom associated with allergic sensitisation and airway inflammation is dominated by increased numbers of lymphocytes, macrophages and neutrophils.

2.1.3 Asthma control and severity

According to the current GINA guidelines (2014), the asthma control level is the extent to which the manifestations of asthma can be observed in the patients, or have been reduced or removed by treatment. It is determined by the interaction between the patient's genetic background, underlying disease processes, the treatment they are receiving, the environment, and psychosocial factors.

The assessment of asthma control suggested by the GINA guidelines has not been formally validated (GINA 2014). It includes both the assessment of current clinical manifestations (symptoms, night waking, reliever medication use, and activity limitation) and control of the expected future risk to the patient such as exacerbations, accelerated decline in lung function, and side-effects of treatment. The assessment should preferably cover a period of four weeks. The level of asthma control is classified as well controlled, partly controlled and uncontrolled. Asthma is controlled if a patient does not have daytime asthma symptoms or the need for reliever medication more than twice a week, no limitation of activities, no nocturnal symptoms or awakenings. A low FEV_1 is an independent predictor of asthma exacerbations and lung function decline. Asthma outcomes have shown to improve after the introduction of control-based guidelines, and currently, control-based asthma management is recommended by GINA (2014) and the Finnish Asthma Current Care Guidelines (2012).

According to the current GINA guidelines asthma severity is evaluated retrospectively from the level of treatment required to control exacerbations and symptoms (GINA 2014). It is possible to assess the severity of asthma when the patient has been on regular controller treatment for

several months. *Mild asthma* can be well controlled with low-intensity asthma treatment, for example low-dose inhaled steroids or leukotriene (LT) antagonists. *Moderate asthma* is well controlled with a low-dose inhaled corticosteroid /long-acting bronchodilator medication, for example. *Severe asthma* requires high-intensity treatment to maintain good control or good control is not achieved despite such medication. Asthma severity is not a permanent feature in an individual, it may change over the months and years.

2.2 Rhinitis

2.2.1 Definition of rhinitis

According to the global guidelines on Allergic Rhinitis and its Impact on Asthma (ARIA) rhinitis is defined as "an inflammation of the lining of the nose and it is characterized by nasal symptoms including anterior or posterior rhinorrhoea, sneezing, nasal blockage and/or itching of the nose. These symptoms occur during two or more consecutive days for more than one hour on most days" (Bousquet et al. 2001; Bousquet et al. 2008b).

2.2.2 Definition and diagnosis of allergic rhinitis

Allergic rhinitis is clinically defined as a symptomatic disorder of the nose induced after allergen exposure by an IgE-mediated inflammation (Bousquet et al. 2008b). Symptoms of allergic rhinitis include rhinorrhoea, nasal obstruction, nasal itching and sneezing which are reversible spontaneously or with treatment. Postnasal drip mainly occurs with profuse anterior rhinorrhoea. Allergic rhinitis is often associated with ocular symptoms. The diagnosis is based on the concordance between typical allergic symptoms and diagnostic tests including allergen specific IgE and SPT.

2.2.3 Classification of allergic rhinitis

Earlier, allergic rhinitis was classified as seasonal, perennial and occupational based on the time of the exposure and symptoms (Dykewicz

et al. 1998). The ARIA document in 2001, introduced a classification including intermittent and persistent allergic rhinitis (Bousquet et al. 2001). Intermittent means that the symptoms are present less than four days a week or for less than four weeks, whereas in persistent allergic rhinitis the symptoms are present for more than four days a week and for more than four weeks. Information on the phenotypes of allergic rhinitis based of unbiased analyses combining clinical features and underlying processes is currently lacking (Anto et al. 2012).

2.2.4 Definition and diagnosis of nonallergic rhinitis

Several nonallergic conditions can cause rhinitis symptoms including infections, hormonal imbalance, physical agents, anatomical anomalities and medications (Bousquet et al. 2008b). In most cases the cause of rhinitis cannot be detected and it is called nonallergic rhinitis or "idiopathic rhinitis", also referred as "nonallergic noninfectious rhinitis" or "vasomotor rhinitis" (Settipane et al. 2013). The terminology is somewhat unestablished. Nonallergic rhinitis is diagnosed when a patient has symptoms that mimic allergic rhinitis, with no definite causal factor and with a lack of demonstrated IgE mediated allergy by SPTs and allergen specific IgE (Bousquet et al. 2008a). It is largely an exclusion diagnosis and thus a heterogeneous condition. Primary symptoms are nasal congestion and rhinorrhoea. However, nasal pruritus, sneezing and conjunctival symptoms are rare (Settipane et al. 2013). Patterns of symptoms may be perennial, persistent or intermittent. Precipitants for nonallergic rhinitis can be changes in climate (temperature, humidity and barometric pressure), strong odours, environmental tobacco smoke, pollutants, chemicals and other occupational exposures, exercise or alcohol ingestion.

Nonallergic rhinitis with eosinophilia syndrome was first described in 1981 (Jacobs et al. 1981). These patients, usually middle aged adults, have perennial nasal symptoms including sneezing, rhinorrhoea, nasal pruritus and reduced sense of smell. Marked eosinophilia is detected in nasal cytology, but no IgE mediated immunologic reaction to common inhalation allergens is seen. This syndrome may be responsible of approximately 30% of all rhinitis cases without allergy (Settipane et al. 1985).

In recent years, a condition called local allergic rhinitis or Entopy has been described. That is a local nasal IgE production and reactivity to allergens without detectable systemic atopy i.e. negative SPTs and specific serum IgE to aeroallergens (Powe et al. 2003).

Other types of nonallergic, noninfectious chronic rhinitis that are not caused by anatomical or mechanical causes or other medical conditions are gustatory rhinitis occurring after indigestion of foods and drinks, atrophic rhinitis, medication associated rhinitis, hormone induced rhinitis and rhinitis of elderly subjects (Settipane et al. 2013).

2.2.5 Severity of rhinitis

Allergic rhinitis symptom severity is classified as mild when no sleep disturbance or bothersome symptoms are present and daily activities and school or work performance is not affected. In the moderate/severe form one or more of the above mentioned items are present (Bousquet et al. 2001). Visual analogue scale (VAS) is an objective, quantitative measure of rhinitis symptom severity (Spector et al. 2003). Separate symptoms such as sneezing, runny nose, nasal congestion and itching or global rhinitis symptoms may be assessed by using a VAS scale. On the VAS scale of 1−10 cm, patients with a VAS of global rhinitis symptoms of <5 cm can be classified as mild in ARIA classification and a VAS of > 6cm is equivalent to moderate/severe AR (Bousquet et al. 2007).

2.3 Prevalence and co-existence of asthma and rhinitis in adults

2.3.1 Prevalence of asthma

There is no single question to define asthma in the questionnaires in epidemiologic studies. Questions about physician-diagnosed asthma and asthma symptoms have been used. The prevalence rates of asthma using both of these definitions are dependent on the awareness of asthma in the population (Eder et al. 2006). It has been estimated, that approximately 300 million people currently have asthma and that it affects 1−18% of the population in different countries (Bahadori et al. 2009; GINA

2014). The World Health Survey included almost 180 000 adults from 70 countries. The prevalence of physician-diagnosed asthma was 4.3%, clinical/ treated asthma was 4.5% and wheezing 8.6% (To et al. 2012). However, the prevalence varied 21-fold in different countries. The highest prevalence was reported in Australia, where the prevalence of physician-diagnosed asthma was 21.0%, clinical/treated asthma 21.0%, and wheezing 27.4%. A high prevalence was seen also in North and West European countries and in Brazil.

The prevalence of asthma has increased worldwide in the second half of the last century (Eder et al. 2006). Some studies have reported that it plateaued thereafter, especially in countries with high asthma rates (Anderson et al. 2007; Lotvall et al. 2009) while other studies suggest that it is still increasing (Gershon et al. 2010). The systematic review of the epidemiological studies concluded that there is no overall signs of a declining trend of asthma prevalence; on the contrary, an increasing trend was suggested in many parts of the world (Anandan et al. 2010).

In Finland the prevalence of asthma has increased during recent decades, signs of levelling off have not been reported. In 1980, the prevalence of asthma was 4.1% in the urban population and 2.7% in the rural population (Heinonen et al. 1987). These figures were based on postal questionnaire answers and a random subset of responders were clinically examined. In 1996, the prevalence of physician-diagnosed asthma among first year university students was 4.2% (Kilpeläinen et al. 2000) and in Päijät-Häme region in southern Finland the non-response adjusted prevalence of physician-diagnosed asthma in the adult population was 4.4% (Hedman et al. 1999). An increase in the asthma prevalence of Finnish men recruited to the army was 20-fold between 1961 and 1989 (Haahtela et al. 1990). After this, no plateauing has been detected, and a 3.5% prevalence was detected in 2003 (Latvala et al. 2005). In the, the prevalence of physician-diagnosed asthma in the adult population was 6.0% in Lapland (Kotaniemi et al. 2002) and 6.8% in the Helsinki area in 1996 (Pallasaho et al. 1999. A recent study of the FinEsS population by Kainu and colleagues (2013) reported that the prevalence of physician-diagnosed asthma had increased in the adult population in the Helsinki area from 6.5% in 1996 to 10.0% in 2006. However, the change in the prevalence of respiratory symptoms suggestive of obstructive airway diseases was less distinct.

2.3.2 Prevalence of rhinitis

The definition of rhinitis has been difficult in epidemiological studies, in which the characterization of rhinitis symptoms is often not a primary objective. A large proportion of subjects defined as having allergic rhinitis in epidemiological studies do not have positive SPT or specific IgE in serum. In the study by Vervloet and colleagues (1991) only 42% of subjects reporting a history of hay fever had an elevation of specific IgE. In addition, many studies of allergic rhinitis focus on questions about hay fever leaving perennial symptoms underestimated. Moreover, sinus imaging is seldom performed, and thus rhinosinusitis cannot be excluded.

It has been estimated that the prevalence of rhinitis including allergic and nonallergic forms in the adolescent/adult population is at least 25% (Leynaert et al. 1999; Zacharasiewicz et al. 2003; Bachert et al. 2006; Molgaard et al. 2007; Bousquet et al. 2008a). Zacharasiewicz and colleagues assessed 22 population-based studies on rhinitis and found that the proportion of rhinitis cases that were attributable to atopy was approximately 50%, suggesting that other half was due to nonallergic mechanisms (Zacharasiewicz et al. 2003). The idiopathic form comprises approximately 71% of nonallergic rhinitis conditions (Settipane 2009). Nonallergic rhinitis is twice as common in women as in men, whereas in allergic rhinitis the gender distribution is more equal (Molgaard et al. 2007). Both allergic rhinitis and nonallergic rhinitis symptoms may exist in the same subject. It has been suggested that this rhinitis subtype may represent 44 to 87% of allergic rhinitis patients and is more common than the pure allergic or nonallergic type of rhinitis (Bernstein 2010). In a Belgian population study on subjects aged over 15 years, the prevalence of allergic rhinitis was 29.8% and that of nonallergic rhinitis 9.6%. Altogether 40.8% of allergic rhinitis patients and 23.5% of nonallergic rhinitis patients had persistent symptoms. Symptom intensity was moderate or severe in 75.4% of allergic rhinitis patients and 53.1% of nonallergic rhinitis patients (Bachert et al. 2006).

The increase in the prevalence of allergic rhinitis has been observed since 1960s (Bousquet et al. 2008b). There is evidence that the increase in the prevalence of allergic rhinitis has levelled off in countries in which the prevalence of allergy and rhinitis is high (Braun-Fahrlander et al. 2004; Eriksson et al. 2012), but in countries where the prevalence is low

a continuing increase is seen. Trends of asthma and rhinitis prevalence are similar, but are not always in line with each other (Bousquet et al. 2008b).

The prevalence of allergic rhinitis has increased in Finland during the last decades among adults; a levelling off in the prevalence has not been detected. In the follow-up study in the population of young and middle-aged twins, the prevalence of physician-diagnosed hay fever was 6.8% in men and 11.8% in women in 1975 and in 1990 it was 9.8% in men and 15.8% in women (Huovinen et al. 1999). In 1980, the prevalence of allergic rhinitis was 26.7% in the urban population and 28.8% the rural population (Heinonen et al. 1987). In the young adult student population studied in 1996, the prevalence of doctor-diagnosed allergic rhinitis was 21.5% (Kilpeläinen et al. 2000). In the adult population in Päijät-Häme region, the prevalence of allergic rhinitis was 37.7% in 1996 (Hedman et al. 1999). In the study of cohort originating from Tampere area, the prevalence of allergic rhinitis was 17.5% at the age of 16 in 1983 and 26% at the age of 32 in 1999 (Huurre et al. 2004). In the cohort of young adults in northern Finland, the prevalence of allergic rhinitis symptoms in past year was 30% in non-farmers and 23% in professional farmers at the age of 31 in 1997 (Lampi et al. 2011). Latvala and colleagues (2005) showed that the prevalence of allergic rhinitis in young men at call up for military service has increased rapidly since 1991, being 8.9% in 2000. In the recent study of Kainu and colleagues (2013) the prevalence of allergic rhinoconjunctivitis increased from 37.2% in 1996 to 44.4% in 2006 in the adult population of Helsinki.

2.3.3 Co-existence of asthma and rhinitis

The co-existence of asthma and rhinitis is common. In the European multi-centre study, European Community Respiratory Health Survey (ECRHS), perennial rhinitis was strongly associated with asthma in atopic subjects (odds ratio 8.1) and also in nonatopic subjects (odds ratio 11.6) (Leynaert et al. 1999). In a recent Swedish study, 63.9% of asthmatic subjects had concomitant allergic rhinitis and 39.8% had chronic rhinitis and the prevalence of asthma in subjects with allergic rhinitis was 19.8% (Eriksson et al. 2011). In outpatient population, concomitant allergic rhinitis was present in 73.9% and nonallergic rhinitis in 13.6% of asthma patients (Vandenplas et al. 2010).

Rhinitis is a risk factor for asthma development. In the 8.8-year follow-up study of ECRHS cohort, the relative risk for development of asthma was 2.71 for nonallergic rhinitis and 3.53 for allergic rhinitis as compared with subjects without rhinitis (Shaaban et al. 2008). In the nested case-control study from Sweden, asthma was also associated with the occurrence of non-infectious rhinitis before asthma onset (odds ratio 5.4) (Toren et al. 2002). In the Finnish 11-year follow-up study the subjects with allergic rhinoconjuctivitis had 2.15 fold risk of developing asthma (Pallasaho et al. 2011). Karjalainen and colleagues (2003) found 4.8 fold risk of asthma in the subjects with occupational rhinitis.

Rhinitis may also have an influence on the asthma control and severity. Magnan and colleagues reported that the severity of allergic rhinitis was associated with the severity of asthma and that allergic rhinitis was associated with worse asthma control (Magnan et al. 2008). Consistent with this, Vandenplas and colleagues (Vandenplas et al. 2010) reported that both allergic and nonallergic rhinitis were associated with the increased risk of uncontrolled asthma. Also, in the study of Eriksson and colleagues (2011) subjects with concomitant allergic rhinitis had more asthma symptoms. In a one year follow-up study moderate/severe rhinitis predicted greater asthma severity and worse asthma control in severe asthma patients (Ponte et al. 2008). On the contrary, in the ECRHS cohort allergic rhinitis was not associated with asthma severity (de Marco et al. 2006). Also, no clear association was detected between asthma and rhinitis severity in mite allergic patients (Antonicelli et al. 2013).

2.4 Allergic airway inflammation

2.4.1 Definition of allergy, allergen and atopy

In 2004 The World Allergy Organization defined allergy as “a hypersensitivity reaction initiated by immunological mechanisms. Allergy can be antibody- or cell-mediated. In the majority of cases the antibody responsible for an allergic reaction belongs to the IgE isotype and these individuals may be referred to as suffering from an IgE-mediated allergy”. Allergens are defined as antigens that cause allergy and atopy is a “personal and/or familial tendency, usually in childhood or adolescence, to become

sensitized and produce IgE antibodies in response to ordinary exposure to allergens, usually proteins. As a consequence, such individuals can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema" (Johansson et al. 2004).

2.4.2 Allergic inflammation process

The allergic inflammatory process in the upper and lower airways in allergic rhinitis and asthma is mainly similar (Bousquet et al. 2008b). The nasal and bronchial mucosa have both pseudostratified epithelium and ciliated and columnar cells resting on the basement membrane. The biggest differences in the histology are in the submucosal level. In the nose a large and highly developed vasculature is seen, whereas bronchial airways are surrounded by smooth muscle bundles. The clinical differences are predominantly due to anatomical differences and the interaction between inflammation and structural cells; vasodilatation in the upper airways leads to nasal blockage and airway smooth muscle cells in the bronchus result in bronchoconstriction.

Allergen exposure in sensitized individuals induces an early phase of allergic inflammation. It involves an acute activation of allergy effector cells (mast cells and basophils) through IgE-allergen interaction and mast cell and basophil degranulation and the release of histamine, tryptase and other mediators including cysteinyl leukotrienes (cysLT) and prostaglandins (PG) (Barnes 2011). The clinical symptoms start within minutes. In the nose histamine and other mediators cause sneezing, pruritus, nasal congestion and mucus secretion. In asthma, vasodilatation, bronchoconstriction and plasma exudation takes place leading to wheezing and dyspnoea. The late phase typically begins within a few hours in the site of allergen challenge. It is characterised by the influx and activation of inflammatory cells including T lymphocytes, eosinophils, basophils, neutrophils and monocytes. In asthma, chronic inflammation leads to airway hyperresponsiveness, globlet cell hyperplasia and airway wall remodeling including subepithelial fibrosis, increased smooth muscle mass, enlargement of glands, neovascularisation and mucus hypersecretion (Bergeron et al. 2009). Also in nasal mucosa in allergic rhinitis remodeling including changes in collagen, proteoglycans and lymphatic vessels has been detected (Kim et al. 2010).

2.4.3 Cells related to allergic inflammation

T-lymphocytes develop in the thymus and circulate between secondary lymphoid tissue and blood. After the first contact with a specific antigen T naïve cells start to proliferate and differentiate into effector T cells. Cytotoxic T cells, which express surface marker CD8 and recognize antigens of major histocompatibility complex (MCH) class I, are important in the defence against intracellular pathogens, especially viruses. Th cells, which express the surface marker CD4 and recognize antigens bound to MHC class II molecules, differentiate into various subsets, Th1, Th2 and Th17, distinguished by the cytokines they produce when activated (Akdis et al. 2011). Activated Th1 cells produce interleukin (IL) 2 and interferon (IFN) γ; they are important in immunoresponses against microbes, in virus defence and also participate in allergic inflammation. Th2 cells are essential in the allergic inflammation and also in direct immunoresponses against intestinal helminths. Th2 polarisation from naïve t cells is initially induced by dendritic cells or exogenous IL-4 from basophils or by IL-25 or IL-33. During allergic inflammation Th2 cells migrate to the site of inflammation to recruit and activate eosinophils, B cells and epithelial cells and switch antibody production to IgE through the actions of the cytokines IL-4, IL-5, IL-9 and IL-13 (Robinson et al. 1992). Th17 cells are a distinct lineage of Th cells expressing IL-17, they may mediate neutrophilic type inflammation and they exacerbate Th2 mediated allergic inflammation (Lloyd et al. 2010; Akdis et al. 2011). T regulatory cells control immune homeostasis, prevent autoimmunity, supress allergic responses and participate in the resolution of inflammation. In allergic inflammation T regulatory cells supress inflammation through the secretion of inhibitory cytokine IL-10, transforming growth factor (TGF) β or by cell surface molecules (Palomares et al. 2010). They also have a direct inhibitory effect on dendritic cells and effector T cells through cell-to-cell contact. Also other subsets of T cells including Th9 cells, T follicular helper, Th22 cells have been proposed to participate in allergic inflammation (Chang et al. 2010; Lloyd et al. 2010; Akdis et al. 2011).

B-lymphocytes mature in the bone marrow and circulate between lymph organisms. However, they are also present in the airway mucosa (Drolet et al. 2010). B-cells are essential in allergic inflammation through

synthesis of IgE. Th2 cells engage cognate B cells through B cell MHC class II and co-stimulatory molecules and through secretion of IL-4 and IL-13 and induce B cells to undergo a class switch to produce IgE. Also other cells may enhance class switch through IL-4 and IL-13 production. In addition, B-cells function as antigen presentation cells and secrete both inflammatory and regulatory cytokines.

Dendritic cells form a network that is localised within the epithelium and submucosa of the entire respiratory tract including both the nasal and bronchial areas (Moller et al. 1996; Hartmann et al. 2006). They are the primary antigen-presenting cells in the airways. In allergic inflammation, dendritic cells engulf allergens, break them into antigenic peptides, and migrate to the lymph nodes where they present these peptides to naïve T lymphocytes or Th2 cells (Savina et al. 2007). Th2 cell activation requires MHC II complexes on the ligation. Dendritic cells can polarise naïve T cells into Th1 or Th2 cells according to their own phenotype and with signals received from processed antigens and from the tissue microenvironment during antigen presentation.

Mast cells are present in the peripheral tissue, the differentiation and maturation of mast cells also takes place there. Along with dendritic cells, mast cells are the first immune cells to interact with allergens. In the early allergic reaction, allergens activate sensitised mast cells by cross-linking surface-bound IgE molecules to release preformed mediators and lipid-derived mediators including histamine, tryptase, PGD2 and LTC4 within minutes (Galli et al. 2012). Other mediators including many cytokines, chemokines and growth factors are produced in mast cells from new transcripts and are secreted in the hours after mast cell activation. Recently, it has been proposed that they also have a role in long-term pathophysiological changes and tissue remodelling in asthma (Galli et al. 2010).

Eosinophils are the most prominent cells in the late-phase allergic airway response (Blanchard et al. 2009). They require IL-5 released by Th2-type cells, granulocyte-macrophage colony stimulating factor (GM-CFS) and eotaxin for their maturation, survival, attraction to the inflammation sites and activation. They release proinflammatory cytokines like IL-4, IL-5 and IL-13, TGF-β and cysLTs. Activated eosinophils release toxic pre-produced products stored in granules, especially major basic protein (MBP), eosinophilic cationic protein (ECP) and eosinophil

peroxidase (EPO), and contribute to tissue damage, inflammation and airway hyperresponsiveness.

Basophils form less than 1% of all the granulocytes in the peripheral blood. In the allergic reaction basophils release histamine and LTC4 to promote inflammation (Siracusa et al. 2013). Basophils have been found to play a role in promoting optimal Th2 cytokine responses, and they may co-operate with dendritic cells to contribute to pathologic airway inflammation.

2.4.4 Mediators related to allergic inflammation

Cytokines are small glycosylated proteins that are involved in cellular growth, differentiation, proliferation, cell-to-cell signaling, chemotaxis, immunomodulation, immunoglobulin isotype switching and apoptosis (Hamid et al. 2009; Akdis et al. 2011). The cytokine actions are mediated through specific cytokine receptors on the target cell surfaces. Depending on the context in which the cytokine is produced or the cell type that responds to the cytokine, they may have different functions. T cells are the major source of cytokines in allergic inflammation, but they also are produced in other inflammatory cells, structural cells and in fibroblasts. Allergic airway inflammation is characterised by the secretion of Th2 cytokines, including IL-4, IL-5 and IL-13 (Robinson et al. 1992; Veldhoen 2009). In addition to Th2 cells, these cytokines are also secreted from mast cells, basophils, eosinophils and structural cells. IL-4 and IL-13 are essential in IgE synthesis through isotope switching of B cells. IL-5 drives eosinophil differentiation, maturation and survival. IL-10 is primarily known as an inhibitory cytokine, but it also has immunostimulary effects. It is produced from naïve T cells, Th1 and Th2 cells and from activated monocytes, mast cells and macrophages. IL-10 reduces the effects of pro-inflammatory cytokines and inhibits eosinophil survival and migration during allergic inflammation. It can also down-regulate IL-4 induced isotype switching of activated B-cells. INF-γ is mainly produced in Th1 cells and has inhibitory effect in Th2 cells. During allergic inflammation it inhibits isotype switching of IgE and IgE production of Th cells and it can also promote cell-mediated cytotoxic responses. Recent asthma studies suggest that IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) predominantly secreted by the

airway epithelium promote Th2 cell function and increase IL-5, IL-9 and IL-13 production (Licona-Limon et al. 2013). Several proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, GM-CSF, and IL-17 have also been linked to allergic inflammation (Akdis et al. 2011; Bhakta et al. 2011).

Chemokines are small cytokines primarily involved in the chemotaxis. Increased levels of chemokines have been detected in bronchial biopsies and bronchoalveolar lavage in asthmatic as well as in the nasal mucosa of subjects with seasonal allergic rhinitis (Miotto et al. 2001; Plewako et al. 2008). They attract and regulate leukocyte trafficking into tissues by binding specific receptors. In addition, they have other functions such as effecting T cell differentiation. Chemokines are classified into four subclasses according to their structure. CC chemokins (or β chemokines) including for example IL-8, eotaxin and RANTES are thought to have the greatest relevance in asthma (Hamid et al. 1993).

Cysteinyl leukotrienes (CysLT) (LTC4, LTD4 and LTE4) are synthetized from arachidonic acid by inflammatory cells such as mast cells, eosinophils, basophils and macrophages. They are released following allergen exposure and they predominate both early and late phases of the allergic response. They are also involved in the maturation and tissue recruitment of inflammatory cells as well as in airway remodeling (Holgate et al. 2003).

Immunoglobin E is synthesised in B lymphocytes in lymph nodes or locally in nasal or bronchial mucosa (KleinJan et al. 2000; Takhar et al. 2007). It has also been suggested that in some asthma and rhinitis patients who have no systemically detectable specific IgE or in whom no triggering antibodies have been identified, IgE may be produced locally in the respiratory mucosa. The production of antigen-specific IgE requires that such antigens are taken up by dendritic cells or other antigen-presenting cells (Burton et al. 2011; Galli et al. 2012) and are presented to Th2 cells. Th2 cells or other cells then secrete IL-4 and IL-13 which induces B cells to undergo a class-switch recombination. It binds to mast cells, basophils and eosinophils facilitating allergen-specific activation of these cells. This has been shown to extend mast cell survival. IgE can also bind to dendritic cells and facilitate the allergen uptake for processing and presentation, and to monocyte macrophages and B lymphocytes.

2.5 Nonallergic components of the pathogenesis of asthma and rhinitis

2.5.1 Inflammation in asthma

Over the past 30 years, it has been believed that asthma is mainly caused by Th1-Th2 imbalance and categorized by eosinophilic inflammation and airway hyperresponsiveness. However, in recent years the knowledge with regard to the asthmatic inflammation has increased. A strong innate immune component is seen in asthma and the important role of epithelium has become evident. In a recent study, the Th2-biased response was detected in only half of the asthma patients (Woodruff et al. 2009). In addition, Th2 cytokine inhibitors have been beneficial for only small subset of patients (Nair et al. 2009). It has been proposed that noneosinophilic asthma is a distinct clinical and pathophysiological phenotype and that the innate immune pathway may play a role in the airway inflammation of this phenotype (Haldar et al. 2007).

Airway epithelium responds to environmental substances including pathogens, allergens, cigarette smoke and pollution. They interact directly with the environment and may be activated by pathogens and endotoxins through pattern recognition receptors and trigger or enhance allergic response. In addition, epithelial cells secrete inflammatory mediators such as IL-25, epithelial cytokines and chemokines, participate in host defence and maintain chronic inflammation (Fahy et al. 2011; Holgate 2011).

Neutrophils are present in airway inflammation in several types of asthma, and in some asthmatics they are the predominant inflammatory cells (Jatakanon et al. 1999). Presence of neutrophils is associated with a worse asthma outcome and inhaled corticosteroids are less effective in these asthma patients. Neutrophils may be recruited to the airways by IL-17 and they may participate in the airway inflammation by releasing reactive oxygen species, cytokines, lipid mediators and enzymes (Borregaard et al. 1997).

2.5.2 Pathogenesis of nonallergic rhinitis

Nonallergic rhinitis is a heterogeneous condition, and the underlying mechanisms are not completely understood. In a subgroup of subjects with nonallergic rhinitis, inflammation have been detected in the nasal mucosa. In patients suffering from nonallergic rhinitis with eosinophilia syndrome, increased numbers of eosinophils and mast cells have been seen in the nasal biopsies (Powe et al. 2001). In some subjects, predominant neutrophilic or mast cell infiltrate can be detected (Maselli Del Giudice et al. 2012). The pathophysiology of local allergic rhinitis is characterised by local production of specific IgE and Th2 cytokine pattern of mucosal cell infiltration (Rondón et al.2009).

Subjects with the noninflammatory form of nonallergic rhinitis are often hyperresponsive to the physical and chemical stimuli such as cold air and strong odours. This may be related to the increase in C-fiber activity (Garay 2004). C-fibers are unmyelinated sensory neurons that innervate vessels, glands and epithelium of nasal mucosa. On stimulation they release neuropeptides such as substance P and calsitonin gene-related protein, leading to increased vascular permeability and nasal secretion.

It has been demonstrated that subjects with nonallergic rhinitis have abnormalities in the autonomic nervous system testing, suggesting an imbalance between the parasympathetic and sympathetic system, or hyporeactivity of the sympathetic system (Jaradeh et al. 2000). In the case of cold induced nasal blockage, a hyperactive afferent cholinergic parasympathetic reflex arc has been detected (Silvers 1991).

2.6 MicroRNAs

The immune system is controlled by the dynamic and multilevel regulation of gene expression in each cell type. Gene expression can be regulated at different levels, from the initiation of the transcription, through RNA processing to the post-translational modification. miRNAs constitute a group of gene expression regulators that post-transcriptionally fine-tune the expression of genes.

In 1993, the first miRNA, named lin-4, was identified in nematode *Caenorhabditis elegans* (Lee et al. 1993). Seven years later the second miRNA, let-7 was detected in the same species (Reinhart et al. 2000).

Following this, a large-scale scale screening and identification of new miRNAs began. Currently, more than 1800 human miRNA sequences are annotated in the miRNA database miRBase. The term "microRNA" was first used in 2001 by Reinhart and colleagues (Lagos-Quintana et al. 2001).

miRNAs are small, 19–25 nucleotides long, single-stranded RNA molecules. They are synthetized in nucleus by RNA polymerase II as longer transcripts called pri-miRNAs. Subsequently, pri-miRNAs are cleaved to small hairpin-like precursors called pre-miRNAs. Pre-miRNAs are exported to the cytoplasm where thy undergo processing by the enzyme Dicer and the generation a short RNA duplex (Bartel 2004). They bind to 3' untranslated region of target messenger RNA (mRNA) and cause gene silencing mainly through degradation of target mRNAs or by inhibition of translation (Guo et al. 2010). They do not switch off the expression of their target genes, but reduce the amount of mRNA and protein. Expression of miRNA is often tissue specific. miRNAs form a complex network; one miRNA can target hundreds of genes and a single gene is typically targeted by multiple miRNAs. They regulate a wide variety of functions including cell proliferation, apoptosis, stress response and immune response (Winter et al. 2009). Several diseases, such as many cancers, metabolic diseases and inflammatory diseases, have been associated with deregulated miRNA expression (Lu et al. 2005; Krutzfeldt et al. 2006; Lu et al. 2009a). The first miRNA-based drugs are already at the clinical trial stage: anti-miR-122 oligonucleotide drug has been tested for hepatitis C virus infection treatment and liposome-formulated miR-34 mimic is being tested for patients with advanced hepatocellular cancer (Lanford et al. 2010; Ling et al. 2013).

Recently, the role of miRNAs in allergic airway diseases has been studied in clinical settings. The expression of one or several predetermined miRNAs, or a panel of several hundreds of miRNAs can be detected from one sample. In 2009, Williams and colleagues (2009) determined the expression of 227 miRNAs in the bronchial biopsies of eight mild asthmatics and eight healthy controls. They found no difference in miRNA expressions between these groups and no changes were detected in the expressions after inhaled steroid treatment of asthmatics. In contrast, Jardim and colleagues (2012) compared bronchial epithelial cells of 16 asthmatics and 16 healthy controls and found 66 differentially

expressed miRNAs. In addition, Solberg and colleagues (Solberg et al. 2012) examined bronchial epithelial cells of steroid naïve asthmatics and found a markedly abnormal pattern of miRNA expression in most of the asthmatics; altogether 217 miRNAs were differentially expressed in the asthmatics. They also studied asthmatics using inhaled steroids and found that inhaled steroids only had a modest effect on these alterations. The discrepancy between the study by Williams colleagues and the more recent studies may be explained by different samples. Williams and colleagues studied miRNA expression in bronchial biopsies, whereas in the more recent studies cultured epithelial cells were investigated.

In addition to bronchial cell miRNA expressions, other biological samples of asthmatics have been studied. Liu and colleagues (2012) identified upregulation of miR-221 and miR-485-3p in the blood samples of six asthmatic children compared to six healthy controls. Tsitsiou and colleagues (2012) found reduced levels of miR-146a/b and miR-28-5p in circulating lymphocytes of patients with severe asthma. It has recently been demonstrated, that the levels of certain miRNAs including miR-21, miR-155 and let-7a are decreased in the exhaled breath condensate of asthmatics when compared to controls (Pinkerton et al. 2013). In addition, 24 miRNAs, including members of let-7 and miR-200 families, have been differentially expressed in the broncholveolar lavage fluid exosomes in the asthma patients when compared to the healthy controls (Levanen et al. 2013).

There are few clinical studies of miRNA expression changes in allergic rhinitis patients. Shaoquing and colleagues (2011) studied nasal biopsy samples of eight patients with allergic rhinitis and eight healthy controls. In the microarray chip of 421 miRNAs, nine miRNAs had more than two-fold change in expression, two miRNAs were up-regulated and seven down-regulated. In another study, a reduced level of miR-21 in human umbrical blood mononuclear leukocytes was associated with antenatal IgE production and development of allergic rhinitis (Chen et al. 2010).

The functional role of the miRNA in allergic diseases has been studied extensively in mouse models and in cultured cell lines. These studies have revealed the importance of some miRNAs in allergic airways diseases, as listed in Table 1. In allergic asthma models, miR-21 has been overexpressed in the lung tissue, the highest levels localized in the macrophages and dendritic cells. Moreover, it has been found to modulate IL-12

expression (Lu et al. 2009b). In the ovalbumin induced asthma model, miR-21 deficient mice had reduced lung eosinophilia, and increased INF-γ level and reduced IL-4 level in bronchoalveolar lavage following an allergen challenge (Lu et al. 2011). In addition, miR-21 was upregulated in the human epithelial cells after IL-13 treatment (Case et al. 2011). Let-7 family miRNAs have been shown to influence the expression of IL-13 in the lung epithelial cell line (Kumar et al. 2011). The intranasal administration of let-7 led to a reduced IL-13 level and reduced bronchial hyperresponsiveness as well as the resolution of allergic inflammation in the ovalbumin-induced mouse model. In contrast, the let-7 inhibitor inhibited the production of allergic cytokines and disease phenotype in the mouse model (Polikepahad et al. 2010). In the acute house dust mite-induced asthma model, miR-126 was upregulated, and inhibition of miR-126 reduced the asthmatic response (Mattes et al. 2009). In addition, miR-126 was up-regulated two weeks after an allergen challenge (Collison et al. 2011a). miR-155 has been shown to modulate the response of human macrophages to IL-13 and is thus essential in the acquisition and maintenance of a Th2 phenotype (Martinez-Nunez et al. 2011). Malmhäll and colleagues (2014) found that miR-155 deficiency resulted in diminished eosinophilic inflammation and Th2 cytokine levels in the mouse model.

Table 1. List of some essential microRNAs and their function in allergic airway inflammation.

miRNA	Function	Reference
miR-21	Upregulated in lung-specific IL-13 expressing transgenic mice with induced allergic airway inflammation.	(Lu et al. 2009b)
	Overexpression reduced TLR2 antagonist-induced lung inflammation in mice.	(Case et al. 2011)
	miR-21 deficiency increased Th1 cytokine levels and reduced eosinophilia in an ovalbumin-induced mouse model of asthma.	(Lu et al. 2011)
	Reduced level in umbrical blood leucocytes associated with development of allergic rhinitis.	(Chen et al. 2010)
let-7 family	Overexpression reduced allergic inflammation in OVA induced mouse model of asthma.	(Kumar et al. 2011)
	let-7 inhibitor inhibited the production of allergic cytokines and disease phenotype in mouse model.	(Polikepahad et al. 2010)
miR-126	Silencing reduced allergic inflammation in a house dust mite induced mouse model of asthma.	(Mattes et al. 2009)
	Reduced eosinophilia in the mouse model of chronic airway inflammation.	(Collison et al. 2011a)
miR-155	Required for Th2 mediated eosinophilic inflammation in the ovalbumin-induced mouse model of asthma.	(Malmhall et al. 2014)
	miR-155-deficient mice developed asthma-like inflammation in the lung.	(Rodriguez et al. 2007)
miR-146a	Increased expression in Th2 lymphocytes in mouse model.	(Feng et al. 2012)
	Reduced cytokine-induced apoptosis in human bronchial epithelial cells.	(Liu et al. 2009)
	Reduced expression in circulating Th1 and Th2 cells in patients with severe asthma.	(Tsitsiou et al. 2012)
miR-145	Up-regulated in the airway wall of house dust mite induced mouse model of asthma and anti-miR-145 reduced allergic inflammation.	(Collison et al. 2011b)
miR-106a	Up-regulated in the lungs of ovalbumin-induced mouse model of asthma, anti-miR-106 reduced allergic inflammation.	(Sharma et al. 2012)
	Regulates IL-10 expression.	(Sharma et al. 2009)

miR-133a	Downregulated in cultured human airway smooth muscle cells after IL-13 stimulation. Anti-miR-133a in bronchial smooth muscle cells increased airway contraction and hyperresponsiveness.	(Chiba et al. 2009)
miR-221	Up-regulated in ovalbumin mouse model of asthma.	(Qin et al. 2012)
	Up-regulated in the peripheral blood of asthmatic children.	(Liu et al. 2012)
miR-1	Down-regulated by vascular endothelial growth factor (VEGF) in the lung endothelium. Intranasal administration inhibited inflammatory responses to ovalbumin and house dust mite.	(Takyar et al. 2013)

2.7 Nitric oxide

2.7.1 Nitric oxide in the airways

Nitric oxide is a small gaseous molecule synthetized from oxygen and the amino acid L-arginine by the nitric oxide synthase (NOS) enzymes (Moncada et al. 1993). In human airways, three isoforms of NOS have been identified (Yates 2001). Endothelial NOS and neuronal NOS, referred as constitutional NOS are calsium-calmodulin dependent and activated in response to a calcium signal. The predominant NOS form in airway epithelia is inducible nitric oxide synthetase (iNOS) (Lundberg et al. 1995). It is calcium independent and activated by proinflammatory cytokines such as inteleukin-1, TNF-α, IFN-γ and bacterial lipopolysaccharides. Up to 1000 times higher levels of nitric oxide can be produced by iNOS compared to constitutional NOS. Nitric oxide is produced by many structural and inflammatory cells including eosinophils, mast cells, epithelial cells, macrophages and smooth muscle cells. In the normal lower airways, iNOS seems to be expressed primarily in the respiratory epithelium.

Nitric oxide has a role in host defence in the airways because of its antimicrobial activities (Lundberg et al. 1999). It has also been shown to regulate ciliary motility (Runer et al. 1998) and it plays a role in the regulation of vasodilatation and neurotransmission (Lundberg et al. 1999; Yates 2001). The continuous expression of iNOS in the normal

epithelium has been suggested to be maintained by IFN-γ (Uetani et al. 2001). It has been proposed that nitric oxide may promote Th2 type inflammation because it reduces INF-γ levels and thus increases proliferation of Th2 cells and Th2 type inflammation. It is also important in eosinophil migration and infiltration (Korhonen et al. 2005). More recently, it has been shown that an increase in nitric oxide in the allergic inflammation of the airways depends primarily on IL-4 and/or IL-13 activity (Guo et al. 1997; Suresh et al. 2007).

Nitric oxide is produced in both the upper and lower airways. However the major source of nitric oxide is the upper airways (Lundberg et al. 1995; Lundberg et al. 1999). Up to 20−100 times higher nitric oxide concentrations can be measured in the upper airways than in the lower airways. Most of the nitric oxide produced in the upper airways originates from the paranasal sinuses (Lundberg et al. 1995).

2.7.2 Measurement

Nitric oxide measurements are usually performed on-line, but off-line measurements of exhaled nitric oxide are also possible. The most common method for measuring nitric oxide has been the chemiluminence technique, which is based on the photochemical reaction between nitric oxide and ozone generated in the analyser. The result is expressed as a fractional concentration, parts per billion (ppb). More recently, hand held devises using electrochemical sensor technology have been developed. These methods show good correlation and reproducibility in both upper and lower airway measurements (Silkoff et al. 1999; Ekroos et al. 2000; Ekroos et al. 2002; Alving et al. 2006; Stark et al. 2007). A recommendation for the standardisation of exhaled and nasal nitric oxide measurements was provided by American Thoracic Society (ATS) and European Respiratory Society (ERS) in 2005 (ATS/ERS 2005).

Exhaled nitric oxide measured using a standardised procedure (ATS/ERS 2005). Subjects inhale nitric-oxide-free air and subsequently exhale near-total lung capacity for at least six seconds against a flow resistor in order to close the soft palate to avoid contamination of the nitric oxide from the upper airways. The plateau concentration is defined as the mean concentration over three seconds during the stable end-expiratory plateau. The procedure is repeated at least twice, and the reproducible

exhalations have plateaus values that agree within 10%. The result of the test is the mean of the plateau values. In order to obtain reproducible measurements, variation of the exhalation flow rate should be small. The recommended flow is 50 ml/s, and during the time of the nitric oxide plateau generation should remain between 45–55 ml/s. Multiple flow rates can be applied to differentiate between alveolar and bronchial components of NO output (Lehtimäki 2003). Several factors influence the exhaled nitric oxide levels. Smoking reduces exhaled nitric oxide level, whereas respiratory tract infections increase the levels (Persson et al. 1994; Kharitonov et al. 1995). In addition, eating nitrate-containing food, drinking coffee and performing spirometric manoeuvres may alter exhaled nitric oxide levels (Deykin et al. 1998; Olin et al. 2001; Bruce et al. 2002).

Nasal nitric oxide measurement requires generation of airflow through the nasal cavity; velum closure is essential to prevent leakage of nitric oxide to the oropharynx or dilution of sample from the gas originating from the lower respiratory tract (ATS/ERS 2005). There is not a single recommended standardised method in use. In the breath-holding method nasal air from the nasal cavity is aspirated with a steady flow by using a nasal olive when the subject is holding his/her breath. In the tidal breathing method, nasal nitric oxide is measured during tidal breathing. The subject breathes with one nostril and the air is sampled from the other nostril at a steady flow. A modification of this method, subject breathing against resistance was recommended by the ATS and ERS Taskforce (ATS/ERS 2005). The humming method, that is, the measurement of nasal nitric oxide during quiet phonation, has been introduced as a tool to measure osteomeatal patency. It has been reported that humming increases nasal nitric oxide levels 15-fold by increasing sinus ventilation and this increase is not detected in patients with occluded sinus ostia (Weitzberg et al. 2002; Lundberg et al. 2003). De Winter and colleagues (2009) compared different methods and concluded that the best reproducibility is achieved by using the breath-holding and humming methods. Nitric oxide concentration is calculated from the steady plateau in the nasal nitric oxide versus time curve, usually reached within 20 seconds. Similar to exhaled nitric oxide measurement, using a constant and controlled flow is essential. There are no reference values for nasal nitric oxide in adults. The values vary even in equivalent patient groups because of the

variation of methods, also a high level of intra-individual variation over time and inter-individual variation in healthy subjects have been detected (Kharitonov et al. 1997; Bartley et al. 1999). Nevertheless, it has been suggested, that the normal nasal nitric oxide range would be 450–900 ppb when the breath-holding method is used (Scadding et al. 2009). Heavy physical exercise, smoking, nasal volume and aerodynamics of the air flow may affect the value (Phillips et al. 1996; Olin et al. 1998; Colantonio et al. 2002)

2.7.3 Exhaled nitric oxide in asthma and rhinitis

Alving and colleagues (1993) first demonstrated higher amount of exhaled nitric oxide in atopic asthma than in healthy subjects. An increase in iNOS expression in bronchial biopsies of asthma patients has been shown (Hamid et al. 1993). Exhaled nitric oxide is commonly regarded as a marker of eosinophilic airway inflammation, or more specifically as a marker of Th2-type local inflammation of the bronchial mucosa rather than general eosinophilic inflammation (Szefler et al. 2012; Bjermer et al. 2014). Exhaled nitric oxide levels are associated with eosinophil numbers in bronchoalveolar lavage fluid, bronchial biopsies and induced sputum (Jatakanon et al. 1998; Payne et al. 2001; Warke et al. 2002; Berry et al. 2005b). Increased levels of exhaled nitric oxide have been reported in subjects with allergic rhinitis without asthma diagnosis or asthma symptoms (Palm et al. 2003). Exhaled nitric oxide level was related to atopy in asthma and rhinitis patients; in subjects with asthma or rhinitis without positive SPTs, the exhaled nitric oxide level did not differ from healthy controls (Gratziou et al. 1999). However, in the study by Rouhos and colleagues (2008) atopic sensitization without airway diseases or symptoms did not increase the exhaled nitric oxide level. The association with markers of eosinophilic inflammation is lost in smokers (Berry et al. 2005b).

Numerous studies have examined the feasibility of exhaled nitric oxide in asthma phenotyping and monitoring. The ATS/ERS Statement recommended that “clinical utility of exhaled nitric oxide based strategies have not been explored extensively. Current available evidence suggest a role in identifying the phenotype in airways disease, particularly in the identification of corticosteroid responsiveness” (Reddel et al. 2009). More

recently, ATS guidelines recommended the use of exhaled nitric oxide in monitoring airway inflammation in patients with asthma (Dweik et al. 2011).

Recent studies have evaluated exhaled nitric oxide levels measured using different flow rates in asthma patients. Lehtimäki and colleagues (2002) originally showed that subjects with nocturnal asthma symptoms had higher alveolar nitric oxide levels compared to asthma patients without nocturnal symptoms and to healthy controls. It has also been shown that alveolar nitric oxide levels correlate with small airway function in severe asthma (van Veen et al. 2006), and that the alveolar nitric oxide levels correlate with the eosinophil count in brochoalveolar lavage, but not with sputum or bronchial wash eosinophils of asthma patients (Berry et al. 2005a).

2.7.4 Nasal nitric oxide in allergic rhinitis

Increased levels of iNOS have been detected in nasal epithelial cells of allergic rhinitis patients (Kawamoto et al. 1999; Takeno et al. 2001), similar to those detected in the bronchial biopsies of asthma patients (Hamid et al. 1993). Studies of nasal nitric oxide levels in allergic rhinitis are controversial. Kharitonow and colleagues (1997) found that the nasal nitric oxide level was significantly elevated in the symptomatic allergic rhinitis patients compared to the healthy controls. Similarly, in the study of Arnial and colleagues (1997) the nasal nitric oxide level was significantly higher in the subjects with allergic rhinitis than in the controls. The nasal nitric oxide level was increased also in those allergic rhinitis patients who did not have rhinitis symptoms on the day of the test. Other studies have shown contradictory results (Henriksen et al. 1999; Palm et al. 2003). In these studies the level of nasal nitric oxide was not significantly increased in the subjects with seasonal allergic rhinitis examined during the pollen season. It has been postulated that these controversial results may be explained by the nitric oxide pathway. In the inflammatory conditions cytokines might induce increased activity of arginase causing reducing bioavailability of L-arginine for iNOS (Meurs et al. 2000). Maniscalco and colleagues (2010) have proposed that “while nitric oxide release from the inflamed nasal mucosa might be increased, at the same time the swelling of the nasal mucosa may lead

to obstruction of the sinus ostia, with less influx of nitric oxide from the sinuses to the nasal cavity, where the nitric oxide is being measured". In most of the studies described above, sinus diseases were not excluded from the study population.

2.8 Induced sputum

2.8.1 Method

In 1992, the induced sputum method was introduced to assess airway inflammation in asthma (Pin et al. 1992b). The method of sputum induction with hypertonic saline and processing has been covered by ERS Task Force in 2002 (Djukanovic et al. 2002; Efthimiadis et al. 2002; Paggiaro et al. 2002). Hypertonic saliva used sputum induction may cause bronchial obstruction. It is regarded as a safe method when it is performed after bronchodilating medication and lung function, usually PEF, is assessed before and after the induction (Wong et al. 1997). The two main methods for sputum processing are in use. In the whole expectorate method the entire sputum expectorate is usually mixed with dithiothreitol (DTT) to reduce disufide bonds in mucus, then homogenized, filtered and finally centrifuged to separate cells and fluid phase. In the sputum plug method mucous plugs are selected using a wooden spatula and the mucous plug material is processed forward similar to the other method. Different methods used for sputum processing may cause differences in the results between the studies. However, in the studies comparing these methods, similar findings in sputum eosinophils and ECP levels have been found (Gershman et al. 1996; Szefler et al. 2012). One of the greatest limitations of the induced sputum method is the difficulty in achieving suitable samples from a substantial proportion of the patients (Matsuoka et al. 2008). Nevertheless, it has been reported that with experienced personnel sputum can be obtained from 80–90% of the patients (Belda et al. 2000; Spanevello et al. 2000). The short term repeatability of induced sputum cell analysis and liquid phase markers is good both in healthy subjects and in asthma patients (Purokivi et al. 2000; Fahy et al. 2001).

The sputum cell count is usually reported as the percentage of non-squamous cells in the sample. Squamous cells are also counted to deter-

mine sample quality. A squamous cell percentage of greater than 80% is taken to indicate inadequate quality of the sputum sample (Fahy et al. 1993). The sputum cell count is a well-validated method for assessing cellular inflammation in the airways (Szefler et al. 2012). It has been shown to reflect the findings in bronchial wash and bronchoalveolar wash samples (Fahy et al. 1995b).

Several biomarkers have been detected in the induced sputum fluid-phase or supernatant of the asthma patients (Dragonieri et al. 2009). However, the validity and reproducibility of techniques to detect fluid-phase markers are not assessed as well as that of the sputum cell analysis, and certain factors (e.g. DTT, proteases in the sputum) may hamper analyses of some of the markers (Efthimiadis et al. 1997; Kelly et al. 2000; Stockley et al. 2000).

2.8.2 Findings in asthma and allergic rhinitis

Sputum eosinophilia has been recognized as a characteristic feature of asthma and shown to predict the response to corticosteroid treatment for more than 50 years (Brown 1958). The eosinophil percentage is related to the airflow obstruction and bronchial hyperresponsiveness in asthmatics, the inverse relationship between the sputum eosinophil percentage and FEV_1 as well as the sputum eosinophil percentage and provocative concentration causing a 20% fall in FEV_1 to metacholine has been observed (Woodruff et al. 2001). The eosinophil count rise in response to the allergen challenge and fall in response to the corticosteroid treatment in asthma (Pin et al. 1992a; Claman et al. 1994). In addition, an increase in the sputum eosinophil count predicts the loss of asthma control (Deykin et al. 2005; Jayaram et al. 2006). An increase in the sputum eosinophil count has also been detected in patients with seasonal allergic rhinitis without asthma (Foresi et al. 1997). The ATS/ERS statement on asthma control and exacerbations concluded that "eosinophil assessment in sputum provides additional, clinically important information about inhaled corticosteroid responsiveness and preventable future risk of exacerbations" (Reddel et al. 2009).

Sputum neutrophilia is a common finding in adult asthma patients with persistent asthma (Gibson et al. 2001). It is also related to severe airway obstruction in asthma; an inverse correlation between sputum

neutrophil count and FEV_1 has been detected (Woodruff et al. 2001). Moreover, an increase in the sputum neutrophil count is seen in asthma exacerbations (Fahy et al. 1995a). Recently, Moore et al. used cluster analysis for over 400 subjects and found that sputum neutrophilia was associated with more severe asthma (Moore et al. 2013).

On the basis of the sputum cell inflammatory profile, asthmatic subjects can be categorised into four groups: eosinophilic (sputum eosinophils ≥2%), neutrophilic (sputum neutrophils >61%), mixed granulocytic (sputum eosinophils ≥2% and neutrophils >61%), pauci-granolocytic type (both eosinophils and neutrophils in normal range) (Simpson et al. 2006; Davies et al. 2013). Sputum inflammatory profiles have been used in phenotyping and endotyping asthma patients. However, recent studies have suggested that the sputum inflammatory profile commonly changes in asthmatics. During a one year follow-up, stabile phenotypes were detected in only one third of subjects with moderate and severe asthma (Al-Samri et al. 2010).

In the fluid phase of sputum, ECP and myeloperoxidase have shown to correlate with eosinophil count in asthmatics (Efthimiadis et al. 1997). The level of IL-8, a chemoatractant of neutrophils has been shown to be elevated in persistent neutrophilic asthma and to associate with sputum neutrophil levels (Gibson et al. 2001). The level of Th2-type cytokine IL-13 has also been found to be elevated in some asthma patients (Saha et al. 2008). Also, Cys-LTs have been increased in asthma patients (Pavord et al. 1999). Recently, the levels of both vascular endothelial growth factor (VEGF) and Cys-LTs were shown to be elevated in asthma and the levels were highest in severe asthma group (Papadaki et al. 2013). In addition, elevated levels of albumin, fibrinogen, nonkinase plasminogen activator, plasminogen activator inhibitor, neurokinin A, 8-isoprostane and matrix metalloproteinasis-9/tissue inhibitor of metalloproteinases rate have been found in the fluid-phase of sputum (Dragonieri et al. 2009).

2.9 Nasal lavage fluid

2.9.1 Method

Proteins and cells cleared from the mucosa tissue of the airways are considered to end up mainly in the airway lumen (Howarth et al. 2005). Nasal lavage is a simple and well tolerated method to investigate nasal inflammation. It is performed by instilling physiologic solution in the nasal cavity and collecting the recovered fluid. Several nasal lavage techniques can be used. In the Naclerio method, subjects sit with their neck extended backward and hold their breath when instilling the fluid and after 10 seconds the subject leans forward and expels the fluid into the container (Naclerio et al. 1983). In Greiff's method, later modified by Grunberg, fluid is introduced with the catheter into anterior part of the nose with the neck flexed forward, with the seal at the anterior nares around the administrative device to prevent the fluid coming out (Greiff et al. 1990; Grunberg et al. 1997). The volume of instilled liquid is usually 2.5−10 ml, with approximately 80% recovery. After collection, the fluid is filtered and centrifuged to separate cells and supernatant. The cell pellet can be suspended in phosphate buffered saline to make microscopic slides for differential cell counts and immunostaining. The repeatability of this test is considered good when evaluating eosinophil count (Belda et al. 2001). However, marked variability in the soluble inflammatory mediators has been detected, and the level of several mediators has been low or undetectable (Roponen et al. 2003; Boot et al. 2008). Nevertheless, significant differences in the levels of inflammatory mediators has been found between healthy subjects and subjects with allergic rhinitis.

2.9.2 Findings in allergic rhinitis and asthma

Nasal lavage fluid has been used to examine nasal inflammation in naturally occurring allergic rhinitis (Howarth et al. 2005). Increased eosinophil counts as well as elevated levels of eosinophil activation markers, ECP and Eosinophil protein X (EPX) have been detected in allergic rhinitis patients compared to healthy controls (Svensson et al. 1990; Knani et al. 1992; Di Lorenzo et al. 1997; Wilson et al. 1998). In addition, levels of markers related to basophil activation, tryptase and

PGD_2, PGE_2 and LT levels have been aggravated in these subjects (Volovitz et al. 1988; Knani et al. 1992; Sugimoto et al. 1994; Di Lorenzo et al. 1997; Wilson et al. 1998). Elevated levels of plasma exudation markers have also been found in allergic rhinitis; an increase in albumin levels have been detected in these patients compared to healthy subjects and an increase in the level of α_2 makroglobulin during pollen season in seasonal allergic rhinitis has been seen (Svensson et al. 1990; Wilson et al. 1998). More recently, Amorom and colleagues showed that lavage fluid eosinophil counts are good predictors of sputum eosinophilia in asthma patients (Amorim et al. 2010). Nasal lavage fluid has also been widely used to study mechanisms of underlying responses to intranasal allergen challenge as well as to challenges with certain stimuli including histamine and methacoline, capsaisin, bradykinin and substace P (Howarth et al. 2005).

2.10 Proteomics

Proteomics is an unbiased method to investigate the entire protein content of the sample, for example, cell, tissue or biofluids, at a certain time point (Coombs 2011). This method utilizes many technologies, including protein extraction and sample fractionation, protein and peptide separation, mass spectrometry to determine the molecular mass of proteins, protein identification and the enrichment analysis of identified proteins. The term proteomics was first introduced in 1995; since then progress in the proteomic field has been rapid, and new mass spectrometry techniques and software for protein identification and expression analysis have been introduced. Proteomics adds information to genetic studies, which are unable to determine whether genes or mRNAs end up being functional proteins. It provides a sensitive tool to detect disease biomarkers and obtain information on protein interactions, the extent of post-translational modifications that contribute to protein function and quantity of a protein in the sample.

2.10.1 Sputum proteomics

During recent years, induced sputum biomarkers have been investigated using proteomics (Wheelock et al. 2013; Viglio et al. 2014). Gray and

colleagues (2008) studied induced sputum samples from 20 healthy controls, 24 subjects with asthma, 24 subjects with chronic obstructive pulmonary disease and 28 with cystic fibrosis. Altogether 105 proteins differentiated significantly in the asthma group compared to healthy controls, and eight of these were identified and confirmed. Gharib and colleagues (2011) reported induced sputum protein abundance identification and quantification and function annotation from five healthy controls and ten asthmatic subjects. They identified altogether 254 proteins in the healthy controls and asthmatics and 17 differentially expressed proteins between these groups. These proteins were associated with protease inhibitory activity, defence response, immunity and inflammation. This method robustly differentiated asthmatics from healthy controls. Lee and colleagues (2013) studied sputum samples of subjects with severe asthma and neutrophilic inflammation with proteomics. They found 13 differentially expressed proteins in the subjects with uncontrolled asthma compared to those with controlled asthma. S100 calcium binding protein A9 was detected to be increased in severe uncontrolled asthma with neutrophilic inflammation.

2.10.2 Nasal lavage fluid proteomics

Similar to induced sputum proteomic studies with asthmatics, the proteomics method has been utilized to study nasal lavage fluid biomarkers in allergic rhinitis. Ghafouri and colleagues (2006) examined the nasal lavage fluid proteome of six patients with seasonal allergic rhinitis and six healthy controls before and during pollen season. They found changed levels of several proteins including one formerly unidentified protein in nasal lavage fluid in the allergic rhinitis patients during pollen season. In addition, one protein (acidic form of α_1-antitrypsin) was increased in allergic rhinitis patients also outside pollen season. Benson and colleagues (2009) studied nine patients with allergic rhinitis and six patients with chronic rhinosinusitis combined with asthma. They identified 197 proteins in the nasal lavage fluid and detected differences in the protein abundances between these two groups. More recently, Wang and colleagues (2011a; 2011b) published two reports on biomarkers related to the effect of glucocorticoid treatment in seasonal allergic rhinitis. In addition to the proteomic analysis of nasal lavage fluid, nasal lavage fluid

cells and biopsies from the nasal mucosa were studied using microarray analysis. They found many new biomarkers for glucocorticoid treatment and showed that it affected wide variety of pathways in allergic rhinitis. A significant change in the expression of several proteins formerly not identified in allergic rhinitis was also detected.

3 AIMS OF THE STUDY

The aim of this thesis was to study airway inflammation biomarkers in asthma and rhinitis in the context of united airway disease hypothesis.

The more specific objectives were:

1. To determine whether airway inflammation and miRNA levels in nasal mucosa differs in symptomatic and non-symptomatic subjects with allergic or nonallergic rhinitis and to examine whether concomitant asthma has an effect on airway inflammation in allergic rhinitis (Study I).

2. To detect potential biomarkers in nasal mucosa related to long term asthma and allergic rhinitis and evaluate whether these markers differ between asthmatics with and without rhinitis or in terms of asthma severity (Study II).

3. To evaluate the association between sinus ostial obstruction and the level of nasal nitric oxide, and the association between nasal nitric oxide and exhaled nitric oxide, sinus opacification, nasal mucosal eosinophilia and Th2-type cytokine levels (Study III).

4. To study the induced sputum proteome of subjects with asthma and rhinitis and to identify differentially expressed proteins and thus potential candidate biomarkers that could be found both in the upper and lower airways (Study IV).

4 MATERIALS AND METHODS

4.1 Subjects and study design

4.1.1 Study population A (I, III, IV)

Study population A comprised of Finnish men and women, who were first year students in Finnish Universities in 1995, aged 18 to 25 years, and took part in a baseline questionnaire study on asthma and allergic diseases and their risk factors. The population is described in detail in the articles of Kilpeläinen and colleagues (2000; 2001a; 2001b; 2002a; 2002b; 2006). A twelve-year follow up study of all participants of the baseline study was performed in 2007, using the same diagnostic questions of asthma, allergic and non-allergic rhinitis. The responders were divided into four groups according to the postal questionnaire answers. The allergic rhinitis group had positive answer to the question of physician-diagnosed allergic rhinitis and negative answers to the questions on asthma or physician-diagnosed asthma. The allergic rhinitis and asthma group had positive answers to the questions of physician-diagnosed asthma and allergic rhinitis. The nonallergic rhinitis group had a positive answer to the question "Have you ever had, recurrently or for a longer period of time, nasal symptoms (such as blocked nose, sneezing, nasal discharge) that are not related to a common cold or other respiratory infection?", and negative answers to the questions on allergic rhinitis and physician-diagnosed allergic rhinitis. The control group had negative answers to the questions concerning nasal symptoms and allergic rhinitis diagnosis, allergic conjunctivitis symptoms and diagnosis, physician-diagnosed atopic dermatitis and asthma diagnosis. They also answered no to the question "Have you ever had attacks of breathlessness with wheezing?".

A sample of respondents from each group was randomly selected to take part in clinical testing at the Finnish Institute of Occupational Health and Turku University Central Hospital according to the postal code. The study design is presented in Figure 1.

After the clinical examination, each subject's group was revised. Subjects in the allergic rhinitis group had one or more positive SPTs to common environmental aeroallergens and rhinitis symptoms related to exposure to the allergen to which they were sensitized. Subjects in the allergic rhinitis and asthma group fulfilled the criteria of the allergic rhinitis group. In addition, they had previous physician-diagnosed asthma or a significant (≥12%) increase in FEV_1 after bronchodilator administration and asthma symptoms. The nonallergic rhinitis group comprised subjects with periodic or perennial rhinitis symptoms. However, they had no positive SPTs to common aeroallergens or in cases of positive SPTs, they had no respiratory symptoms related to the allergens tested prick positive. Subjects in the control group did not have respiratory diseases, nor recurrent or constant respiratory symptoms. They did not have positive SPTs to common aeroallergens or in cases of positive tests, no respiratory symptoms related to the allergens tested positive. A total of 179 subjects participated the clinical examinations. After the examinations, the control group consisted of 42 subjects, the allergic rhinitis group of 52 subjects, the allergic rhinitis and asthma group of 40 subjects and the nonallergic rhinitis group of 44 subjects. One subject with nonallergic asthma without rhinitis symptoms did not fulfil the criteria for any group. The subjects were further divided into two subgroups: subjects with current rhinitis symptoms and subjects without current symptoms. In Study III the allergic rhinitis group and the allergic rhinitis and asthma group were combined and analysed as one allergic rhinitis group.

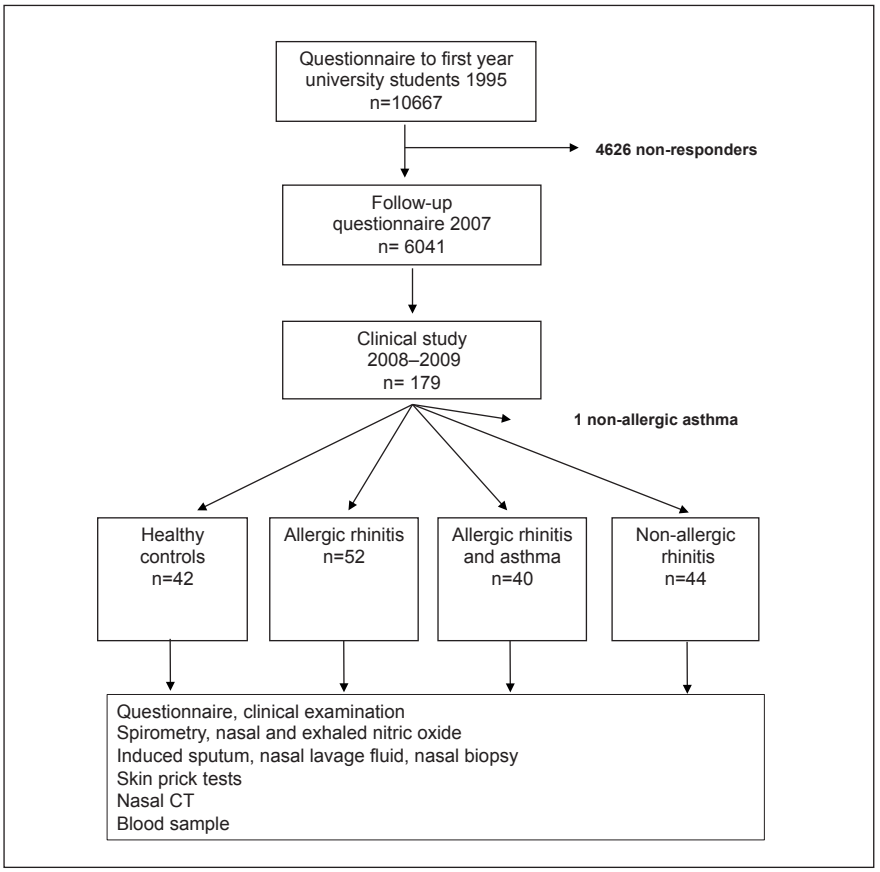

Figure 1. Description of Study population A and the clinical examinations (Studies I, III, IV).

4.1.2 Study population B (II)

The subjects in Study population B (Study II) were men who had performed their military service between 1986 and 1997. The subjects in the asthma group had been referred to the Central Military Hospital in 1987–1990 due to a diagnosis of asthma or symptoms suggestive of asthma. One or more of the following asthma criteria were met by the participants during their time of hospitalisation: a significant reversibility of bronchial obstruction in spirometry (12% and 200ml in FEV_1),

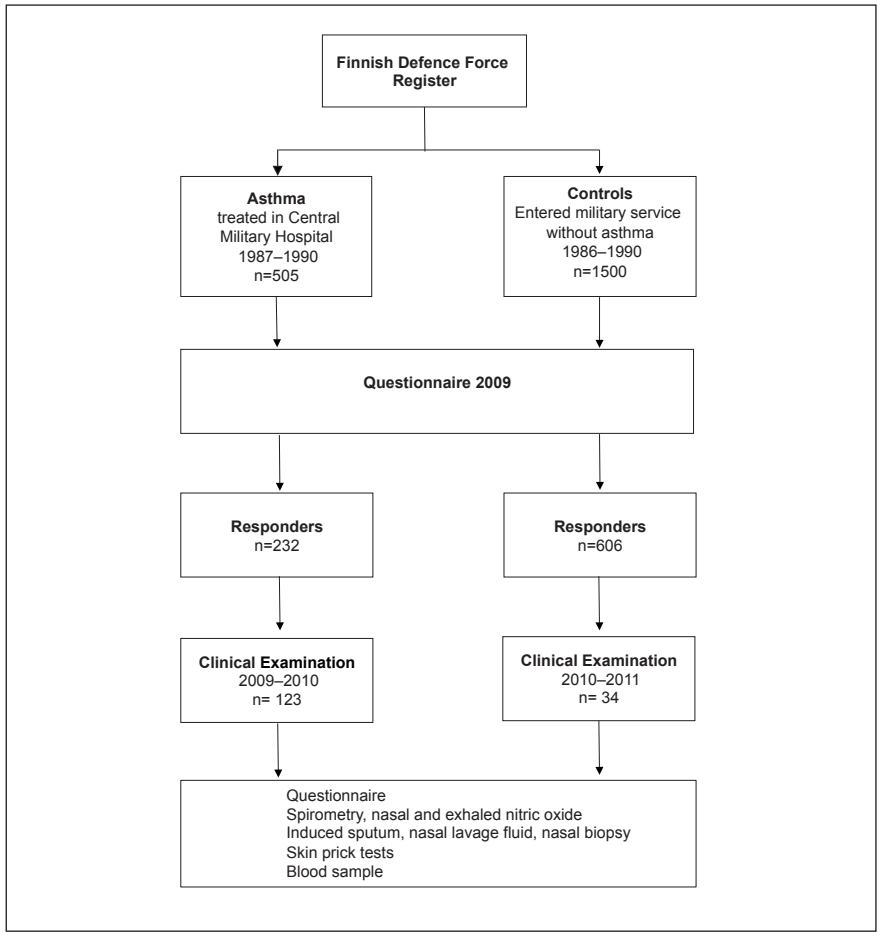

Figure 2. Description of Study population B and the clinical examinations (Study II).

airway hyperresponsiveness detected in the histamine challenge (dose of histamine causing a 15% decrease in FEV_1 or PEF ≤ 0.39 mg), a positive exercise test (a decrease of at least 15% in PEF in the 30-minute follow-up after exercise), PEF recording showing repeated significant bronchodilation responses (≥15%) or significant daily variation (≥20%) at least three times or evidence of the earlier diagnosis of asthma (Lindström et al. 2012). A subset of subjects had borderline diagnostic findings. The

control group consisted of men who entered their military service in 1986–1990 without asthma, and asthma was not diagnosed during their military service. The study subjects responded to the postal questionnaire sent out in spring 2009, described in detail in the publications of Lindström and colleagues (2011; 2012; 2013a; 2013b). The flowchart describing Study population B is shown in Figure 2. A total of 123 subjects in the asthma group and 34 subjects in the control group participated the clinical examinations. Based on postal questionnaire answers subjects with allergic rhinitis or asthma were excluded from the control group. Subjects with asthma were analysed in the subgroups of different asthma severities as well as in terms of concomitant allergic rhinitis.

4.2 Classification of asthma (II)

Current asthma severity was assessed using a combination of GINA 2002 classifications of symptoms and FEV_1 and current medications as described by Liard and colleagues (2000). Clinical severity was classified on four grades based on the frequency of nocturnal and diurnal symptoms during the past 12 months and the prebrochodilator FEV_1 percentage of the predicted value (Lindström et al. 2012). Asthma treatment was classified on four grades based on daily medication use. Asthma severity was a combination of clinical severity and asthma treatment classifications, categories were 1) remission, 2) intermittent, 3) mild persistent, 4) moderate persistent and 5) severe persistent. These categories were combined in the analysis as follows: *nonpersistent asthma* included asthma remission and intermittent asthma, *persistent asthma* included mild, moderate and severe persistent asthma.

4.3 Clinical methods

In Study population A, the clinical examinations were carried out at the Finnish Institute of Occupational Health and at Turku University Central Hospital in 2008–2009. In Study population B, the subjects were investigated in 2009–2011 at the Finnish Institute of Occupational Health. All participants were interviewed and examined by a physician specialised in ear, nose and throat diseases and a specialist in respiratory diseases.

4.3.1 Questionnaire

All the subjects completed in a questionnaire including questions about their medical history, respiratory symptoms, medication and smoking. They also filled a VAS scale of the severity of nasal symptoms with the separate scales for symptoms of rhinorrhoea, nasal congestion and nasal itchiness. VAS scale was a 10 cm line in which the subjects marked the severity of their nasal symptoms. 0 cm stood for no symptoms and 10 cm for very severe symptoms.

4.3.2 Skin prick tests

SPTs were performed using both a negative control and histamine as a positive control. A panel of birch, alder, timothy grass and mugwort pollen, Alternaria alternata, cat, dog and house dust mite Dermatophagoides pteronyssimus were tested in both populations. In addition, Aspergillus fumigatus and horse epithelium were tested in Study population A and meadow fescue, orchard grass and Cladosporium herbarum were tested in Study population B (ALK-Abello, Nieuwegein, the Netherlands). A wheal diameter of ≥3 mm was considered positive. In case of dermographismus (the wheal of negative control was ≥2 mm) existed, the SPT was not accepted for evaluation and the subject was tested with specific IgE to aeroallergens.

4.2.3 IgE

We measured serum total IgE and specific IgE to common environmental allergens with Phadia UniCAP system (Phadia, Uppsala, Sweden). Specific IgE was measured to specific allergen (birtch, timothy grass, cat) in cases of dermographismus in SPT. The result of specific IgE <0.35 kU/l was regarded as normal.

4.3.4 Spirometry

Lung function was measured with flow volume spirometry combined with a bronchodilation test by using a standard spirometer (Spirostar USB Medikro, Kuopio, Finland). The test was performed according to the ATS and ERS guidelines (Miller et al. 2005) and interpreted using the predictive values assessed for the Finnish population (Viljanen 1982).

4.3.5 Exhaled and nasal nitric oxide

An on-line chemiluminescence analyser equipped with nasal nitric oxide software (NIOX, Aerocrine AB, Solna, Sweden) was used to measure exhaled and nasal nitric oxide. The measurements were performed according to the ATS and ERS recommendations (ATS/ERS 2005).

Exhaled nitric oxide was measured while the subjects exhaled against a flow resistor slowly from total lung capacity. The exhalation flow was 50 ml/s. The mean value was recorded for a three-second period from the end-exhaled nitric oxide plateau. The mean value of two or three consecutive measurements within 10% or 2.5 ppb variation was used in the analysis.

Nasal nitric oxide was measured from the nostril while the subject was holding his/her breath for 40 seconds. The aspiration flow was 5 ml/s. The value was recorded from the nitric oxide plateau and the mean value was calculated similar to the exhaled nitric oxide of the measurements within the range of 10%.

4.3.6 Nasal CT (III)

Computed tomography (CT) of the nasal region was obtained from Study population A. Both coronal and axial scans were obtained from the subjects examined at Turku University Central Hospital and coronary scan was obtained from the group of patients examined at the Finnish Institute of Occupational Health. The CT scans were scored using the Zinreich methodology (Kennedy et al. 2005). This is a modification of the Lund-Mackay scoring system, originally developed to score CT changes in chronic rhinosinusitis (Meltzer et al. 2006). Sinus opacification (mucosal swelling and/or fluid) was scored from frontal, maxillary, anterior and posterior ethmoid and sphenoid sinuses both sides. Opacification of each sinus was scored on a six-point scale, 0 was scored for 0%, 1 for 1%–25%, 2 for 26–50%, 3 for 51–75% and 4 for 76–99% and 5 for 100% opacification, resulting in a maximum sum score of 50. Sinonasal obstructions of the frontal recess, middle meatus, infundibulum and sphenoethmoid recess were scored from both sides independently. No obstruction was scored as 0, partial or suspected obstruction was scored as 0.5 and total or definitive obstruction as 1. The maximum sum score

was 8. Additional natural or man-made ostia were also taken into account in scoring. The radiologists who scored the CT images were blinded with the patient information. A subset of images were scored by two radiologists (T. Vehmas and M. Varpula) in order to assess the intra and inter-reader consistency.

4.4 Collection and preparation of biological samples

4.4.1 Nasal biopsies (I, II, III)

Nasal biopsies were taken from the anterior superior part of the inferior conchae. For histological analysis they were fixed in 10% buffered formalin and embedded in paraffin. The samples taken for the polymerase chain reaction (PCR) analysis were immediately quickly-frozen and kept in -70°C.

4.4.2 Induced sputum (IV)

The sputum was induced by inhaling hypertonic saline according to the current ERS Task Force guidelines (Djukanovic et al. 2002). PEF was measured before and after the procedure and bronchodilating medication was administered before sputum induction. The entire expectorant was processed. The sample was immediately processed. Prior to further processing, a smear sample was obtained for cell analysis. DTT (Sputolysin reagent, KGaA /Calbiochem, Darmstadt, Germany) and water was added to the samples and they were incubated for 45 min, pre-filtered through nylon cloth and centrifuged (500g x) to remove cells. The liquid phase was filtered through 0.45 μm (Millex-hv PVDF, Millipore) and frozen to -80°C. Protein concentration approximations were calculated from the protein gel band intensities by comparing the intensities (Image Quant 1D TL 7.0 software, GE Healthcare, Uppsala, Sweden) to known amounts of molecular weight markers. Sputum samples were concentrated five-fold with in ultracentrifugal concentrator tubes (VivaSpin 4, 5000 MWCO PES, Sartorius Stedim Biotech, Goettingen, Germany). For proteomic analysis, 100 μg of each concentrated sample was used, and for Western blot analysis 3 μg of the untreated sample was used.

4.4.3 Nasal lavage fluid (IV)

The nasal lavage fluid sample was obtained by introducing a catheter into the anterior part of the nose with the neck flexed forward, and with the fingers pressing from outside, sealing the nostril. A total of 7.5 ml of physiologic solution was instilled into the nose with a syringe, and the procedure was repeated twice with the same liquid. The lavation was performed on both nostrils and the samples were combined. Immediately after sampling, the fluid was centrifuged at 500g x to separate cells from the liquid phase. After this, the supernatant was centrifuged again at 4000g x and filtered through a 0.45 μm membrane (Millex-hv PVDF, Merk Millipore, County Cork, Ireland). Samples were frozen to -70°C. Protein concentrations were measured with RC DC™ Protein Assay (BioRad, Hercules, CA, US). For the proteomic analysis, each sample was concentrated to 250 μl with ultrafiltration (Amicon Ultra-15 5000 MWCO, Merk Millipore, County Cork, Ireland). Untreated samples were used for Western Blot, whereas ELISA analysis samples were concentrated by five-fold in SpeedVac (Thermo Scientific, Waltham, MA, US).

4.5 Assessment of inflammatory cells

Nasal biopsies (Studies I, II, III) were cut in 2.5 μm thick sections, which were stained with hematoxylin and eosin. The samples were examined under light microscopy (Leica DM LB, Wetzlar, Germany). The numbers of eosinophils and neutrophils were counted in three high-power fields at x 400 magnification.

Induced sputum cells (Study IV) were analysed from the smear sample obtained from the untreated sputum. The sample was stained with Tryptan blue and the total cell count and cell viability was assessed using hemocytometer. The cytospin preparation was made and the slides were stained with May-Grunwald-Giemsa stain. Thereafter, a differential cell count of 200 non-squamous cells was performed. The criteria for good quality sample was <60 squamous cells/ 200 non-squamous cells.

4.6 Real-time PCR analysis (I, II, III)

Real-time PCR was used to measure mRNA levels of cytokines in the nasal biopsy samples. Total cellular RNA was extracted using Trisure Reagent (Bioline, London, UK) according to the manufacturer's protocol. The RNA content was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Extracted RNA was used as a template for complementary DNA (cDNA) synthesis. cDNA was synthesized from 0.5 μg of total RNA in a 25 μl reaction mixture using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, US). The quantitative Real Time-PCR was performed in a 96-well optical reaction plate in ABI PRISM 7500 Fast Sequence Detector (Applied Biosystems, Carlsbad, CA, US) using predeveloped primers and probes from Applied Biosystems. The gene expression between samples was normalised with endogenous ribosomal 18S. The cycle threshold value (C_T) of a sample was determined according to manufacturer's instructions (Applied Biosystems, Carlsbad, CA, US). The results were calculated with $2^{-\Delta\Delta CT}$ method (Applied Biosystems, Carlsbad, CA, US) using SDS 1.4 Software. We assessed mRNA expressions of the following cytokines in both study populations: IL-4, IL-5, IL-13, IL-17 and IFN-γ. In Study population A the expressions of TNF-α, IL-22, IL-6 and IL-10 were also detected.

4.7 MicroRNA assay (I, II)

miRNA quantification was carried out as described by the manufacturer (Applied Biosystems, Carlsbad, CA, US) using TaqMan real-time PCR. Altogether 4 ng of total RNA containing the small RNA fraction was reverse transcribed by using the TaqMan MicroRNA Reverse Transcription Kit and the miRNA-specific stem-loop primers (Applied Biosystems CA, US). The reverse transcription product (1.0 μl) was introduced into the 15-μl PCR reaction mixtures. Reaction mixtures were incubated in 96-well plates on the ABI 7500Fast thermocycler (Applied Biosystems, Carlsbad, CA, US). Target gene expression was normalized between different samples based on RNU48 small nuclear RNA expression values. The results were calculated with the $2^{-\Delta\Delta CT}$ method with SDS 1.4

Software (Applied Biosystems, Carlsbad, CA, US). The expressions of the following miRNAs were assessed in both study populations: miR-7, miR-143, miR-187, miR-224, miR-498, miR-767-5p, miR-874 and miR-886-3p, let-7e, miR-18a miR-126, miR-146a, miR-155, miR-205, miR-210, miR-233 and miR-326. In addition, the following miRNAs were studied in Study population A: miR-17, miR-19a, miR-19-b1, miR-20a, miR-21, miR-26, miR-101, miR-125b, miR-132, miR-133, miR-142-3p, miR-146b, miR-147, miR-148b, miR-150, miR-152, miR-181a and miR-203.

4.8 2D-DIGE (IV)

Before two-dimensional (2D) differential gel electrophoresis (DIGE) labelling, contaminants such as salts, detergents and lipids were removed from the sputum samples using ReadyPrep 2-D Cleanup kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. A total of 50 μg of protein from each sample was used for analysis. The labelling was also performed according to the manufacturer's instructions (Amersham CyDye DIGE flours (minimal dye) for Ettan DIGE (GE Healthcare Biosciences, Pittsburgh, PA, US). The protein samples were suspended in 2 μg/μL DIGE labelling buffer and they were labelled randomly with fluorescence dyes Cy3 and Cy5. A pooled internal standard, containing equal amounts of protein from all samples was labelled with Cy2 in order to decrease technical variation and to align spots during analysis. The labelled samples were absorbed into 18 cm long pH interval 3–10 non-linear Immobiline DryStrips (GE Healthcare, Biosciences, Pittsburgh, PA, US). The strips were rehydrated for six hours with a rehydration buffer, after which the proteins were absorbed on the strips with the cup loading method. Proteins were separated vertically using isoelectric focusing with ETTAN IPGphor II (Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing started with direct current from 150 volts and raising volts in five steps within 19 hours to 8000 volts. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins by their size; the runs were performed with Ettan DALT (GE Healthcare Biosciences, Pittsburgh, PA, US) using 15 Watts per gel. After electrophoresis, the fluorescence of

the protein gels was scanned with the Ettan DIGE imager (GE Healthcare Biosciences, Pittsburgh, PA, US) with a resolution of 40 μm using specific wavelengths for each of the three dyes. DeCyder 2-D Differential Analysis Software v.7.0 (GE Healthcare Biosciences, Pittsburgh, PA, US) was used for spot detection, quantification and the search for abundance differences. Protein spots with ≥ |1.5| fold change in abundance and with Student's *t*-test *p*-value ≤ 0.05 between different study groups were chosen for mass spectrometry identification.

4.9 Tandem Mass Spectrometry (IV)

The gels were silver stained and the significantly up-or down-regulated protein spots were cut from the gel, reduced with DTT, and alkylated with iodoacetamide to prepare them for in-gel digestion with trypsin for 16 hours. The formed peptides were extracted from the gel pieces, dried in a vacuum centrifuge, and dissolved in 2% formic acid. Each peptide mixture was analysed with automated nanoflow capillary liquid chromatography (LC) – tandem mass spectrometry (MS) using CapLC system (Waters, Milford, MS, US) coupled to an electronspray ionization quadrupole time-of-flight mass spectrometer (Waters). The obtained mass fragment spectra were analysed using Mascot v.2.1 Software (Matrix Science Ltd., London, UK) and compared against human entries in the NCBInr database. Hierarchical clustering and principal component analysis of the identified proteins were performed with DeCyder Extended Data Analysis software (GE Healthcare Biosciences, Pittsburgh, PA, US).

4.10 Immunological validation (IV)

4.10.1 Western blot

Sputum or nasal lavage fluid samples were loaded into precast 26-well 12% Criterion TGX gels (Bio-Rad laboratories, Hercules, CA, US) and separated at 100V for 5 minutes and 200V for 60 minutes in buffer. A pool of all test samples was used as an internal standard on each gel to provide a reference for the normalisation of the results. Proteins were

transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, US) using the Criterion Blotter (Bio-Rad laboratories, Hercules, CA, US) at 300 mA for 2 hours in a transfer buffer. Anti-FABP5 (Abcam #ab84028) primary antibody dilution was 1:800. Immonoblot staining was performed with anti-rabbit peroxidase-conjugated immunoglobulin (1:1000) (Dako Cytomaton, Glostrup, Denmark) and chemiluminescent HRP-substrate ECL detection reagent (Perkin Elmer, Waltham, MA, US). They were visualised using Image Quant LAS 4000 mini quantitative imager (GE Healthcare Biosciences, Pittsburgh, PA, US) and the intensities of the protein band were analysed with ImageQuant TL (GE Healthcare Biosciences, Pittsburgh, PA, US).

4.10.2 ELISA

Commercial ELISA immunoassays (Invitrogen, Camarillo, CA, US) were used to measure protein levels of VEGF and CysLT in the nasal lavage fluid. The CysLT kit measured all three CysLT metabolites: TLC_4, LTD_4 and LTE_4. The analysis was performed according to the manufacturer's instructions with five-fold concentrated nasal lavage fluid.

4.11 Statistical methods

Data consisted of both continuous and categorical values. We expressed continuous variables and expressed them as means ± standard deviation (SD), or medians and quartiles (Q) 1–3. Categorical values were expressed as percentages. When exploring differences between the groups we applied Chi-square test for categorical variables. For the continuous normally distributed variables we used ANOVA and for the non-normally distributed variables Kruskal-Wallis test. Student's t-test or the Mann-Whitney U-test were used as post-hoc tests for continuous variables and Bonferroni correction was used for multiple comparisons. We computed Pearson or Spearman's correlation between the continuous variables and analysed the intra- and inter-reader agreements by using the single measures intraclass correlation (weighted kappa). The effects of the confounding factors were studied by using multinomial logistic regression model and general linear model. A p-value of <0.05 was considered statistically significant.

The analyses were carried out with IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY, US) software and GraphPad Prism 5 software (GraphPad, San Diego, CA, US). For the Gene Ontology annotations and hierarchical clustering analyses of the proteins (Study IV) we used DeCyder 2-D Differential Analysis Software v.7.0, (GE Healthcare Biosciences, Pittsburgh, PA, US).

4.12 Ethics

All the subjects signed an informed consent document. Studies I, III and IV were approved by Turku University and the Turku University Central Hospital Ethics Committee (approval number 19/180/2008). Study II was approved by the Ethics Committee of the Department of Medicine of Helsinki University Central Hospital (approval number 284/13/03/00/08).

5 RESULTS

5.1 Clinical characteristics of the study subjects

5.1.1 Study population A (I, III, IV)

The main characteristics of Study population A are shown in Table 2. We detected no significant differences between the study groups (control, allergic rhinitis, allergic rhinitis and asthma, nonallergic rhinitis) in terms of age, smoking or BMI. Gender distribution was not equal, there were more women than men in all groups, and the biggest proportion of women was in the nonallergic rhinitis group. Based on the SPT results, most of the subjects in the allergic rhinitis and asthma group were sensitized to multiple allergens, both to perennial and seasonal allergens. After the subjects with current respiratory infection symptoms were excluded (Study I) we detected a significant difference in the total IgE level between the groups ($p<0.01$), the level of IgE was higher in allergic rhinitis (mean 213.6, SD 416.5) and allergic rhinitis and asthma (mean 195.9, SD 220.3) groups compared to nonallergic rhinitis (mean 43.9, SD 69.6) and control groups (mean 32.1, SD 37.2). We detected no significant differences between the groups in FVC% ($p=0.09$), FEV_1% ($p=0.52$) or FEV_1/FVC ($p=0.11$). Instead, we found significant differences in the VAS scores in rhinorrhoea, nasal congestion and itchiness between the groups. The highest score for rhinorrhoea was found in the allergic rhinitis and asthma group (median 24.0 mm Q_1–Q_3 9.3–38) and in the nonallergic rhinitis group (median 24.0 mm, Q_1–Q_3 6.8–50). The highest nasal congestion score was detected in the nonallergic rhinitis group (median 18.5 mm, Q_1–Q_3 7.8–60) and the score for nasal itchiness was most elevated in the allergic rhinitis group (median 16.0 mm, Q_1–Q_3 3.8–50).

Table 2. Main characteristics of Study population A and B.

	Study population A (n=179)	Study population B (n=157)
Men, n (%)	63 (35)	157 (100)
Age, year, mean (SD)	33.1 (1.5)	41.2 (1.9)
Current smokers, n (%)	14 (7.8)	44 (28)
BMI, kg/m^2 (SD)	23.9 (3.8)	27.5 (4.9)

SD= standard deviation, BMI=body mass index

5.1.2 Study population B (II)

In Table 2, the essential characteristics of study population are shown. We did not detect statistically significant differences in smoking or BMI between the study groups. Four subjects with respiratory infection, one subject with diaphragm relaxation and one subject on whom SPTs were not performed were excluded from the analysis. The asthma group was divided into two subgroups, persistent (n=63) and nonpersistent (n=54) based on the asthma severity. Allergic rhinitis was detected in 79% of the subjects of the nonpersistent asthma group and in 85% of the subjects in the persistent asthma group. Altogether 84% of the subjects in the nonpersistent asthma group and 89% of the subjects in persistent asthma group were sensitized to common environmental allergens, most of them both to seasonal and perennial allergens. In the control group, 24% were sensitized to environmental allergens, but they did not report respiratory symptoms related to these allergens. Total IgE and blood eosinophils were significantly increased in both nonpersistent and persistent asthma compared to controls ($p<0.01$). The persistent asthma group had significantly lower lung function parameters (FVC% of predicted, FEV_1% of predicted or FEV_1/FVC) compared to the controls and the nonpersistent asthma group ($p<0.01$). In Table 3, the VAS scores of nasal symptoms are presented. A significant difference between the groups was detected in rhinorrhoea, nasal congestion as well as in nasal itching.

Table 3. Visual analogue scale (VAS) rhinitis scores of Study population B.

	Total (n=156)	Control (n=34)	Non persistent asthma (n=65)	Persistent asthma (n=57)	P-value
VAS score rhinorrhoea, mm, mean (SD)	27.3 (24.6)	20.9 (18.7)	23.9 (22.6)	35.2 (28.1)	0.027
VAS score nasal congestion, mm, mean (SD)	31.1 (27.1)	19.0 (21.5)	28.2 (25.8)	41.9 (27.9)	<0.01
VAS score nasal itchiness, mm, mean (SD)	19.1 (21.7)	10.7 (16.5)	18.8 (19.8)	24.4 (24.9)	<0.01

5.2 Nasal cytokine and microRNA expressions (I, II)

5.2.1 Study I

When mRNA expressions of cytokines in nasal biopsies were compared between the four study groups (control, allergic rhinitis, allergic rhinitis combined with asthma, nonallergic rhinitis), we detected significant differences in the levels of Th2 cytokines. When compared to controls, we detected up-regulation of IL-13 mRNA in the allergic rhinitis ($p<0.05$) and in the allergic rhinitis and asthma group ($p<0.05$). Similarly, when compared to the nonallergic rhinitis group, IL-13 mRNA was up-regulated in the allergic rhinitis group ($p<0.05$) and the allergic rhinitis and asthma groups ($p<0.05$). In addition, we detected up-regulation of IL-4 mRNA in the allergic rhinitis group compared to the control ($p<0.05$) and nonallergic rhinitis group ($p<0.05$) and an up-regulation of IL-5 mRNA in the allergic rhinitis and asthma group compared to the controls ($p<0.05$). A slight increase in nasal nitric oxide level in the allergic rhinitis and allergic rhinitis and asthma groups as well as in the number of eosinophils in nasal biopsies in the allergic rhinitis and asthma group was also detected, but the differences were not statistically significant.

We also detected an upward trend in the levels of miR-205, miR-155 and miR-498 in the allergic rhinitis group and a downward trend in let-7e in the allergic rhinitis and asthma groups. When subgroups of subjects with non-current rhinitis symptoms (n=87) and current symptoms (n=38) were compared between the groups, we found increased levels of miR-205 ($p<0.01$), miR-155 ($p<0.01$) and miR-498 ($p<0.05$) in the currently symptomatic allergic rhinitis group compared to the controls. These miRNAs were also significantly up-regulated in the allergic rhinitis group compared to the nonallergic rhinitis group. Instead, let-7e was significantly down-regulated in the allergic rhinitis and asthma group without current rhinitis symptoms compared to the controls ($p<0.05$) and nonallergic rhinitis group ($p<0.05$). We also detected that let-7e was down-regulated ($p<0.05$) whereas miR-205 ($p<0.05$) and mR-155 ($p<0.05$) up-regulated in subjects with positive SPTs compared to those with negative SPTs.

5.2.2 Study II

In Study population B we detected an increased level of exhaled nitric oxide and a decreased level of IFN-γ mRNA in nasal biopsy in both asthma groups compared to the controls. No significant differences were detected in the nasal nitric oxide level, the eosinophil count or the Th2 cytokine mRNA levels in the nasal mucosa.

Altogether ten miRNAs were differentially expressed in the nasal mucosa of the asthma patients compared to the controls (all $p<0.05$). The levels of miR-18a, miR-126, let 7e, miR-155 and miR-224 were down-regulated, and miR-498, miR-187, miR-874, miR-143 and miR-886-3 up-regulated in the asthmatics. There was no significant differences between the non-persistent and persistent asthma groups in the expressions of these miRNAs. However, there was a tendency for more distinct up-regulation in the persistent asthma group whereas a tendency for more distinct down-regulation in the non-persistent asthma group was seen.

When a subgroup of asthmatics with concomitant allergic rhinitis were compared with the controls, a down-regulation of three miRNAs: miR-18a, miR-126, miR-155 and an up-regulation of two miRNAs: miR-498 and miR-187 was detected. miR-18a, miR-126, miR-155 and miR-498 were also differentially expressed in asthmatics without concomitant allergic rhinitis compared to the controls. We also found a

positive correlation between miR-155 and exhaled nitric oxide ($r=0.32$, $p<0.01$), miR-155 and nasal nitric oxide ($r=0.36$, $p<0.01$) and miR-155 and IL-13 mRNA ($r=0.38$, $p<0.01$) miR-155 and IgE ($r=0.21$, $p=0.024$), and an inverse correlation between miR-489 and IFN-γ levels ($r=-0.39$, $p<0.01$) in asthmatics.

5.3 Nasal nitric oxide and nasal CT findings (III)

In Study population A, we examined associations between nasal nitric oxide levels and nasal CT scores of sinus opacification and obstruction. A total of 175 subjects were included in the analysis and divided into three groups: controls (n=42), subjects with allergic rhinitis (including subjects with allergic rhinitis with and without asthma) (n=89) and subjects with nonallergic rhinitis (n=44). One subject with asthma but no rhinitis and three subjects who had used inhaled steroids in the previous two weeks were excluded from the analysis.

Based on Zinreich CT scoring, partial or total obstruction of the ostia was detected in 15.3% of the subjects in the frontal recesses, 6.3% in the middle meatus, 22.2% in the infundibula and in 11.4% in the sphenoethmoid recess. We did not detect statistically significant differences in the obstruction score ($p=0.60$) or in the opacification score ($p=0.25$) between the study groups. A significant correlation between the opacification and obstruction scores ($r= 0.60$, $p< 0.01$) was detected. The level of nasal nitric oxide was increased in the allergic rhinitis group ($p=0.035$) compared to the control group, and it was negatively associated with sinus obstruction ($p<0.01$). When we blotted nasal nitric oxide on the total obstruction score, we found that nasal nitric oxide started to decrease in the allergic rhinitis group when the total obstruction score exceeded 2 (Figure 3). In the control group and nonallergic rhinitis group this phenomenon was not as distinct.

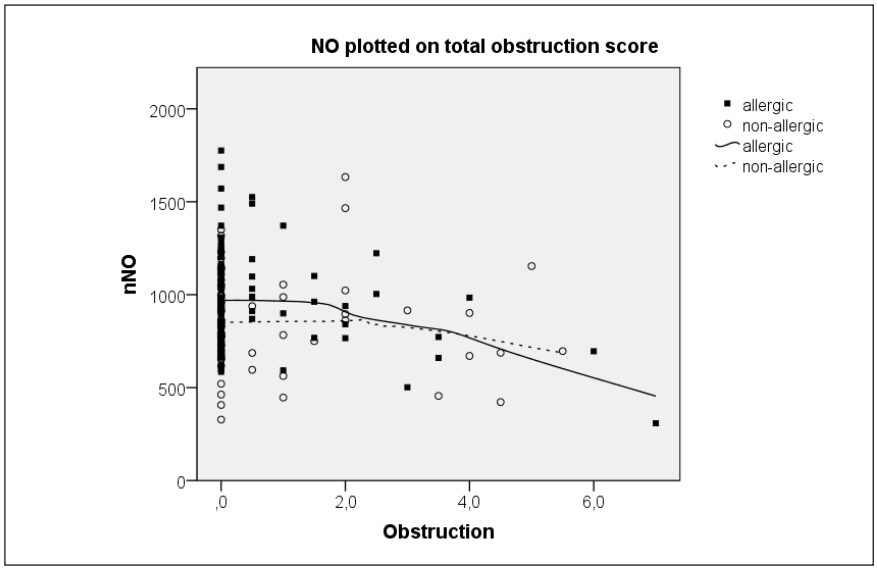

Figure 3. Nasal nitric oxide (nNO) plotted on total obstruction score separately in subjects with allergic rhinitis (AR) and in subjects with nonallergic rhinitis and controls (NAR+ Control).

When allergic rhinitis patients without significant obstruction in ostia, i.e. subjects with obstruction score <2 (n=79), were analysed, we found a significant correlation between nasal nitric oxide and exhaled nitric oxide ($r=0.28$, $p< 0.05$), nasal nitric oxide and opacification score ($r=0.25$, $p< 0.05$), and nasal nitric oxide and nasal eosinophils ($r=0.29$, $p< 0.05$). However, the correlation between nasal nitric oxide and IL-13 mRNA was not statistically significant ($r=-0.05$, $p>0.05$).

5.4 Sputum proteomics (IV)

Altogether 172 subjects in Study population A were included in Study IV; seven subjects with nonallergic asthma were excluded. Induced sputum samples of 21 subjects (4 from the allergic rhinitis group, 6 from the allergic rhinitis and asthma group, 6 from the nonallergic rhinitis group and 5 from the control group) were selected for the proteomic analysis.

No statistically significant differences in the sputum protein concentration were detected between the groups. The protein concentrations of the nasal lavage fluid samples varied between 0.03–0.19 mg/ml, and no statistically significant differences were detected between the groups.

The proteomic analysis revealed 80 differentially expressed (at least 1.5 fold change in expression and $p<0.05$ in the student's t-test) proteins in sputum and 63 proteins in nasal lavage fluid. Altogether 31 different sputum proteins were identified; these were also identified in the nasal lavage fluid. The hierarchical clustering showed differences in expression patterns, especially between the asthma with concomitant allergic rhinitis group and the control group as well as between the allergic rhinitis group and the control group. The Gene Ontology (GO) classification of the biological processes associated with the proteins found are shown in Figure 4. Response to stimulus was the most enriched among the identifications.

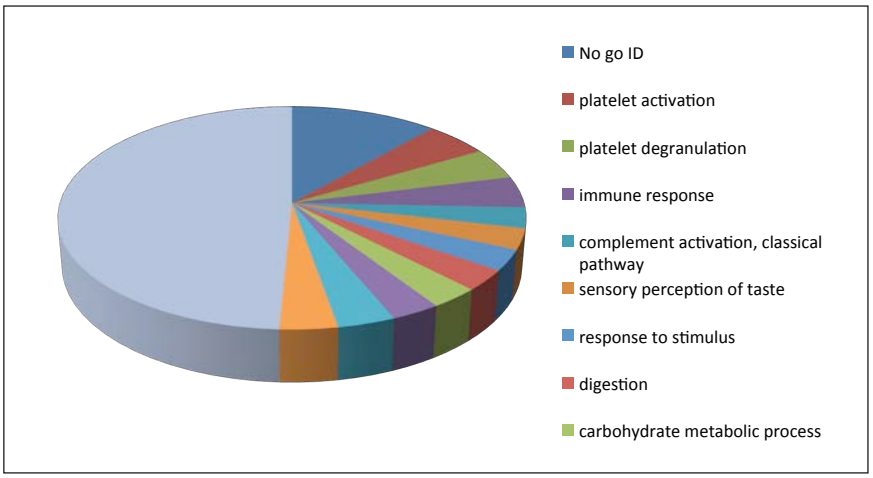

Figure 4. The gene ontology (GO) on biological processes connected to the identified induced sputum proteins.

In 2D-DIGE, we detected significant differences between the groups in the protein abundances in several proteins including FABP5, in Chinatinase-3-like-protein 2 (CH3L2), Carbonic anhydrase 6 (CAH6) Cysteine-rich secretory protein 3 (CRISP3). These proteins were validated in the whole study population using immunoblotting. The level of FABP5 was significantly increased in the asthmatics both in the sputum and in the nasal lavage fluid compared to the controls, no statistically significant differences between the study groups were detected in other assessed proteins. The level of FABP5 significantly positively correlated with both VEGF and CysLT levels in nasal lavage fluid.

6 DISCUSSION

6.1 Study populations and clinical findings

Study population A (studies I, III, IV) was a young adult population, consisting of men and women aged 31 to 38 years. The study group was randomly selected on the basis of the answers to the follow-up postal questionnaire. The gender distribution was concordant with that of the study of Molgaard and colleagues (2007), which reported that nonallergic rhinitis was twice as common among women than among men, whereas the gender distribution is more equal in allergic rhinitis. No significant differences were detected between the study groups in smoking habits, BMI or age.

The mean VAS scores of nasal symptoms in the four groups were low, reflecting mild rhinitis severity. The symptom scores of rhinorrhoea and nasal congestion were similar in the allergic rhinitis and nonallergic rhinitis groups, whereas the nasal itching score was lower in the nonallergic rhinitis group. Our findings are similar to the earlier reports (Molgaard et al. 2007). The asthma group had normal mean spirometric values (FVC%, FEV_1% and FEV_1/FVC) and exhaled nitric oxide level. Most of the asthmatics did not take regular asthma medication. These results indicate that the asthma severity in the asthma group was mainly low.

Study population B (study II) comprised middle-aged men who had been in military service approximately 20 years ago and thus the results cannot be generalised in women and other age groups. The study population is rather homogenous in terms of age, nationality, sex and smoking habits diminishing the confounding factors. The asthma group mainly represents the early onset allergic phenotype (Wenzel 2012). However, there is a subgroup of asthmatics without concomitant allergic rhinitis

(23 subjects, 18%) and without allergic sensitization (15 subjects, 13%) in the population. We can presume that we studied mainly chronic changes in the nasal mucosa instead of acute allergic reactions, because the subjects were examined outside pollen season and the VAS scores of nasal symptoms were low. The symptoms of nasal congestion and itching were increased in the asthma and allergic rhinitis group and also in the asthmatics without concomitant allergic rhinitis. Our results are in line with a previous study showing that asthma is associated with rhinitis also in non-atopic subjects (Leynaert et al. 2004). The VAS scores of nasal symptoms were significantly increased in subjects with persistent asthma symptoms compared to those with nonpersistent symptoms. These findings are concordant with studies showing an association between asthma and rhinitis severity and increased severity of asthma and an increase in nasal symptoms (Magnan et al. 2008; Eriksson et al. 2011). However, some studies have not found a clear association between asthma and rhinitis severity (de Marco et al. 2006; Antonicelli et al. 2013).

Current asthma severity was assessed on the basis of symptoms, lung function and treatment using the GINA guidelines 2002 version, as in previous studies (de Marco et al. 2006), and is described in more detail in the former study of Lindström and colleagues (2012). Approximately half of the subjects had asthma remission or intermittent asthma and the other half mild, moderate or severe persistent asthma. The distribution of asthma severity was in line with a previous population-based report on the same age group (de Marco et al. 2006).

6.2 Nasal microRNAs (I, II)

We studied the miRNA expressions in nasal biopsies of the two study populations (A and B). The microarray analysis was performed similarly in both studies. Based on knowledge at the time of the analysis, we selected for the analysis of 28 miRNAs in Study I and 14 miRNAs in Study II, linked to allergic rhinitis and asthma, allergy, inflammation or immunological responses. Information regarding miRNAs has increased rapidly in recent years, and some miRNAs currently linked to allergic inflammation were not included in the analysis.

We found some differentially expressed miRNAs in the nasal mucosa of the allergic rhinitis and asthma patients, but no changes in the miRNA expression in the mucosa of the nonallergic rhinitis patients compared to the controls. As a whole, the differences in the miRNA expressions were rather modest in Study population A (Study I), with mild asthma and rhinitis. In this population, statistically significant differences in four miRNA expressions were found in the subgroups of subjects with currently symptomatic and non-symptomatic allergic rhinitis. We also found increased levels of Th2 cytokines in subjects with allergic rhinitis and asthma. In nonallergic rhinitis, other than inflammatory mechanisms are thought to be essential. Thus, it is not surprising that the expressions of assessed miRNAs of the nonallergic rhinitis group and the control did not differ.

In Study population B (Study II) 11 differentially expressed miRNAs were detected in asthmatic subjects. We also found an increase in blood eosinophil count and decreased level of IFN-γ in the nasal mucosa. However, we did not find an increase in the Th2-type cytokines. The differences between the findings of these studies may reflect the longer duration of asthma and rhinitis and more severe asthma in population B (study II) and on the other hand more symptomatic subgroup of allergic rhinitis patients in the population A (Study I).

We studied biopsy samples of nasal mucosa including several cell types. Similarly, Williams and colleagues (2009) examined bronchial biopsies of mild asthmatics and healthy controls and found no differences in the miRNA expressions between these groups or after the inhaled steroid treatment of asthmatics. Whereas, Solberg and colleagues (2012) investigated miRNA expressions of bronchial epithelial cells obtained from steroid naïve asthmatics and healthy controls and found markedly abnormal pattern of miRNAs in the asthmatics, altogether 217 differentially expressed miRNAs were detected. In addition, Jardim and colleagues (2012) found 66 differentially expressed miRNAs in the cultured epithelial cells of asthmatics when compared to controls. The differences in the numbers of differentially detected miRNAs may reflect the differences in the studied sample types.

Shaoquing and colleagues (2011) used a microarray chip of 421 miRNAs to analyse nasal biopsies of allergic rhinitis patients. They detected nine miRNAs with more than a two-fold change in expression

in the allergic rhinitis patients. We included eight of these miRNAs in the analysis in both of the studies. We found difference in the expression in only one of these miRNAs. Interestingly, miR-498 was up-regulated in the allergic rhinitis group, whereas in the former study it was down-regulated. In study II, six of these miRNAs were differentially expressed in the asthmatics. Similarly, five of the miRNAs up-regulated in our study, were down-regulated in the study of Shaoquing and colleagues (2011). These differences may be explained by the complex functions and networks of miRNAs as well as differences in the study populations. The population of Shaoquing and colleagues (2011) comprised subjects undergoing surgery for nasal obstruction. We may assume that the nasal disease in that population was more severe or complicated than in our populations.

We detected three miRNAs differentially expressed in the both studies: miR-155, miR-498 and let 7e. In study I, miR-155 was up-regulated in the symptomatic allergic rhinitis patients and in the atopic subjects compared to non-atopic ones. In study II, it was down-regulated in asthmatics with and without allergic rhinitis and a weak positive correlation between miR-155 and exhaled and nasal nitric oxide was detected. MiR-155 has been found to play an important role in the Th2 inflammation by modifying macrophage reaction to IL-13 (Martinez-Nunez et al. 2011) and in mouse models miR-155 deficiency has been shown to result in decreased Th2 cytokine levels and eosinophilic inflammation (Malmhall et al. 2014). Let 7e was down-regulated in asthmatic subjects in both studies and also in atopic subjects compared to non-atopic in study I. It belongs to a let-7 family, which has been demonstrated to influence the expression of IL-13 in lung epithelial cell line and intranasal administration of let-7 has been shown to reduce IL-13 level and hyperresponsiveness and lead to resolution of allergic inflammation in the mouse model (Kumar et al. 2011). However, the inhibitor of let-7 inhibited the allergic cytokine production and disease phenotype in the mouse model (Polikepahad et al. 2010). mir-498 was up-regulated in symptomatic allergic rhinitis patients (study I) and in subjects with allergic rhinitis and asthma, and also in asthmatics without allergic rhinitis (Study II). It also inversely correlated with the IFN-γ level in asthmatics. miR-498 was also down-regulated in the previous study on allergic rhinitis patients (Shaoqing et al. 2011) but the function of

miR-498 in allergic inflammation is not known. It is highly expressed in some cancers (Schepeler et al. 2008) and recently depletion of T-cell intracellular antigen has been shown to cause up-regulation of miR-498 (Sanchez-Jimenez et al. 2013).

We found some differences between the miRNA expressions in the nasal mucosa of subjects with asthma and allergic rhinitis and those of the controls. No differences in nasal eosinophil count was found between those groups. These findings may indicate, that panel of miRNAs might be more sensitive in assessing of allergic inflammation than traditional markers. Moreover, differences in the miRNA expressions in nasal mucosa were detected in asthmatics with and without concomitant allergic rhinitis, suggesting that nasal mucosa could be as useful as a surrogate of bronchial epithelium in assessing inflammation in asthma.

6.3 Nasal nitric oxide in rhinitis (III)

We found a slight increase in the nasal nitric oxide level in allergic rhinitis. This finding is in line with early studies by Kahritonow and colleagues and Arnial and colleagues (Arnal et al. 1997; Kharitonov et al. 1997). In contrast, other studies have found no significant increases in nasal nitric oxide levels in allergic rhinitis (Henriksen et al. 1999; Palm et al. 2003). The discrepancy between the studies may partly be due to obstruction of the sinus ostia caused by mucosal swelling (Maniscalco 2010). We used the breath-holding method to collect nasal nitric oxide. Nitric oxide collected with this method has been proposed to reflect the nitric oxide level in nasal mucosa, whereas the humming method releases nitric oxide from sinuses (Maniscalco et al. 2004). By using the humming method we could have detected bigger differences between subjects with and without ostial obstruction. The level of nasal nitric oxide correlated with the level of exhaled nitric oxide as shown in the previous studies (Williamson et al. 2010).

We used Zinreich classification to score opacification of the sinuses and obstruction of the sinus ostia in nasal CT. It is a more sensitive scoring method to score sinus opacification than the Lund-Mackay score, which has been widely used to score changes nasal CT changes (Meltzer et al. 2006). The levels of opacification and obstruction were low in all

the groups. We detected no differences between the study groups, which is in line with the previous studies and supports the view that limited mucosal changes in CT scans are not indicative for allergy (Ono et al. 2011; Shusterman et al. 2012).

When we studied a subgroup of allergic rhinitis patients without marked sinus ostia obstruction, we detected a positive correlation between the nasal nitric oxide level and sinus opacification. In the previous studies using the Lund-Mackay scoring system in allergic rhinitis and healthy controls a negative correlation has been found (Shusterman et al. 2012). This discrepancy may be due to the more sensitive scoring system in our study and an interaction between opacification and obstruction in the previous studies. We also found a significant positive correlation with the nasal nitric oxide level and the nasal eosinophil count in these subjects. This finding is in line with studies showing associations between sputum eosinophils and exhaled nitric oxide (Berry et al. 2005b) and supports the view that nasal nitric oxide reflects eosinophilic inflammation in the nasal mucosa, but is affected by sinus ostia obstruction in allergic rhinitis. This reduces the feasibility of nasal nitric oxide in the assessment of airway inflammation and monitoring allergic rhinitis in clinical work. When a high level of nasal nitric oxide is detected in an allergic rhinitis patient, it might be taken as an indicator of allergic inflammation and suggest that distinct ostial obstruction is present. However, several factors including exercise, medication and respiratory viral or bacterial infections affect nasal and exhaled nitric oxide levels (ATS/ERS 2005). It is important to pay attention to respiratory infection symptoms before measuring nasal or exhaled nitric oxide.

6.4 Sputum proteomics (IV)

We assessed induced sputum proteome in a subgroup of 21 non-smoking subjects with allergic rhinitis, asthmatics with allergic rhinitis, nonallergic rhinitis and healthy controls. We identified 31 differentially expressed proteins, which were also found in nasal lavage fluid. This is in line with the findings of the previous study, in which eosinophilia in the nasal lavage fluid was found to be a good predictor of sputum eosinophilia (Amorim et al. 2010). The nasal lavage fluid method has some advantages

compared to the induced sputum. It is well tolerated and a sample can be obtained from almost all adults. Instead, the induced sputum sample cannot be obtained from at least 10–20% of the subjects (Belda et al. 2000; Spanevello et al. 2000; Matsuoka et al. 2008). Moreover, the nasal lavage fluid cells can be separated from the liquid phase without chemical treatment, whereas induced sputum treatment with DTT may harm the analysis of some mediators (Efthimiadis et al. 1997; Kelly et al. 2000). However, the validity and the reproducibility of techniques to detect fluid-phase markers in sputum or nasal lavage fluid are not assessed as well as in cell analysis, and considerable variability has been observed in some markers (Stockley et al. 2000; Boot et al. 2008).

In the sputum proteomic analysis, the abundance of FABP5 was increased in the asthmatic subjects when compared to the subjects with allergic rhinitis and those with nonallergic rhinitis. In the immunoblot validation in the whole study population, we found increased levels of FABP5 in both induced sputum and nasal lavage fluid in asthmatics. FABP5 belongs to the family of small cytosolic lipid-binding proteins, fatty acid binding proteins, which regulate glucose and lipid homeostasis and inflammation through their actions in adiposytes and immune cells (Makowski et al. 2005a; Yamamoto et al. 2008). It has been linked to Th17 and T-regulatory cell differentiation, inflammatory responses and oxidative damage (Makowski et al. 2005b; Rolph et al. 2006; Li et al. 2009). We detected a positive correlation between VEGF and FABP5. VEGF is and epithelium derived cytokine associated with Th2 inflammation and airway remodeling (Lee et al. 2004; Chetta et al. 2005). In addition, we detected a significant correlation between FABP5 and CysLTs, which has been shown to up-regulate VEGF production (Poulin et al. 2011). Our findings suggest that FABP5 may participate in the regulation of airway inflammation and remodelling by CysLTs and VEGF mediated cell signalling cascades, and thus might be useful as a biomarker of allergic asthma phenotype in the future.

6.5 Limitations of the studies

There are some limitations related to the study population. The population comprised men and women aged around 30–50 years. Most of

the asthmatics represented the early onset allergic phenotype. Thus, the findings of this thesis cannot be generalized to other age groups or other asthma phenotypes. In Study population A, the subjects mainly had mild asthma and rhinitis and currently had mild symptoms. In Study population B, the samples were taken outside the pollen season and the rhinitis symptoms of the subjects were mainly mild. We may have found more distinct differences in the biomarkers between the groups if the subjects we studied had had more severe diseases (i.e. patients from outpatient policlinics). Many of the subjects had earlier used inhaled or nasal steroid medications. Even though the medication was mainly withheld before the samples were taken, it may have had some effect on the results. We could have found bigger differences in evaluated markers between the groups if we had analysed samples of steroid naïve subjects.

There are also some limitations in the sample techniques and analysis. miRNAs were analysed from the biopsy samples, in which cellular heterogeneity may mask the differences in the miRNA expressions in the individual cell types. Based on current knowledge, analysis of the cultured epithelial cells of the study subjects and the use of a panel of multiple miRNAs might have revealed more differentially expressed miRNAs. However, these methods were not technically achievable at the time of the analysis. In Study III, more distinct differences may have been found between the nasal nitric oxide of the groups if the humming method had been used. Similarly in Study IV, the protein separation and mass spectrometry technique we used results in fewer protein identifications than more advanced techniques.

7 SUMMARY AND CONCLUSIONS

The main findings and conclusions of the study:

1. Some differences were detected between the miRNA expressions in the nasal biopsies of the subjects with symptomatic allergic rhinitis and the healthy controls as well as between the non-symptomatic subjects with allergic rhinitis and asthma the healthy controls. No differences were found between the subjects with nonallergic rhinitis and the healthy controls. The miRNA expressions were relatively similar in the subjects with allergic rhinitis without asthma and in those with concomitant asthma. As a whole, the differences in miRNA expression were rather modest.

2. Differences were detected between the miRNA expression in the nasal biopsies of subjects with long term asthma and allergic rhinitis and those of the controls. Only suggestive differences in the miRNA expressions were found on the basis of asthma severity. Some changes in the miRNA expressions in the nasal biopsies were also found in the asthmatics without concomitant allergic rhinitis. Differences in miRNA expressions were also found in the nasal mucosa of the asthmatics, when differences in the eosinophil count could not be detected. This finding suggests that a panel of miRNAs might be a more sensitive marker of allergic inflammation than traditional markers.

3. Nasal nitric oxide was slightly elevated in allergic rhinitis patients compared to healthy controls, and it associated with exhaled nitric oxide. Sinus ostial obstruction negatively correlated with the level

of nasal nitric oxide in subjects with allergic rhinitis. In subjects without marked ostial obstruction, we found a positive correlation of nasal nitric oxide with sinus opacification and with nasal eosinophil count. We did not, however, detect an association with nasal nitric oxide and Th2 type cytokine IL-13. The feasibility of nasal nitric oxide assessment in clinical practice is limited, because sinus ostial obstruction may effect nitric oxide level. Nevertheless, a high level of nasal nitric oxide in an allergic rhinitis patient indicates eosinophilic inflammation in the nasal cavity and the absence of marked sinus ostial obstruction.

4. Proteomic analysis of sputum and nasal lavage fluid identified the same proteins in both samples. We found an increased abundance of FABP5 in the induced sputum of allergic asthmatics. Elevated levels of FABP5 were detected in the immunoblot validation in both the sputum and nasal lavage fluid. The FABP5 level also correlated with VEGF and CysLT levels, indicating its potential role in inflammation and remodelling in asthma. These findings suggest that sputum proteome analysis is a useful tool when searching for potential candidate asthma biomarkers, and that nasal lavage fluid might be useful in assessing asthma markers.

Our findings support the view of asthma and rhinitis as manifestations of one airway disease and that inflammation of the upper and lower airways in allergic rhinitis and asthma is similar. Biological samples are easier to obtain from upper than lower airways. Thus, markers obtained from the upper airways could be used in assessing lower airway inflammation in the future. Our results suggest that new sensitive biomarker panels for the clinical evaluation of allergic airway inflammation may be found by using miRNA analysis and proteomics.

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Airway inflammation in asthma and allergic rhinitis share common characteristics, and these conditions can be seen as manifestations of one disease. The aim was to investigate airway inflammation biomarkers in the light of this view.

We dected differences in microRNA expression in the nasal mucosa of asthma and allergic rhinitis patients when no significant differences in the inflammatory cells and cytokines were found. A biomarker was found in the sputum of the asthmatic patients, and this marker was also detected in the nasal lavage fluid of these patiens.

Our results suggest that new sensitive biomarker panels for the clinical evaluation of allergic airway inflammation may be found using miRNA analysis and proteomics. Upper airways could be used as a surrogate of lower airways when assessing airway inflammation in asthma.

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