Non-invasive sampling methods of inflammatory biomarkers in asthma and allergic rhinitis

DOOR

Johan Diderik Boot, geboren te Chertsey, Engeland in 1977

INTRODUCTION

General introduction and outline of the thesis

Allergic airway inflammation

Asthma and allergic rhinitis are common, chronic diseases of the respiratory tract. Both conditions often coexist and show systemic manifestations including atopy and blood eosinophilia (1). Worldwide, the prevalence of asthma is estimated at 1-18% and allergic rhinitis at 10-25% of the population, respectively (1,2). In the past 20 years, the incidence of the allergic airways disease has increased, especially among children. In the US, the annual direct and indirect expenses for asthma are estimated at $13 billion (1998 values) and for allergic rhinitis at $2 to 5 billion (2003 values) (1,3). Although severe or life-threatening in only a minority, allergic airways disease affects the quality of daily life of many patients with impact on school attendance and productivity at work. The World Health Organization has estimated that 15 million disability adjusted life years (DALYs) are lost annually due to asthma, representing 1% of the global disease burden. Despite modern medications, still too many patients are not adequately controlled. In addition, asthma and AR are chronic conditions that cannot be cured and most patients require lifelong controller medication and lifestyle adjustments. Hence, there is still an unmet need for novel targeted treatments and more accurate monitoring methods of the disease process (4,5).

Pathophysiology – early and late allergic reaction

In spite of recent progress in elucidating several inflammatory mechanisms of allergic airway disease still many etiological and pathophysiological questions remain unanswered. The pathogenesis of asthma is multifactorial and its expression depends on the interactions of several susceptibility genes and environmental factors. Atopy is the strongest identifiable predisposing factor for developing allergic airways disease (6). Overall, the allergic inflammation within bronchial and nasal tissues is very similar with some local differences (figure 1) (7,8). Exposure to a new allergen results in uptake and processing by dendritic cells. In genetically predisposed individuals, the presentation of processed allergen by dendritic cells to naïve T helper (Th) cells induces the development of Th2 cells (9). Subsequently, Th2 cells release interleukins (IL)-4 and IL-13 which results in differentiation of B cells into allergen specific immunoglobulin (Ig)-E-producing plasma cells (10). The airway epithelium also participates in the allergic response by producing thymic stromal lymphopoietin (TSLP) which is thought to stimulate dendritic cells, B cells and mast cells (11,12). The newly synthesized IgE subsequently binds to surface receptors of mast cells and basophils inducing 'priming' (sensitization).

Upon re-exposure, the allergen binds to the cell surface-bound IgE resulting in cross-linking of the receptors causing degranulation of mast cells releasing preformed pro-inflammatory mediators (histamine, chymase and tryptase) and *de novo* synthesis of other pro-inflammatory mediators (leukotrienes, prostaglandins, platelet activating factor and bradykinin) (13). These mediators have been shown to possess bronchoactive and pro-inflammatory properties in various species, causing airway smooth muscle contraction, vasodilatation, increased vascular permeability, mucus hypersecretion, and the recruitment of pro-inflammatory cells (14,15). Inhalation of a relevant allergen by sensitized subjects produces an early airway response (EAR) in both allergic asthma and allergic rhinitis (8). The EAR-related events are organ-specific and include bronchoconstriction, dyspnea, wheezing and cough within the lower airways and itching, sneezing, rhinorrhea, and congestion within the upper airways accompanied by ocular symptoms (16).

Evidence points to the involvement of early pro-inflammatory mediators in the development of the late allergic response (LAR) which follows in approximately 50% of patients, usually occurring between 3 to 12 hours post-allergen (7,17). Within the lower airways, the LAR is characterized by persistent broncho-obstruction, allergen-induced airway hyperresponsiveness (AHR) and structural airway changes (remodeling) (8,17). In this inflammatory process, several effector cells and their products participate and interact. Epithelial and inflammatory cells are stimulated to produce chemoattractants (e.g. eotaxin, RANTES) (13,18). Pro-inflammatory mediators including tumor necrosis factor (TNF)α, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-4 and IL-13 stimulate the expression of vascular adhesion molecules on endothelial cells. These events result in an increased recruitment of leukocytes (mostly eosinophils, but also basophils and neutrophils) and lymphocytes into the bronchial and nasal mucosa. In addition, IL-4 and IL-13 have the ability to induce production of transforming growth factor alpha (TGF-α) by epithelial cells. TGF-α through autocrine signaling results in mucous metaplasia and fibroblast proliferation (19). Simultaneously, the secretion of IL-5 by Th2-cells produces further activation and infiltration of eosinophils (20). Allergens can also trigger the release of pro-inflammatory neuropeptides (e.g. the tachykinins: neurokinin A and substance P) from sensory nerves within the airways causing the so-called neurogenic inflammation (figure 1). Substance P in particular has been shown to possess pro-inflammatory properties inducing vasodilation, microvascular leakage, and mucus hypersecretion within both airway compartments (21,22). Similarly, within the upper airways, the LAR is characterized by a long-lasting nasal congestion, accompanied by nasal eosinophilia and increased hyperreactivity (7).
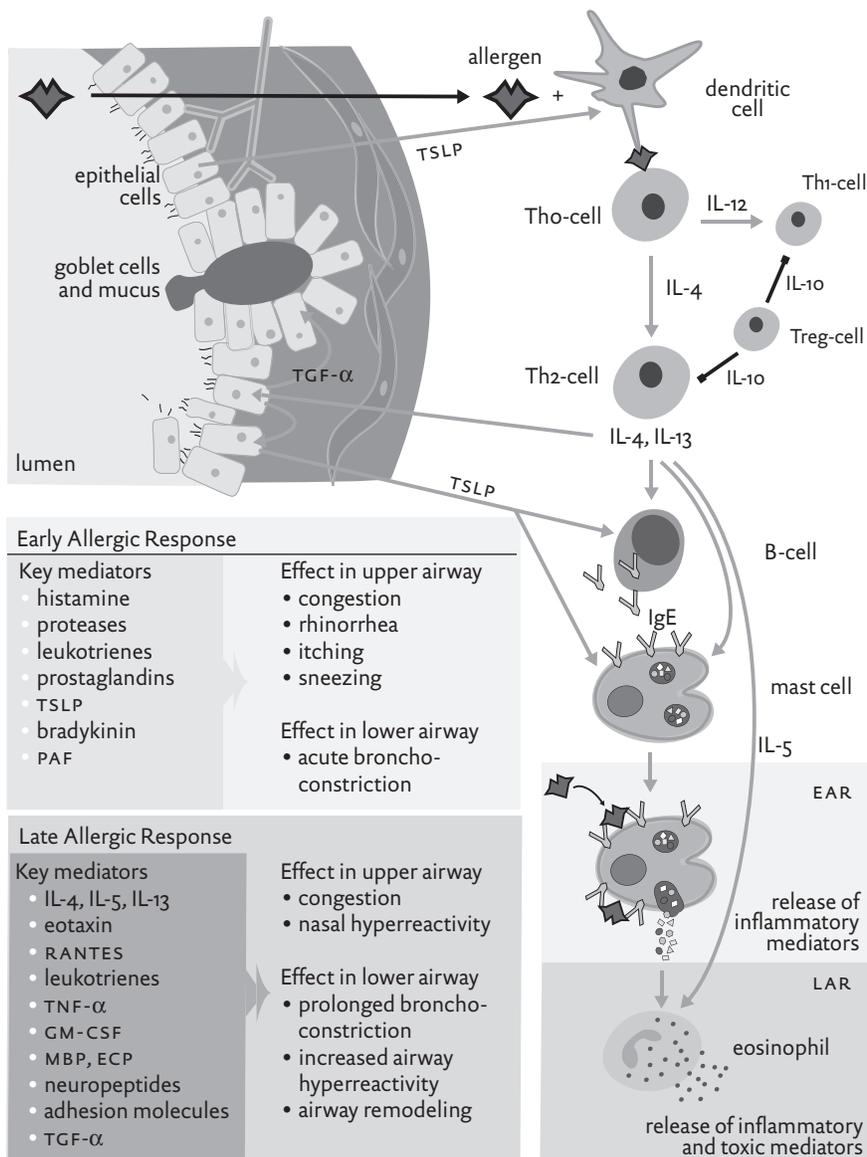
FIGURE 1 Cells and mediators involved in the early and late allergic response in allergic asthma and allergic rhinitis. ECP = eosinophilic cationic protein, GM-CSF = granulocyte-macrophage colony stimulating factor, IGE = immunoglobulin-e, IL = interleukin, MBP = major basic protein, PAF = platelet activating factor, TGF−α = transforming growth factor alpha, TH = T helper, TNF-α = tumor necrosis factor alpha, TSLP = thymic stromal lymphopoietin.

One airway disease

In addition to similarities in airway responses and components of inflammation, several studies provided evidence for a systemic, bidirectional cross-talk between the two airway compartments. Segmental allergen challenge in non-asthmatic patients with allergic rhinitis caused increased inflammatory cell numbers in both bronchial and nasal mucosa in association with symptoms of allergic rhinitis and blood eosinophilia (23,24). Inversely, a nasal allergen challenge in non-asthmatics with allergic rhinitis resulted in increased expression of adhesion molecules and uptake of eosinophils in the bronchial mucosa, while topical treatment with nasal corticosteroids reduced markers of lower airway inflammation (25,26). Supported by this data, asthma and allergic rhinitis are considered manifestations of the same allergic airway syndrome, the so-called combined allergic rhinitis and asthma syndrome (CARAS) (27). Based on (non)-invasive sampling techniques, the list of players involved in allergic airway disease is continuously expanding and offers still novel targets for drug development and clinical monitoring. Examples comprise novel approaches including CCR3 antagonists and toll like receptor agonists as potential targeted treatment for allergic airway disease (28,29).

Heterogeneity of asthma and allergic rhinitis

In view of the heterogeneity of both asthma and rhinitis, assessment of traditional disease markers, including symptoms and lung function, does not suffice for clinical monitoring and drug development. Especially in asthma, these measures appeared to be poorly related to the underlying airway inflammation (30). In addition, several factor analyses revealed that symptoms and lung function, markers of airway inflammation and airway hyperresponsiveness provide complementary information on the disease severity and activity of asthma in both adults and children (31,32). Hence, the combination of (as much as possible of) these outcome parameters is a prerequisite for future disease management and early drug development (33,34).

Biomarkers as efficacy measures in drug development/clinical monitoring

A biological marker (biomarker) is a physical sign or laboratory measurement that can serve as an indicator of normal biological processes, pathophysiological processes or pharmacological response to a therapeutic intervention (35). There is an ongoing exploration of new biomarkers that are closely

linked to asthma and allergic rhinitis. In principle, all biological compounds of the inflammatory cascade could serve as biomarkers.

Biomarkers can be employed for various purposes, including diagnosis, staging, indicator of disease activity/progression or predictor of a treatment response. Validated biomarkers are of major value in early clinical trials to establish "proof of mechanism" of novel drugs (36). Ideally, a biomarker should have the following characteristics (35):

- Clinical relevance: i.e. there is a clear relationship between the biomarker and the pathophysiological events leading to a clinical endpoint.
- Sensitivity and specificity for intervention effects.
- Reliability and repeatability: the biomarker should be measured in a preciseand reproducible way.
- Simplicity of sampling methodology (preferably via non- or semi-invasive sampling techniques) and measurement to promote widespread use.

We propose that a combination of these properties make a biomarker "applicable" for research and development purposes. Implementation of biomarkers in early drug development has several advantages. They can be used as substitutes for clinical endpoints to demonstrate a significant treatment effect in studies requiring long-term treatment or large study populations. Biomarkers also allow the possibility to explore the pathophysiological mechanism of novel interventions. However, since one biomarker may – if at all - capture only a small fraction of the intervention effect, it is important to sample multiple biomarkers whenever possible. Regulatory authorities, such as the EMEA and the FDA, advocate incorporation of validated biomarkers into early clinical studies to speed up timelines of drug development (Critical Path Initiative; FDA 2004).

When implementing biomarkers in clinical trials or monitoring of asthma and allergic rhinitis, it is important to consider the heterogeneous nature of the inflammatory response which may affect the selection of adequate biomarkers (37). In addition, for proof of mechanism studies, one should bear in mind that airway inflammation is only present in patients and, not in non-atopic, healthy subjects. Hence, to assess efficacy of novel anti-asthma/allergy therapy, it is mandatory to move into patients as soon as possible in early drug development.

Applicability of inflammatory biomarkers in allergic asthma and allergic rhinitis

As described previously, multiple pro-inflammatory mediators are involved in the early and late allergic responses and a summary of the most important players is listed in Figure 1.

Consequently, sampling the airways for the measurement of inflammatory components adds novel information on the pathophysiology of disease, helps to validate novel biomarkers and generates a rationale for novel targets in drug development. For example, in the beginning of the 20th century, histamine was isolated from ergot extracts and its role in the pathophysiology of anaphylaxis and allergy was elucidated in the subsequent years (38). Four decades later, this knowledge translated into the development of anti-histamines. To date, anti-histamines are still the cornerstone of targeted pharmacotherapy for allergic rhinitis and other allergic disorders (39,40). More recent examples of targeted drugs are anti-leukotrienes and anti-IgE. In 1940 Kellaway and Trethewie discovered the "slow reacting substance of anaphylaxis", which appeared to constitute of leukotrienes (41). In the following decades, the important role of leukotrienes in inflammatory processes, including asthmatic airway inflammation was established. In 1982, Bengt Samuelsson received the Nobel Prize for his extensive research in this field. This discovery lead to the development of anti-leukotrienes (leukotriene synthesis inhibitors and leukotriene receptor antagonists) for the treatment of asthma. In the second half of the 1990s, these drugs were launched as a novel targeted class of anti-asthma therapy since 25 years (10).

IgE is a hallmark of allergic disease and high serum levels are associated with an increased risk for the development of asthma in later life (42). Omalizumab (a humanized monoclonal antibody directed against circulating IgE) decreases levels of serum IgE. Based on its specific anti-inflammatory properties, Omalizumab effectively improved disease control allowing reduction of the daily ICS dose in two-thirds of patients with allergic asthma and/or allergic rhinitis (43,44). Recent GINA and ARIA guidelines implicated this drug as add-on therapy for the treatment of therapy-resistant, severe allergic asthma and AR (2,40,45). The therapeutic dose of Omalizumab is based on the body weight and should be guided by total serum IgE levels (46). However, not all mediators involved in the inflammatory cascade qualify for biomarkers or drug targets. It was anticipated that IL-5, being the primary interleukin involved in eosinophil activation and recruitment would provide a new target for anti-inflammatory therapy (47). However, while a single intravenous dose of anti-IL-5 (mepolizumab) produced a near-complete depletion of serum eosinophils, it failed to protect against allergen-induced LAR and the associated AHR in patients with mild persistent asthma (48). Likewise, another anti-IL-5 antibody, SCH55700 failed to show any clinical efficacy in terms of symptoms, airway obstruction and AHR following a single intravenous dose (49). Based on a bronchial biopsy study evaluating the effects of 20 weeks of treatment with anti-IL-5 therapy, it has been speculated that subtotal reduction in bronchial eosinophils may possibly account for the lack of clinical effect (50). In conclusion, these data underscore the importance

of ensuring that changes in the selected inflammatory biomarker translate into a clinically significant effect and targeting this biomarker with novel treatment should result in clinically meaningful improvements. In addition, samplings of the biomarker should preferably be conducted in the most relevant environment, i.e. in the target organs, being the lung and nose, instead of the serum. Ideally, one mediator could serve as a biomarker of both asthma and AR. Cleaved Secretory Leukocyte Protein (CSLPI) could potentially be such a mediator. It is present in both the lower and upper airways and is a biomarker of chymase activity in vitro (51). Chymase is a protease, released from mast cells, important effector-cells in the pathophysiology of allergic airway inflammation, AHR and airway remodeling (52,53). The in vivo role of CSLPI is explored in this thesis.

Currently applied biomarkers in asthma

In line with these observations, in recent years, several biomarkers derived from the airways were tested as predictors for disease control. An example, is the trial by Sont and colleagues comparing a treatment strategy aimed at reducing airway hyperresponsiveness (AHR strategy) with the asthma management according to current guidelines (reference strategy) to achieve long-term disease control in patients with mild to moderate persistent asthma (54). After 24 months, patients in the AHR group had better asthma control (expressed as improvement in lung function, exacerbation rate and AHR) albeit with a significantly higher dose of inhaled steroids as compared to the reference strategy group. Green and colleagues compared sputum eosinophils as a biomarker of asthma control versus the traditional disease parameters: symptoms and lung function in patients with moderate to severe persistent asthma (55). After 12 months it appeared that the treatment strategy guided by sputum eosinophils was superior to the traditional approach, significantly reducing severe asthma exacerbations by over 60%. Interestingly, these effects were achieved at a comparable dose of inhaled corticosteroids as in the control arm. The development and validation of non-invasive sputum sampling thus enables studying and monitoring of the (kinetics of the) components of airway inflammation. However, this technique requires multidisciplinary expertise and read-outs usually take several days or weeks. Therefore, sputum induction and analysis may be only feasible for a patient population regularly attending a specialized hospital and hampers its implementation into a primary care setting. This stresses the need of disease-related and treatment-responsive biomarkers, in combination with simple, non-invasive and reproducible, preferably online measuring capability. More recently, several studies in children and adults with chronic asthma

provided evidence for the applicability of exhaled nitric oxide (eNO) measurements, a still–invasive, simple, online sampling method, as a biomarker of asthma control (56,57). As a result, measurements of eNO have been implicated in the monitoring of asthma control according to current GINA guidelines (2). These data underscore the usefulness of inflammometry in early clinical trials and in disease monitoring, warranting the development and validation of non-invasive sampling techniques such as exhaled breath condensate (EBC) analysis and the search for suitable biomarkers including the detection of smell prints (58-60).

The methodology of sampling techniques including their respective yield of inflammatory biomarkers from the lower airways are discussed in more detail in chapter 2 and evaluated in clinical trials in chapters 3-6.

Currently applied biomarkers in allergic rhinitis

Sampling inflammatory mediators from the upper airway compartment may be equally valuable. Although no prospective long-term clinical trials for guiding anti-allergic therapy have been performed thus far, numerous studies have investigated the relationship between several components of upper airway inflammation, symptoms and response to treatment in patients with allergic rhinitis (61,62). Similar to asthma, in patients with seasonal allergic rhinitis, an increased numbers of effector cells, i.e. mainly mast cells and eosinophils, have been found in the nasal mucosa during pollen season (61). As compared with placebo, treatment with intranasal corticosteroids reduced the influx of these inflammatory cells together with improvement in symptoms. Likewise, in another study, treatment with intranasal corticosteroids decreased eosinophilic cationic protein (ECP), a marker of eosinophilic degranulation, in patients with allergic rhinitis (62). This decrease was correlated with the decrease in symptom scores. Measuring eosinophils or ECP requires semi-invasive sampling techniques, like nasal lavage or nasal brushings. Although valuable, less-invasive sampling techniques are preferable. Similar to the lower airways, NO can be measured in exhaled nasal air in a completely non-invasive manner. So far, it has been found that nasal NO (nNO) is increased in patients with AR compared to healthy volunteers and is decreased following anti-inflammatory therapy (63). However, long term reproducibility of nNO, its relationship to clinical symptoms and applicability in disease monitoring need to be further elucidated.

In summary, there are several non- and semi-invasive sampling techniques of the upper airways; many resembling the techniques used to sample the lower airways. However, unlike in the lower airways, many of the upper airways sampling techniques still await validation. These techniques and biomarkers have been investigated in chapters 7, 8 and 9.

Aim and outlines of the present studies

Aim

The general aim of this thesis was to evaluate the feasibility and applicability of several non-invasive sampling methods and quantification techniques for the assessment of inflammatory biomarkers in allergic asthma and allergic rhinitis.

Outlines

INTRODUCTION

In the introduction (chapter 1), the rationale for the thesis has been provided in view of the current need of novel biomarkers for novel, targeted drug entities and to guide disease control. In addition, immunological and pathophysiological characteristics of allergic asthma and allergic rhinitis are discussed. The inflammatory responses within allergic airways following allergen challenge are outlined, since this exacerbation model provides potential targets for (monitoring the response to) drug treatment. In addition, the advantages of incorporating biomarkers in early drug development and disease monitoring are clarified. Chapter 2 provides an extensive overview of non-invasive sampling techniques (established and experimental) of inflammatory biomarkers and quantification techniques that can be used in early clinical development of novel drugs for asthma and potentially in disease monitoring.

CLINICAL STUDIES – ALLERGIC ASTHMA

In the first clinical studies, we focus on allergic asthma to evaluate the feasibility and applicability of induced sputum, eNO and EBC as sampling methods in allergic asthma. Chapter 3 is a good example of a methodologically sound approach to drug development. First, a proof of concept was established using the tachykinin NK1/NK2 receptor antagonist against its agonist, the neurokinin A challenge, in patients with mild persistent asthma. Subsequently, the proof of mechanism of this drug was tested in an allergen exacerbation model in patients with similar asthma characteristics. Apart from the more traditional allergen-induced airway responses (EAR, LAR and AHR), eNO and sputum inflammatory markers were used to assess the efficacy of the drug. In Chapters 4 and 5 the validity and applicability of eNO as a biomarker of airway inflammation in asthma was extended. In Chapter 4, we investigated the effect of vigorous bronchodilation on eNO levels following

an allergen-induced LAR confirming evidence previously provided by Silkoff *et al* for the effect of airway diameter on eNO (64). In chapter 5 the reliability and applicability of a novel hand-held device for measurement of exhaled NO (Niox Mino®) in several subject populations was compared with a standardized stationary chemoluminescence analyzer. In Chapter 6, we assessed the reproducibility of several novel sputum and EBC inflammatory markers from asthmatic patients quantified by novel processing- and detection techniques. Before implementation into clinical trials, novel techniques (sampling, processing and detection) need to be tested for their validity. These validation steps have been summarized in a recent conference report on bioanalytical method validation (65). In our pilot study a first step is made towards validation of Luminex, Mesoscale and mRNA quantification in sputum and EBC samples of asthmatics.

CLINICAL STUDIES — ALLERGIC RHINITIS

In the second part of this thesis, the clinical studies focused on AR to evaluate the feasibility and applicability of nasal lavage, nasal brush and nNO as sampling methods in allergic rhinitis. In Chapter 7 the reproducibility of common markers of allergy, including serum IgE levels and skin prick test, was tested in combination with inflammatory markers in nasal lavage and nasal brush. Subsequently, the effect of a nasal allergen challenge versus the allergen's diluent (=placebo) was tested on the kinetics of these biomarkers. In Chapter 8, the applicability of nasal NO measurements was evaluated for the monitoring of allergic upper airway inflammation following a nasal allergen challenge in subjects with allergic rhinitis.

CLINICAL STUDIES — ALLERGIC ASTHMA & ALLERGIC RHINITIS

Finally, Chapter 9 extends the biomarker search to Secretory Leukocyte Protease Inhibitor (SLPI). The function of SLPI is to protect tissues through its anti-protease properties and it may serve as a possible biomarker of chymase activity in allergic upper and lower airway disease.

DISCUSSION

Chapter 10 covers the discussion and conclusion sections and includes a critical evaluation of our data in relation to current literature offering a guideline for the selection of a relevant biomarker. In addition, speculations are made for future directions for biomarker research in asthma and allergic rhinitis.

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A critical appraisal of methods used in early clinical development of novel drugs for the treatment of asthma

J.D. Boot [1], P. Panzner [2], Z. Diamant [1]

1   Centre for Human Drug Research, Leiden, The Netherlands
2   Charles University Prague, Medical Faculty, Pilsen, Czech Republic

Introduction

Asthma is a chronic disorder of the airways characterized by at least partly reversible airways obstruction, airway inflammation, hyperresponsiveness and remodeling. Furthermore, there is a systemic link to atopy, predisposing for IgE-related comorbidities, such as atopic dermatitis, allergic rhinitis/rhinosinusitis and blood eosinophilia (1;2). Presently, asthma and related disorders are considered complex and heterogeneous diseases. This has resulted in a paradigm-shift from a general to an individualized, and from a local to a systemic therapeutic approach for this 'systemic airways disease'.

Measurement of airway inflammation being the hallmark of asthma is crucial to assess the disease's activity and severity. Although bronchial mucosal biopsies are still the gold standard (3), there are many disadvantages to this invasive and costly procedure. Therefore, an increasing number of non-invasive sampling methods have been developed closely approaching or complementary to the gold standard (Figure 1) (4). Some of these methods have been validated and even included into clinical evaluation and therapy monitoring, whereas others are still in a more explorative phase.

Airway hyperresponsiveness (AHR) has been shown to correlate with the degree of airway inflammation and remodeling and can be quantified by a direct bronchoprovocation test (5, 6). Such tests have been standardized and validated for the diagnosis and evaluation of asthma (7). In addition, some indirect bronchoprovocation tests (BPTs), mimicking specific features of asthma, serve as disease models in early drug development trials.

With this review we aim to evaluate standardized and upcoming non-invasive and semi-invasive sampling methods and (in)direct BPTs providing practical recommendations on their applicability for clinical monitoring or early clinical trials. This review provides an extension on the recommendations for clinical intervention trials in asthma (8).

```
┌──────────┐
│ eNO      │
│ EBC      │        Airway                    Airway
│ Sputum   │────    inflammation  ◄──────►   remodeling   ──
│ BAL      │                                                 
│ Biopsy   │                          │
│ AMP BPT  │                          ▼
└──────────┘                       Airway
                               hyperresponsiveness

                                  Asthma symptoms
```
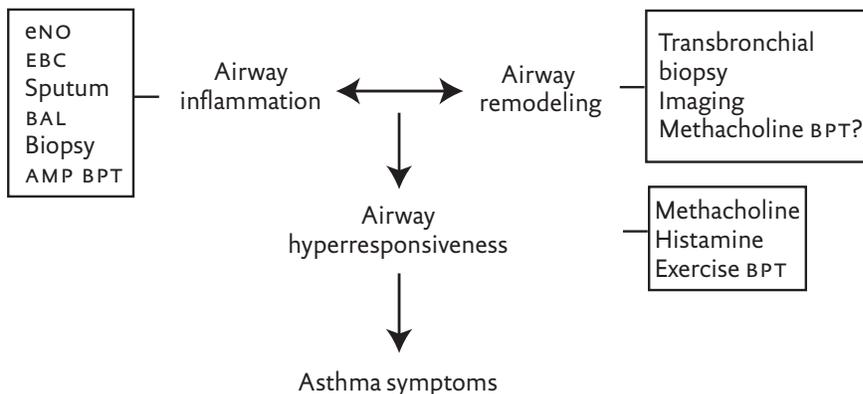
FIGURE 1   Key features of asthma and possible sampling methods. eNO = exhaled NO, EBC = exhaled breath condensate, BAL = bronchoalveolar lavage, AMP = adenosine 5' monophosphate, BPT = bronchoprovocation test.

Sputum

Sputum induction and processing technique

Sputum induction has been explored since the 1990s and in 2002 the European Respiratory Society (ERS) issued guidelines to harmonize the different techniques used worldwide (9). The recommended induction technique can be summarized as follows: prior to sputum induction, 200-400 µg salbutamol is administered as a safety measure, followed by spirometry to assess baseline Forced Expiratory Volume in one second (FEV_1). Subsequently, the entire procedure is carefully explained to the subject prior to commencing. The procedure has to be performed in a quiet, secluded environment to obtain the subject's full cooperation. The hypertonic saline (NaCl 4.5%) aerosols are generated by an ultrasonic nebuliser with an average output of 1 ml/min hence producing a dose of about 5-7 ml per inhalation. During the procedure, subjects perform three to four inhalations by tidal breathing through a mouthpiece for 5-7 minutes each. According to guidelines, the same inhalation time should be maintained throughout the procedure with a total duration of 15-20 minutes. After each induction, subjects are instructed to blow their nose, rinse their mouth and take a sip of water to minimize nasal and saliva contamination before expectoration of sputum.

To further minimize saliva contamination, subjects are either requested to spit saliva into another cup or sputum and saliva can be separated directly after collection but before processing. Both methods yield satisfactory results but are not interchangeable (10;11). During the procedure, all expectorations can be pooled in a pre-weighed plastic cup. After every inhalation, spirometry should be performed as a safety procedure. If the FEV$_1$ decreases by more than 20% from baseline, further induction should be discontinued and salbutamol administered. After the entire collection procedure, the obtained secretions should be processed within 2 hours according to standardized guidelines by a laboratory technician (11;12).

Biomarkers in sputum

CELLULAR PHASE

When performed according to ERS guidelines, sputum cell counts can be performed in a reproducible and validated manner (13, 14). This applies especially for eosinophil and neutrophil counts (15). Eosinophils (and neutrophils in severe persistent asthma) are considered as key effector cells of the asthmatic airway inflammation (16, 17). Increased eosinophil counts have been demonstrated in sputum samples both in validated models of asthma, such as allergen-induced late response, and in the actual disease (18). As compared with non-asthmatic volunteers, Louis *et al* showed increased sputum eosinophils and neutrophils in asthmatics (19). Moreover, the percentage inflammatory cells appeared to be related to disease severity, with further increase during exacerbations (20, 21). Conversely, anti-inflammatory interventions reduced sputum eosinophils both following allergen challenge and in 'wild type' asthma (22-27). In most studies, the reduction in sputum eosinophils was accompanied by an improvement in symptoms scores and lung function parameters. Green *et al* achieved superior asthma control applying a treatment regimen aimed at reducing sputum eosinophils rather than aiming at improving symptom scores and lung function parameters (28). In general, sputum eosinophil count is a validated biomarker to sample airway inflammation that can be employed both in clinical setting and in early drug development.

FLUID PHASE

Presently, numerous inflammatory mediators can be measured in the fluid phase of sputum ('supernatant'), however the validity and reproducibility of several techniques has not yet been determined. Apart from the induction

technique, there are at least two other reasons that can account for this. First, processing of sputum may affect mediator measurements. Dithiothreitol (DTT) is added to the sputum sample, in most processing protocols, to free mediators by dispersing the mucus layer through cleavage of the disulphide bonds (11). However, DTT may also affect the disulphide bonds in the mediators (29). Second, varying dilutions may account for inaccurate measurements among the samples and presently there is not yet a validated dilution factor to correct for this (12). ERS guidelines recommend immunoassays as the method of choice to quantify mediators in sputum due to their reproducibility, specificity and improving sensitivity (12).

GRANULOCYTE PROTEINS

Eosinophil cationic protein (ECP) and myeloperoxidase (MPO) are granulocyte proteins that can serve as activation markers of eosinophils and neutrophils, respectively. In sputum of asthmatics, (increased levels of) ECP have been found to be well-correlated with the eosinophil cell counts (30). In addition, anti-inflammatory treatment decreases both the eosinophils and ECP within the airways (25, 31). While measurements of ECP in sputum have been shown to be reproducible, MPO concentrations appear to be affected by sputum induction and/or processing technique and therefore immunoassays are not always reproducible (29, 32, 33).

LEAKAGE MARKERS

Microvascular leakage is another aspect of airway inflammation that can be assessed by measuring the concentrations of leakage markers in the sputum of asthmatic patients. Based on several studies applying different induction and processing techniques, albumin and fibrinogen have been shown to be reproducible leakage markers correlating with the degree of airway inflammation. Pizzichini *et al* demonstrated increased levels of albumin and fibrinogen in sputum of asthmatics as compared with healthy controls (14). Two other studies found that increases in albumin and fibrinogen corresponded with asthma severity (20, 33). Apart from albumin and fibrinogen, another potential leakage marker has been studied in asthmatic subjects is $\alpha 2$-macroglobulin. Applying BPTS with pro-inflammatory tachykinins in patients with asthma, van Rensen *et al* showed in patients with asthma that $\alpha 2$-macroglobulin (and albumin) appeared the best leakage markers (34).

CYTOKINES AND CHEMOKINES

These biomarkers are degraded by DTT. An extensive overview on cytokine and chemokine recovery from sputum is reported in the ERS guidelines (13).

Several research groups have investigated modified sputum processing techniques to optimize recovery of these biomarkers with overall good results (35-37). However, these processing techniques are not fully validated and most of them prevented recovery of other mediators from the samples. As an exception, IL-8, potent neutrophil chemoattractant, seems less affected by DTT and can be quantified by a validated immunoassay (29, 38). In several studies, increased reproducible levels of IL-8 have been demonstrated during asthma exacerbations and in patients with severe persistent asthma (38, 39). Hence, IL-8 is a validated marker for the assessment of drug efficacy in more severe asthma and for monitoring of asthma exacerbations.

EICOSANOIDS

Leukotrienes (LTs) and isoprostanes are derivates of arachidonic acid. Both groups of inflammatory mediators are involved in the pathophysiology of asthma (40, 41). Cysteinyl leukotrienes (Cys-LTs) are mainly released from activated mast cells and eosinophils. As compared to healthy controls, increased levels of these bronchoactive mediators have been measured in several body fluids of asthmatic subjects, including sputum (42, 43). Moreover, the concentration of Cys-LTs was found to correlate with disease-severity and was not affected by corticosteroids (57).

F_2-isoprostanes are considered specific markers of oxidative stress (44). These mediators are involved in the pathophysiology of inflammatory diseases, including asthma and COPD. 8-Isoprostane is the most extensively studied isoprostane, and reproducible levels have been measured in sputum and exhaled breath condensate (EBC) of both healthy and asthmatic subjects, with higher levels in those with more severe disease (45). In agreement with these data, increased levels of this eicosanoid have been reported during asthma exacerbations (45). Similar to Cys-LTs, 8-isoprostane is relatively inert to treatment with corticosteroids (46). Hence, both Cys-LTs and 8-isoprostane are useful markers of airway inflammation and oxidative stress in disorders including asthma.

PROTEASES

Matrix metalloproteinases (MMPs) are members of a large family consisting of calcium and zinc dependent enzymes. Several MMPs are involved in the process of extracellular matrix degradation and are considered key players in airway remodeling occurring in e.g. asthma, COPD and lung fibrosis (47). In the process of remodeling, there is a critical balance between MMP-9 and its counterpart, tissue inhibitor of metalloproteinases (TIMP). In asthma, increased levels of MMP-9 have been found in sputum, BAL and bronchial

biopsies (48-52). In addition, several investigators reported an imbalance between MMP-9 and TIMP, resulting in a disease-severity dependent increase of the MMP-9/TIMP ratio (48, 49, 53). In agreement with these data, allergen challenge has been shown to induce further increase in MMP-9, without increasing TIMP levels both in sputum and bronchoalveolar lavage (BAL) of asthmatic subjects (48, 49, 52). This MMP-9/TIMP imbalance may at least partly account for the allergen-induced airway remodeling in asthma. In conclusion, MMP-9/TIMP ratio in sputum is a potential marker for monitoring effects of interventions directed against airway remodeling.

Limitations of induced sputum

Only trained personnel should perform sputum inductions and careful patient instruction is needed prior to the procedure to ensure safety of the subject. Processing and analysis of the sputum samples are time-consuming and expensive procedures which require a well-equipped laboratory including a technician and an experienced cytopathologist. Furthermore, the results are not immediately available.

Not all patients are able to expectorate sputum and not all sputum samples are suitable for analysis. On average, sputum induction is successful in only 80-90% of adult patients and approximately 30% of asthmatic patients have normal sputum eosinophil counts (19, 28, 33, 54-56). In children (6 years and older) the percentage of successful inductions is significantly lower, around 80% (57, 58). In addition, the procedure should not be performed in patients with unstable or severe persistent asthma and those with moderate to severe disease should be carefully monitored during the induction process for the occurrence of sudden severe bronchoconstriction (59, 60).

Since sputum induction may affect the composition of the inflammatory cells and mediators within the airways, serial sputum inductions cannot be readily performed within a short time-interval, which may be a disadvantage in a clinical trial setting (61, 62). According to recommendations, a wash-out interval of at least 2 days should be allowed between two serial inductions to prevent potential carry-over effects (9, 63). Another disadvantage of the procedure is that most patients find the procedure strenuous and sometimes even embarrassing.

Summary and recommendations - sputum

Sputum is defined as secretion originating from the lower airways. Sputum induction by inhalations of hypertonic saline promoting expectoration is a validated method both for research and diagnosis. The obtained sputum

samples can be divided into a 'solid' phase consisting of cells, and a fluid phase containing soluble mediators. Both components can be quantified to assess the presence and activity of inflammatory markers. Sputum induction can be regarded as a semi-invasive procedure and is safer, cheaper and generally easier to perform than bronchial biopsy or BAL but more troublesome than exhaled nitric oxide (eNO) or EBC. Over the last fifteen years, a large amount of research has contributed to validation and standardization of the technique. A recent ERS Task Force document has been issued relating on recommendation and guidelines for standardized induction, collection, processing and analysis of sputum (13). Although airway sputum, BAL and bronchial mucosal biopsy provide samples from different lower airway compartments (64), a reasonable relationship has been found between these techniques providing similar information on the inflammatory airway components (54, 65). In addition, the recommended sputum induction protocol can be modified to enable differentiation between inflammatory cells from central and distal airways (66). In conclusion, induced sputum is a validated tool suitable for monitoring lower airway inflammation both in patient care and in early drug development. The pros and cons of sputum induction are summarized in Table 1.

Nitric Oxide (NO)

NO measurement technique

In 2005, the American Thoracic Society (ATS) issued updated recommendations for the measurements of NO from the upper and lower respiratory tract (67). Although various methods have been reported, the online measurement during a single-breath exhalation against a fixed resistance is currently the recommended sampling technique. This highly reproducible method has been standardized and is now widely used (68, 69). This technique can be summarized as follows: subjects are seated in front of a PC-screen while wearing a nose-clip, and instructed to blow into the NO-analyser with a constant flow rate (50 mL/s) for approximately 20 seconds. Blowing against a resistance ensures soft palate closure and prevents contamination with NO from the upper respiratory tract. A constant flow rate is important for a representative measurements, since NO is markedly flow dependent (70). After several seconds of expiration, an NO plateau is reached and the NO level is measured online by a chemoluminescence analyser. NO is expressed in parts per billion (ppb) and measurements are repeated until three reproducible values are obtained within 10%. Repeated measures do not affect the results.

TABLE 1 OVERVIEW OF THE MOST RELEVANT ADVANTAGES AND DISADVANTAGES OF THE DISCUSSED TECHNIQUES.

	SPUTUM INDUCTION	EXHALED NO	EXHALED BREATH CONDENSATE	BRONCHOPROVO-CATION TESTS
Pro's	-Multiple biomarkers -Reproducible cell differentials on cyto-spins -Valid tool for assessment of anti-inflammatory therapy	-Non-invasive -Reproducible -Inexpensive measurements -Direct results -Allows serial measurements -Tool for assessment of anti-inflammatory therapy	-Non-invasive -Multiple biomarkers -Allows serial measurements -Potential tool for assessment of anti-inflammatory therapy	-Reproducible -Direct results -Valid tools/models for assessment of anti-inflammatory therapy
Contra's	-Semi-invasive -Expensive & time-consuming procedure & analysis -Representative samples available in approx. 80-90% of subjects -Soluble markers subject to dilution -Non-repeatable over short time-period (<48 h) -Experienced personnel needed (MD/cytopatho-logist/lab) -Rescue medication needed -Contraindicated in severe persistent asthma/COPD/active cardiovascular disorders	-Expensive equipment -Many perturbing factors -Longitudinal samplings within 1 patient are more informative than single measurements	-Assays not fully reproducible -Expensive & time-consuming proce-dure/assays -Soluble markers subject to dilution -Specialized lab needed	-Semi-invasive -Experienced personnel (MD/technician) needed -Non-repeatable over short time-period (>6h-3wk) -Rescue medication needed -Contraindicated in severe persis-tent asthma/COPD/active cardiovascular disorders
Overall assessment	-Validated tool for monitoring of anti-inflammatory drug-effects - Lengthy, expensive procedure - Not suitable for patients with severe bronchoconstric-tion/comorbidities	-Validated tool for monitoring of anti-inflammatory drug-effects - Patient & researcher-friendly method	- Procedure awaits further validation - Patient & researcher-friendly method	-Validated tools for monitoring of AHR and anti-inflammatory drug-effects -Some BPTs are standardized models of asthma - Not suitable for patients with severe bronchoconstric-tion/comorbidities
Refs*	(9, 11, 12, 14, 60, 208)	(4, 67, 108, 209)	(110, 112, 113, 148, 210)	(7, 153, 154)

* Position papers and reviews/AHR: airway hyperresponsiveness/BPTS: bronchoprovocation tests

Although ambient NO appears to have no effect on eNO, most analyzers are equipped with a scrubber to ensure subjects inhale NO-free air prior to the exhalation manoeuvre. The results can be viewed online, which allows incorrect manoeuvres and values to be discarded. Correct operation of the equipment is relatively easy and does not require extensive training. In addition, performing (serial) measurements does not impose a great burden on patients and can be easily explained, which is ideal for young children (applicable from 4-5 years).

NO as a biomarker of airway inflammation

Exhaled NO is a sensitive marker of acute airway inflammation. In asthma, acute airway inflammation implies either loss of control or exacerbation. In clinical trials, this can be induced either by allergen challenge or by tapering off anti-inflammatory therapy (mainly corticosteroids). Allergen challenge, especially the late asthmatic response (LAR), is a well-known inducer of airway inflammation (71). Kharitonov *et al* reported a clear correlation between the size of the LAR and allergen-induced increase in eNO 10 hours post-allergen (72). Similarly, several tapering studies have shown that loss of asthma control is associated with an increase in eNO (21, 58, 73). In addition, these studies demonstrated that the change in eNO is a better predictor for loss of asthma control than baseline eNO per se. However, Leuppi *et al* found no increase in eNO during asthma exacerbations as a result of tapering off inhaled corticosteroids (ICS) (74). This aberrant observation may be due to measuring eNO offline in contrast with online measurements used in other studies.

ExhaledNO is very responsive to anti-inflammatory therapy. ICS have been shown to produce a dose-dependent reduction in eNO, preceding the decline of other disease-related parameters (75, 76). Other anti-inflammatory therapies for asthma, including leukotriene receptor antagonists (LTRA) and anti-IgE, have also been shown to reduce eNO both in children and adults (77, 78). Several studies report a correlation between eNO and other markers of airway inflammation and responsiveness in asthma which adds to its applicability as a valid biomarker for clinical monitoring and early drug development. Jatakanon *et al* showed significant correlations between eNO, sputum eosinophils and PC_{20} methacholine in steroid naïve patients with mild persistent asthma (79). These data have been confirmed and extended by Dupont *et al* who found a correlation between eNO and PC_{20} histamine in patients with similar asthma characteristics (80). However in asthmatics using ICS, the correlation between the different markers of airway inflammation and responsiveness is lost (81, 82). This is due to a fast decrease of eNO attaining

a maximal response even on low dose ICS therapy, resulting in almost normal eNO levels, while airway inflammation and hyperresponsiveness are still present. Therefore, eNO should probably not be used as the sole marker of airway inflammation in asthmatics using corticosteroids.

TABLE 2   REFERENCE ENO VALUES MEASURED IN CLINICAL STUDIES

SUBJECTS	N* (NUMBER OF SUBJECTS)	ENO MEAN** (PPB)	INTERQUAR- TILE RANGE (PPB)	REFERENCE
Atopic asthmatic adults***	44	58.5	30.4 – 85.5	(68, 211)
Non atopic asthmatic adults	30	18.9	14.6 - 33.4	(211, 212)
Atopic asthmatic children***	118	25.7	11.4 – 56.2	(68, 105, 106)
Atopic non asthmatic adults	67	27.6	11.3 – 49.3	(211, 212)
Non atopic non asthmatic adults	158	15.9	11.5-21.7	(68, 211, 212)
Non atopic non asthmatic children	332	8.8	N.A.	(213)
Cystic fibrosis	83	5.3	3.3 – 8.5	(214, 215)

Effect of corticosteroid intervention on eNO levels in patients with asthma

SUBJECTS		ENO BEFORE	ENO AFTER	% CHANGE	REFERENCE
Corticosteroid treatment > stable asthma	52	75.2	34.0	-54.8%	(75, 216, 217)
Tapering off corticosteroids > unstable asthma	67	9.8	21.8	+122.4%	(21, 73)

All measurements were performed with an online chemoluminescence analyzer.
N.A. = not applicable, eNO = exhaled NO. *Total number of subjects from all studies combined, **Median was used as mean when necessary, ***Patients with mild to moderate persistent asthma without maintenance therapy.

Limitations of eNO

The equipment used for online measurement of eNO is bulky and expensive. Recently, a handheld and less costly NO meter, NIOX MINO®, has been introduced, yielding reproducible NO measurements similar to the stationary chemoluminescence NO-analyzer (83). The availability of smaller and cheaper devices will enable future NO measurements to be applied at larger scale, including non-academic hospitals and extramural care.
Exhaled NO appears to be dependent on airway calibre. Recent studies have shown decreased eNO on bronchoconstriction, e.g. following allergen

challenge, with an increase after bronchodilatation (84-87). As asthmatic patients often have unperceived bronchoconstriction, eNO values may be underestimated. Applying maximal bronchodilation with short-acting β2-agonists before eNO measurements, may be a good option to correct for this perturbing factor (84).

Unlike EBC and sputum, eNO depicts only one single component of the inflammatory response within the airways, while its origin is difficult to assess. Recent studies have proposed a two-compartment model to discriminate between alveolar (representing the small airways) and conducting airways contribution (88, 89). Using this model, Lehtimaki *et al* demonstrated that increased eNO is mainly derived from the larger airways, as in both steroid-naïve patients with mild-to-moderate persistent asthma and nonasthmatic controls comparable alveolar NO levels were measured. Moreover, treating these patients with ICS established a decrease in airway NO levels but failed to reduce alveolar NO (90, 91). Similar findings have been reported in patients with mild-to-moderate persistent asthma and nonasthmatic controls, whereas in those with severe disease, alveolar NO was increased despite ICS and reduced by oral prednisolon (92). In this study alveolar NO appeared to be well correlated with eosinophils in BAL. Hence, these findings suggest that in severe persistent asthma alveolar NO is a potential marker for distal airway inflammation that cannot be easily reached by ICS.

Many factors appear to affect eNO levels including atopy status, airway caliber, medication, nitrate rich food, smoking and airway infections (67, 93-96). Both in clinical practice and in clinical trials, these perturbing factors should be anticipated or corrected for to enable comparison of representative eNO values within one subject, among subjects and across studies.

Summary and recommendations - NO

Nasal NO is produced in the upper respiratory compartment, while eNO mainly comes from the lower airways following synthesis from L-arginine by constitutive (cNOS) and inducible NO (iNOS) synthases (97). cNOS is expressed in neuronal, endothelial and epithelial cells under baseline conditions, whereas iNOS is mainly expressed in inflammatory, epithelial and airway smooth muscle cells and can be further upregulated by inflammatory cytokines (98). Exhaled NO can be measured in exhaled breath of several species, including humans (99, 100). Increased iNOS expression has been demonstrated in bronchial biopsies of asthmatic patients as compared with healthy controls (101). In agreement with these data, increased NO levels occur in exhaled air from patients with asthma with further increase during

exacerbations (72,102). In addition, iNOS inhibitors, such as alcohol and corticosteroids, have been shown to reduce the expression of iNOS and consequently reduce eNO levels in asthmatic subjects, but not in (non) atopic, non-asthmatic controls (103,104). Furthermore, eNO concentrations appeared to correlate with IgE levels and the number of positive skin prick test wheals (105). Similarly, atopic asthmatics have been shown to produce higher levels of eNO than non-atopic asthmatics and higher eNO levels have been reported in atopic subjects as compared with non-atopic controls (Table 2) (93,106,107).

Taken together, upregulated iNOS expression accounts for high eNO concentrations in asthma and eNO levels have been shown to relate to atopy status and disease severity. Although increased eNO is not a prerequisite for asthma only, measuring eNO is an established tool for the assessment of airway inflammation and a validated marker in interventional trials with anti-inflammatory asthma therapies. The pros and cons of eNO measurements are summarized in Table 1.

Exhaled Breath Condensate

Collection of EBC

Several collectors and condensers are currently available (109-111). All devices are easy to use and both young and old subjects can easily perform the procedure (Figure 2). In most protocols, subjects have their nose clipped while breathing through a mouthpiece into a non-rebreathing valve connected to a tube of variable length for approximately 15-30 minutes (112). During the procedure, the exhaled breath travels through the tube that serves as a cooling chamber and the thus formed condensate is collected (usually around 2 mL/sample) in a cooled collection chamber. Cooling of the samples is advised to preserve "thermo-labile" markers (112). Subsequently, samples can be directly analysed or frozen pending analysis. The non-rebreathing valve ensures separation of in- and expiratory air and prevents rebreathing of exhaled samples. Exhalation flow affects the composition of EBC. Therefore, flow variations should be limited during each procedure. The long duration of sample collection makes fixed exhalation flow rate - commonly applied in eNO measurements - impossible. Hence, following subject's acclimatization, tidal breathing over a predefined time-interval is probably the best way to minimize influence of exhalation flow rate on the EBC composition. Using a fixed exhaled volume may be a good alternative.

ICE

Non rebreathing valve

Subject

Cooled tube

Collection chamber
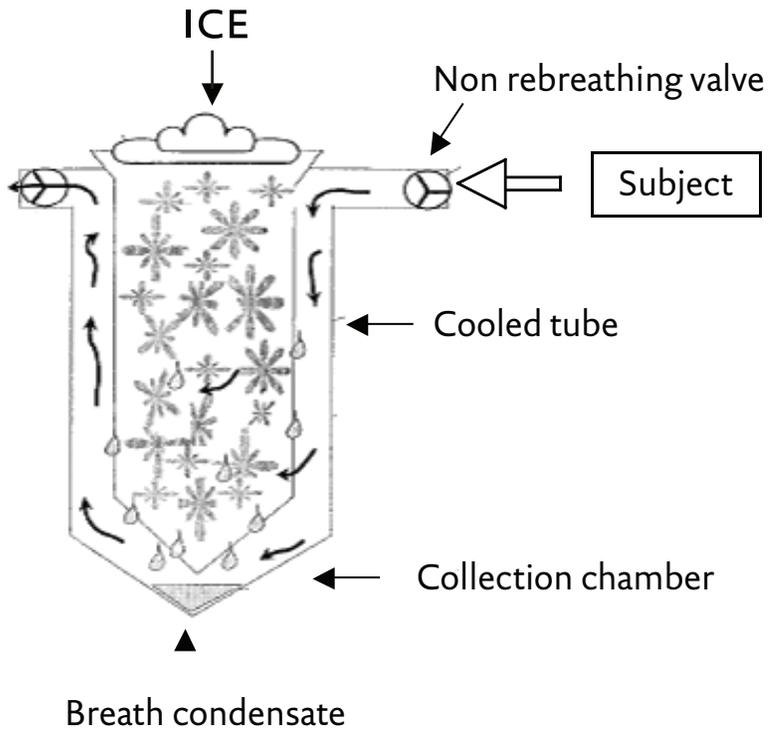
Breath condensate

FIGURE 2   Schematic diagram of an exhaled breath condensate collecting device (reproduced and modified with permission from (207)

Biomarkers in EBC

HYDROGEN PEROXIDE

Hydrogen peroxide (H_2O_2) is formed when O_2- released by activated inflammatory cells such as neutrophils, macrophages and eosinophils, undergoes spontaneous or enzymatic dismutation. Hence, H_2O_2 is a potential marker of oxidative stress common in many inflammatory conditions (113). Indeed, reproducible increased concentrations of H_2O_2 have been measured in active smokers and patients with more severe asthma and an inverse correlation between FEV_1 and H_2O_2 has been found (114-117). In these patients anti-inflammatory drugs, such as ICS, have been shown to reduce exhaled H_2O_2 accompanied by improvements in FEV_1 (114, 118).

The stability after collection is an important advantage of measuring H_2O_2 (119, 120). However, in several healthy subjects and in some asthmatics with

mild disease H_2O_2 concentrations remained below the detection limit (116, 120). Therefore, more sensitive assays are required to study airway inflammation in these patients.

EICOSANOIDS

Leukotrienes and isoprostanes are involved in the pathophysiology of inflammatory disorders including asthma and COPD. These eicosanoids can be measured in EBC by an enzyme immunoassay (EIA) or by gas chromatography/mass spectrometry (121). As compared with non-asthmatic controls, increased levels of cys-LTs have been detected in asthmatic subjects. Similar with measurements in sputum, cys-LTs levels in EBC appeared to be correlated with disease severity and could be reduced by anti-inflammatory drugs (122, 123). Leukotriene B4 (LTB_4) is released by activated neutrophils; increased levels have been found in patients with severe persistent asthma and COPD (122,124, 125). In these patients, sputum LTB_4 levels have been shown to correlate with those in EBC.

Conform with sputum, in EBC 8-isoprostane is the most extensively studied prostanoid for its stability and good detectability in both healthy state and disease (126). In asthma, 8-isoprostane EBC concentrations have been found to be correlated with levels of eNO (127). However, 8-isoprostane levels appeared to correlate with asthma severity while eNO does not. Unlike eNO, 8-isoprostane is not completely suppressed by corticosteroid treatment. Hence, 8-isoprostane is a potential indicator for ongoing airway inflammation despite anti-inflammatory treatment and hence may be a useful marker for asthma control (123, 127, 128). Moreover, recent data suggest a link between 8-isoprostane concentration in EBC and small airways inflammation (129).

NO DERIVED PRODUCTS

NO within the airways can react with O_2 yielding NO-derived products, NO_2^- and NO_3^-. Unsurprisingly, NO_2^- and NO_3^- levels are increased in EBC of asthmatics and corticosteroids have been shown to reduce these inflammatory markers (130, 131).

Nitrotyrosine is another stable end-product of NO and O_2^-, detectable in EBC of healthy subjects and three-fold increased in steroid-naïve patients with mild persistent asthma (124). In correspondence with other NO-derivates, inhaled and oral corticosteroids reduced nitrotyrosine levels in asthma below those of healthy controls (124). Unsurprisingly, nitrotyrosine and NO appeared to be correlated (124).

Nitrosothiol a metabolite of NO and gluthatione is yet another NO-derivate. Elevated concentrations of this biomarker have been found in EBC of patients with severe persistent asthma, as compared with mild asthmatics and

healthy, non-asthmatic controls (132). Similar to other NO-derived products, nitrosothiol is a potential marker of asthma severity and control.

PH

Measurement of pH in EBC is relatively simple and inexpensive as compared with other biomarkers. Furthermore, several studies have reported that pH measurements are reproducible and not affected by temperature or duration of collection, airway obstruction, oral ammonia or storage (133, 134). Using different collection devices, a number of research groups have measured pH in EBC of healthy subjects yielding an average pH of around 7.8, whereas in asthma the average pH was found below 7.5 (133-137). Asthma exacerbations result in further decline of pH with reversal following corticosteroid treatment (137). This corresponds with other studies showing a higher pH in steroid-treated patients as compared with steroid-naive asthma controls. The low costs, good reproducibility in combination with the availability of reference values are major advantages of pH measurements over other inflammatory markers in EBC.

CYTOKINES AND CHEMOKINES

Several cytokines and chemokines including IL-4, TNF-α, macrophage-derived chemokine, and eotaxin have been detected in EBC of asthmatics (138, 139). The most striking EBC data so far have been reported in steroid-naïve asthmatic children by Shahid *et al* showing an increase in IL-4 combined with a decrease of IFN-Y (140). Following treatment with ICS, the IL-4 and IFN-Y levels returned within the ranges of healthy controls. These data clearly demonstrate the Th2-driven cytokine profile in human asthma and the modulating effects of anti-inflammatory therapy. However, in all these studies some cytokine and chemokine measurements could not be reproduced in all subjects and other investigators fail altogether in measuring cytokines in EBC (141). Hence, the methodology awaits validation and more sensitive assays are needed.

Limitations of EBC and potential remedies

EBC has several limitations. Until recently there has been no consensus on the collection and storage techniques which complicates correct comparison and interpretation of data across different studies. Commercially available condensers in combination with a recent Task Force publication providing guidelines for standardization of collection and storage procedures are expected to solve most of these issues in the future (112, 142-144). The ERS/ATS task force document provides a review of several protocols using differ-

ent condensers yielding reproducible data of specific measurements in EBC. Using the RTube® (Respiratory Research, Inc., Charlottesville, VA, USA), Vaughan *et al* showed reproducible pH measurements in EBC of asthmatics (134). The Cryocond® (Boehringer Ingelheim, Burlington, Canada) has been used in several studies yielding reproducible H_2O_2 measurements in EBC of subjects with asthma and allergic rhinitis (77, 145). Ecoscreen® (Jaeger GmbH, VIASYS Healthcare, Hoechberg, Germany) is still another commercially available condenser providing reproducible levels of sodium and chloride in EBC of healthy volunteers, children with asthma and cystic fibrosis (146).

An even greater concern is the poor availability of sensitive assays yielding reproducible measurements (116, 146). Ideally, biomarkers should present well within the concentration range of sensitive and reproducible assays. Moreover, there should be a well-defined concentration range between healthy state and disease. However, partly due to dilution, the concentration of the majority of the inflammatory markers in EBC is generally in the range of the lower detection limit of most assays. Tufvesson *et al* report two potential processing strategies to improve measurements of several inflammatory markers in EBC, i.e. sample concentration and coating of collectors to minimize absorption to plastic material (147). Both methods await standardization.

Another obstacle in interpreting EBC data is the dilution factor. The airway lining fluid captured in EBC is variably diluted during the collection process (148). Extrapolation to the original concentrations within the airways is only possible applying a dilution factor (149, 150). Presently there is no validated dilution factor.

Yet another inconsistency is the composition of an EBC sample including various components of different origin, coming both from different parts of the respiratory and from the digestive tract. Unfortunately, it is not possible to designate the relative contribution of these sites to the exhaled mediators. However, there are some devices and methods being applied to prevent excessive saliva contamination, such as a saliva trap or allowing the subject to rinse the oro-pharynx prior to collection and swallow accumulated saliva during collection. Furthermore, applying different flow-rates may enable discrimination between central and peripheral airways (151).

Summary and recommendations – EBC

EBC is a new sampling technique for various markers of inflammation in inflammatory airway diseases including asthma. EBC is a potential tool for monitoring anti-inflammatory effects of novel drugs. Being a simple, non-invasive

procedure requiring uncomplicated and inexpensive equipment are its main advantages over bronchial biopsies, BAL and induced sputum (134, 148). Exhaled breath consists of two phases: the gaseous phase, containing volatile substances such as NO and carbon dioxide (CO_2), and a liquid phase containing non-volatile components including water-soluble inflammatory markers (113). The non-volatile ions and proteins originate from the airway lining fluid. These entities are aerosolized due to local turbulence to become liquid constituents of EBC (143).

So far, there has been no standardization of EBC sample collection or analysis and this needs to be solved before results can be interpreted, compared and its clinical applicability can be assessed. These issues have recently been largely addressed by an ATS/ERS task force resulting in novel EBC guidelines (112). Overall, EBC seems a promising tool for both research and clinical monitoring. So far, several markers of airway inflammation have been detected in the EBC of asthmatics, including hydrogen peroxide, leukotrienes, isoprostanes, NO-derived products, pH, chemokines, cytokines and adenosine (113, 148,152). In addition, measurement of drug concentrations in EBC may allow studying the link between pharmacokinetic properties and pharmacodynamic effects in the future. The pros and cons of EBC measurements are summarized in Table 1.

Bronchoprovocation tests

Methodology of BPTs

The ATS and ERS have issued several guidelines for standardized BPTs or challenges with both direct and indirect challenges. These guidelines address methodology, recommended equipment and preparation of pharmacologic agents (7, 153, 154).

Methacholine is the most common direct bronchoconstrictor stimulus applied for both diagnostic and research purposes (Figure 3 and Table 3). Two challenge methods are being recommended in the guidelines: the 2-minutes tidal breathing method with 2-fold concentration increases and the 5-breath dosimeter method with 4-fold increases (7). In the 2-min tidal breathing method, up to 10 consecutive doubling concentrations of methacholine are placed in a jet-nebulizer and aerosolized (0.13 mL/min) with a constant-output compressor. Patients are instructed to perform tidal breathing for 2 min through the mouth with the nose clipped. Airway response is measured after each concentration step over the subsequent 3-min period by FEV_1 and expressed as % fall from baseline FEV_1. In the 5-breath dosimeter method,

patients slowly inhale 5 breaths from functional residual capacity to total lung capacity (i.e. a slow deep breath) through a dosimeter-driven nebulizer containing up to 5 fourfold concentration increases of methacholine. After each manoeuvre, patients are instructed to hold the breath for 3-5 seconds. Airway response is measured by FEV$_1$ before and 3 min after each concentration step. In both protocols, challenges are repeated at 5 min intervals until the FEV$_1$ falls by at least 20% from baseline FEV$_1$. The provocative concentration causing a 20% fall in FEV$_1$ (PC$_{20}$) is calculated by extrapolation and is indicative of the degree of AHR (range: no AHR, mild, moderate, severe AHR, respectively). After challenge, a short-acting β2-agonist is administered for immediate bronchodilation and FEV$_1$ is measured after 10 minutes to assure lung function returns within baseline values. BPTs usually last up to 60-70 minutes, depending on the degree of AHR and the protocol used.

Although both methods are reported to be reproducible in clinically stable subjects, there are important differences that may affect the outcome in some asthma patients (7, 153). Cockcroft and co-workers found that specifically in subjects in whom mild AHR was found using the 2-min tidal breathing method, no measurable AHR could be detected using the 5-breaths dosimeter method (155). This difference can be explained by the bronchodilator effect of the dosimeter method, which reduces the sensitivity of the test specifically in patients with mild AHR.

SMOOTH MUSCLE	BRONCHIAL VESSELS	NEUROGENIC PATHWAYS	INFLAMMATORY MEDIATORS

Methacholine
Histamine
Neurokinin A
Leukotriene D4
PAF
Substance P
Bradykinin
Methabisulphite
AMP
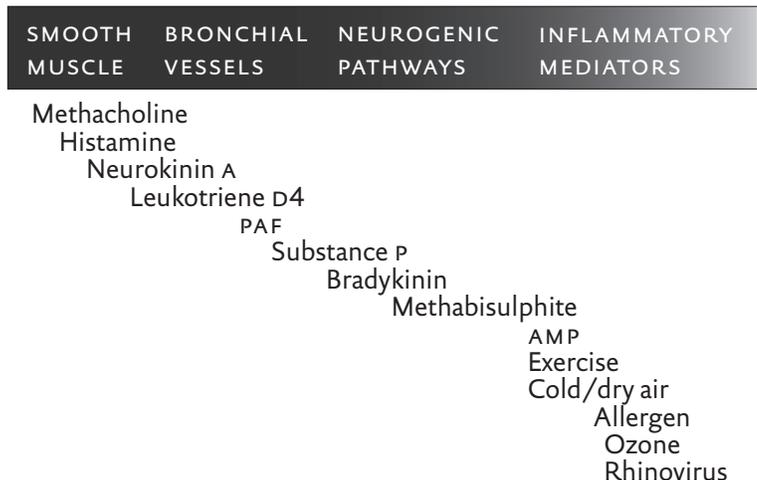Exercise
Cold/dry air
Allergen
Ozone
Rhinovirus

FIGURE 3   Overview of direct and indirect stimuli and their mechanism of action. PAF = platelet activating factor, AMP = adenosine 5' monophosphate. direct challenges

Indirect challenges comprise a heterogeneous group of pro-inflammatory stimuli. The methodologies slightly differ across the spectrum of the challenges. The majority of indirect challenges has been standardized and basically follows the same principle as a direct challenge. During these tests subjects are exposed to serial increasing concentrations of a pharmacological agent or increasing exposure to exercise resulting in a fall in FEV_1 as compared with baseline. Airway response measurements and safety precautions are similar to those in methacholine challenge and are described in the ERS guidelines and a recent ERS task force report (7, 154). The most commonly used indirect challenges have been standardized and validated (Table 3).

Bronchoprovocation tests and their clinical relevance

PC_{20} METHACHOLINE: MARKER OF AHR AND AIRWAY REMODELING VERSUS AIRWAY INFLAMMATION

Methacholine BPT is the preferential test to assess and quantify AHR by PC_{20}. According to previous evidence, the wash-out period between two consecutive methacholine challenges varies from zero up to 24 hours in asthma and up to 6 hours in nonasthmatic subjects (156, 157). Two landmark studies investigated the relationship between methacholine-induced AHR and airway inflammation and/or remodeling (5, 6). In patients with mild-to-moderate persistent asthma, Sont *et al* compared a treatment strategy aimed at improving AHR with the existing strategy aimed at improving symptoms and lung function (5). After 24 months, patients treated according to the AHR-strategy had a lower exacerbation rate corresponding with a reduced number of eosinophils in bronchial biopsies as compared with the reference strategy. Furthermore, subepithelial reticular basement membrane (rbm) thickness, a feature of airway remodeling, was significantly reduced in the AHR-group as compared with the reference group. Comparable observations were reported in patients with similar asthma characteristics by Ward *et al* after 12 months of treatment with high doses of ICS (6). Following longterm anti-inflammatory therapy, the changes in PC_{20} methacholine and rbm-thickness, being one of the characteristics of airway remodeling, appear to be interrelated (6).

In contrast with these data, the relationship between PC_{20} methacholine and markers of airway inflammation has not been unanimously demonstrated. Although several studies reported a good correlation between methacholine sensitivity and markers of airway inflammation (5, 28, 158, 159), other

studies did not find such relationship (160). These contradictive data may be the result of different patient characteristics, methodologies and/or treatment regimes and remain to be clarified.

INDIRECT CHALLENGES: INDUCERS OF AIRWAY INFLAMMATION AND MODELS OF ASTHMA

In contrast with the direct stimuli, there is a variety of indirect airway challenges interfering with different pathophysiological mechanisms within the airways. These indirect stimuli possess varying mechanisms of action with a gliding scale between direct to indirect as schematically depicted in figure 3. Depending on their (specific) mode of action, several indirect challenges can be applied for various purposes, including monitoring or research of pathophysiological mechanisms and proof of principle/concept studies in asthma. In the following paragraphs, the most important and validated indirect challenges will be addressed (7, 153, 154).

AMP CHALLENGE

Adenosine 5' monophosphate (AMP) releases inflammatory mediators from activated mast cells. Recent studies in asthma have shown that airway responsiveness to AMP - expressed as PC_{20} AMP - is a valid marker of airway inflammation (154). Furthermore, PC_{20} AMP has been shown to be more sensitive to ICS and high altitude than PC_{20} of other direct or indirect agents including methacholine or bradykinin (6, 154, 161, 162). In several intervention studies, there was a fast-onset, ICS-dose-dependent improvement in PC_{20} AMP which correlated with a significant reduction in inflammatory markers and improvement in asthma symptom scores (163-165). Thus, AMP is a sensitive marker of fluctuations in airway inflammation during a relatively short time interval. Whether this is relevant for monitoring chronic inflammation remains to be established.

EXERCISE CHALLENGE AND COLD, DRY AIR CHALLENGE

Exercise and the related cold, dry air challenge have been shown to induce bronchoconstriction in 70-80% of asthmatics (166). Although the underlying mechanisms are still incompletely understood, there is evidence of mast-cell triggered mediator release and neural reflexes contributing to the pathophysiology of these airway responses (153, 167). Indeed, intervention with antileukotrienes, antihistamines and neurokinin receptor antagonists have been shown to protect against exercise/cold dry air-induced bronchoconstriction in patients with asthma (168-171). Presently, these indirect challenges are applied as standardized models of mast-cell mediated mechanisms in asthma in proof of concept studies with new drugs or for diagnostic purposes (7, 154).

ALLERGEN CHALLENGE

Inhalation of serial increasing concentrations of a standardized allergen extract, the so-called allergen BPTs or allergen challenge, is the most useful preclinical model of asthma (7). This indirect challenge can be conducted in two ways: inhalation of aerosolized allergen or local instillation of allergen into a pulmonary segment via bronchoscope. The first method allows investigation of the relation between the allergen-induced airway and inflammatory responses, whereas the latter only allows to study the allergen-induced inflammatory response (7, 18, 172). The characteristics of the inhalational allergen challenge include early asthmatic response (EAR) occurring within 10 minutes after inhalation of an effective dose of a relevant allergen, and a LAR which is associated by allergen-induced AHR, occurring in approx. 50% of the subjects. The EAR is a mast-cell-triggered event, resulting in acute, transient airway smooth muscle (ASM) contraction (0-3 h), whereas the LAR is characterized by a chronic inflammatory response (over 24 h), in which eosinophils and their mediators prevail and induce the associated AHR (up to 3 weeks). Recent evidence has been provided that allergen challenge may induce features of airway remodeling in both animals and humans (173, 174). These characteristics render this model a suitable tool to study both the acute and chronic sequelae of asthma.

STEROID TAPERING

Steroid tapering is an exacerbation model in patients with moderate-to-severe persistent asthma in which gradual reduction of corticosteroid therapy results in a gradual deterioration of FEV_1 and PC_{20} accompanied by an increase in symptom scores and inflammatory markers (21, 175, 176). In clinical trials, steroid tapering is a validated tool to test the effect of potential anti-inflammatory therapy on asthma control. Recent intervention studies applying tapering off corticosteroids have been performed with leukotriene receptor antagonists and anti-IgE (177-180).

RHINOVIRUS AND OZONE CHALLENGES

Rhinovirus and ozone challenge tests are still other, more experimental, exacerbation models of asthma. Similar to allergens, virus infections are important causative agents of asthma exacerbations. Rhinoviruses have been detected in 10-44% cases of adults and 23-86% cases of children with asthma exacerbations (181, 182). Grünberg *et al* showed that experimental rhinovirus infections induce airway inflammation and associated AHR in asthmatics, closely resembling spontaneous asthma exacerbations (183, 184). The inflammatory influx caused by a rhinovirus mainly consists of eosinophils,

neutrophils and their respective mediators (183-186). In analogy with the airway inflammation in severe persistent asthma, the neutrophil component may – at least partly – explain why corticosteroids do not effectively protect against rhinovirus-induced airway inflammation (187, 188, 189). Experimental viral infections are exacerbation models of asthma suitable to study pathophysiological mechanisms and for early drug development (190). Ozone is another indirect, bronchoactive stimulus inducing acute neutrophil inflammation and oxidative stress into the airways of (non) asthmatic subjects (191-193). Consequently, ozone may be a useful tool to investigate new drugs with anti-oxidant properties (194, 195).

Limitations of bronchoprovocation tests and models

Overall, airway challenges with direct stimuli are valuable tools to assess AHR, while indirect challenges are useful to study the relationship between a specific stimulus and the airway inflammatory response. Due to different modes of action, care should be taken when selecting a challenge for a specific protocol.

If performed according to guidelines by adequately trained technicians, BPTs are safe and generally well-tolerated (7). The most common adverse reactions are transient, usually mild respiratory problems (dyspnea, chest tightness, cough) and sometimes headache. The most important health-related contraindications for challenges are active cardiovascular diseases and uncontrolled or severe persistent asthma with an FEV_1 less than 1.2 L (7).

In clinical trials, it should be taken into account that indirect airway challenges may affect markers of inflammation, which also applies for histamine that has been shown to induce an airway exudative response (196). When performed serially, an adequate washout period should be allowed to correct for these "carry-over effects". The length of a washout period is dependent on the pro-inflammatory response of the indirect stimulus and varies between at least 6 hours for histamine and as long as 6 weeks for a rhinovirus challenge (197, 198).

Summary and recommendations - Bronchoprovocation tests

AHR and chronic airway inflammation are the hallmarks of asthma (199). Several interventional trials have demonstrated that targeting either component resulted in better asthma control (5, 28). AHR is defined as an exaggerated bronchoconstrictor response to (non)specific stimuli such as cigarette smoke, exercise, viruses or allergens.

TABLE 3   OVERVIEW OF DIRECT AND INDIRECT CHALLENGES/ASTHMA MODELS

STIMULUS	MODE OF ACTION	CHARACTERISTICS	SPECIFIC DRAWBACKS	REFS
Metha-choline	Directly on ASM	Gold standard to quantify AHR by PC_{20} methacholine Potential marker of airway remodeling	Less related to inflammation Not specific for asthma	(7, 153, 155)
AMP	Release of mast cell-derived mediators	Sensitive marker of airway inflammation	No sharp cut-off between atopic asthmatics and atopic controls	(7, 154, 200)
Allergen	Acute release of mast cell-derived mediators (EAR), in ±50% of cases followed by a more chronic inflammatory response primarily mediated by activated eosinophils and their products (LAR)	Most suitable model with both acute and chronic features of asthma (EAR, LAR and AHR)	Strenuous and long-lasting procedure Extra safety needed Long washout period Contraindicated in severe persistent asthma/COPD/ active cardiovascular disorders	(7, 18, 172)
Exercise/ cold dry air	Release of mast cell-derived mediators	Diagnostic tool Suitable model to test efficacy of therapy directed against mast cell-derived mechanisms	Strenuous test, not suitable for elderly or handicapped	(7, 153, 154)
Virus/ Ozone	Activated eosinophils and neutrophils and their mediators	Experimental models to test therapy interfering with virus-induced mechanisms of asthma, including airway eosinophils/ neutrophils; unresponsive to ICS	Extra safety and laboratory procedures needed due to viral inoculation	(183, 194, 218)
Steroid tapering	Induction of exacerbation by withdrawal of anti-inflammatory treatment and subsequent flare up of airway inflammation	Validated model to test efficacy of potential anti-inflammatory therapy on asthma control	Stepwise dose reduction necessitates lengthy trials	(175, 176)

ASM = Airway Smooth Muscle, AHR = airway hyperresponsiveness, AMP = Adenosine 5' mono-phosphate, EAR = early asthmatic response, LAR = late asthmatic response, ICS = inhaled cortico-steroids

Bronchoactive stimuli can either directly or indirectly act on ASM cells and consequently induce bronchoconstriction. Methacholine and histamine are direct stimuli, inducing bronchoconstriction through direct interaction with specific receptors on ASM cells (7, 200). AMP, non-isotonic aerosols, exercise and allergen challenges are examples of indirect stimuli causing ASM contraction through the release of bronchoactive mediators from inflammatory cells and neurons (Figure 3). Challenges with methacholine, histamine and exercise can test the (severity of) AHR, while challenges with most indirect stimuli may serve as functional estimate of airway inflammation in a clinical or research setting. Furthermore, some of these tests have been standardized and have become diagnostic tools or validated models of asthma. For example, AMP challenge appeared a sensitive test to assess (the severity) of airway inflammation (162, 201), while challenges with exercise, allergen, ozone and rhinovirus can induce specific features of asthma, including a transient worsening of airway inflammation and AHR. These challenges are used as validated models for early drug development to test specific pharmacological activity. The pros and cons of BPTs have been summarized in Table 1 and 3.

Recommendations

Chronic airway inflammation is the hallmark of asthma and related disorders, resulting in various features including airway hyperresponsiveness and airway remodeling. Several studies have shown that symptoms and lung function parameters do not reflect the activity of the airway inflammation in asthma, and hence do not provide adequate information on asthma control (5, 28, 203, 204). Alternatively, inflammatory indices, such as sputum eosinophils and eNO, appeared to be good indicators of asthma severity and control (205). Therefore, sampling of airway inflammation is becoming an integral part of the diagnosis, and monitoring of asthma and a valuable tool to test the pharmacological efficacy of anti-inflammatory therapy in early clinical trials. Presently, there is an increasing array of non-invasive methods aimed at sampling the airway inflammation. Standardized and validated markers of airway inflammation include eNO and sputum eosinophils, while exhaled breath condensate is a promising method that still awaits refinement of technique and more sensitive assays. In addition, there are more or less standardized and validated bronchial provocation tests for quantification and or assessment of airway hyperresponsiveness (methacholine, histamine, exercise challenge), airway inflammation (adenosine monophosphate), or to mimic

specific pathways during a flare up of asthma – mostly applied in early clinical trials (exercise, allergen, rhinovirus, ozone). Although the methacholine BPTs has been shown to be correlated with some features of airway remodeling, there is still a paucity of non-invasive markers for this complicated asthma feature, except imaging (Figure1).

The most promising future biomarker for asthma remains to be identified and should be disease-specific, non-invasive, reproducible, inexpensive, fast and simple and well- responding to the pharmacological activity of anti-asthma therapy. The most recent developments point towards the potential application of so-called 'omics' techniques to blood, sputum and recently even to exhaled breath (206, 207). Such systems may drastically change the screening and monitoring of airway disease.

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NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

Effect of an NK1/NK2 receptor antagonist on airway responses and inflammation to allergen in asthma

J.D. Boot [1], S. de Haas [1], S. Tarasevych [1], C. Roy [3], L. Wang [4], D. Amin [4], J. Cohen [1],
P.J. Sterk [2], B. Miller [4], A. Paccaly [4] J. Burggraaf [1], A.F. Cohen [1], Z. Diamant [1].

1   Centre for Human Drug Research, Leiden, The Netherlands
2   Leiden University Medical Center, Leiden, The Netherlands
3   sanofi-aventis Pharma Recherche-Developpement, France
4   sanofi-aventis U.S. Inc., Bridgewater, NJ, USA

Abstract

Rationale: The tachykinins substance P and neurokinin A (NKA) are implicated in the pathophysiology of asthma.

Objective: We tested the safety, tolerability, pharmacological and biological efficacy of a tachykinin NK1/NK2 receptor antagonist, AVE5883, in asthmatics in 2 double-blind, placebo-controlled, cross-over studies.

Methods: The pharmacological efficacy of a single inhaled dose (4.8 mg) of AVE5883 was tested against inhaled NKA in 20 asthmatics. Subsequently, we studied the biological efficacy of the pharmacologically effective dose on inhaled allergen in a multiple dose trial (4.8 mg TID, 9 days) in 12 asthmatics with dual responses to inhaled house dust mite. On day 8, an allergen challenge was conducted and airway response was measured by FEV_1 until 9 h post-allergen. Exhaled NO, $PC_{20}FEV_1$(Methacholine) and induced sputum were performed on days 1, 7 and 9.

Results: AVE5883 had a bad taste and transient bronchospasm occurred in some subjects. A single inhaled dose shifted the dose-response to NKA by 1.2 doubling doses. Unexpectedly, pretreatment with multiple doses of AVE5883 enhanced the allergen-induced early and late airway responses. However, there were no significant differences in the allergen-induced changes in exhaled NO, $PC_{20}FEV_1$(Methacholine) and sputum cell differentials between placebo and AVE5883.

Conclusions: Despite its demonstrated pharmacological activity against inhaled NKA, multiple doses of AVE5883 increased the allergen-induced airway responses without affecting markers of airway hyperresponsiveness and airway inflammation. Our data question the prominent role of neurogenic inflammation in asthma and, consequently, the therapeutic potential of dual tachykinin antagonists.

Introduction

Asthma is a chronic inflammatory disease of the lower airways associated with various comorbidities and characterized by variable, often reversible, airflow obstruction (1). Pathophysiologically, airway hyperresponsiveness (AHR) to various bronchoconstrictor stimuli is the hallmark of asthma, which appears to be related to chronic airway inflammation (2). Hence, anti-inflammatory therapy with inhaled corticosteroids is the cornerstone of pharmacotherapy of persistent asthma (1). However, longterm use of (high doses of) inhaled corticosteroids may induce troublesome local and/or systemic side effects (3). Furthermore, despite a good, overall clinical efficacy, even high doses of inhaled corticosteroids do not fully suppress the airway inflammation in all asthmatic patients (4-6). Therefore, novel therapeutic options are being explored targeting various aspects of airway inflammation.

Within human airways, the tachykinins Substance P (SP) and Neurokinin A (NKA) are the predominant neuropeptides released from the nonadrenergic-noncholinergic (NANC) system by mechanical, thermal, chemical or inflammatory stimuli (7;8). It appears, that SP exerts its pro-inflammatory effects mainly by stimulation of the tachykinin NK1 receptors, whereas NKA mainly causes tachykinin NK2 receptor-mediated effects (9). Upon inhalation, SP induces AHR and the so-called 'neurogenic inflammation' within the airways of both non-asthmatic and asthmatic individuals, characterized by microvascular leakage, mucus secretion, and inflammatory cell responses (9-11), whereas inhaled NKA mainly causes bronchoconstriction (12,13). Furthermore, in contrast with non-asthmatic controls, increased tachykinin NK1 and NK2 receptor mRNA expression has been demonstrated within the airways of asthmatic patients (14,15). Correspondingly, in subjects with allergic asthma, increased concentrations of SP have been found in sputum and bronchoalveolar lavage (BAL) at baseline, with further increase following segmental allergen challenge (8,16). Similarly, in still another study in allergic asthma, increased NKA levels have been detected 4 h following an allergen bronchoprovocation test (7).

These observations provided evidence that the tachykinins SP and NKA may contribute to airway inflammation and hence may be implicated in the pathophysiology of asthma. Therefore, several tachykinin NK1 and/or NK2 receptor antagonists have been developed and tested against indirect challenges including NKA, adenosine monophosphate and hypertonic saline in patients with asthma (17-21). However, until recently, there are no published studies on clinical efficacy of dual tachykinin NK1/NK2 receptor antagonists in asthma nor on their biological efficacy in allergen challenge, being the most representative model of asthma.

AVE5883 is a non-peptidyl, dual tachykinin NK1/NK2 receptor antagonist with high specificity and affinity for tachykinin NK1 and NK2 receptors (Ki = 5.6 and 3.1 nM for the NK1 and NK2 receptor respectively, and >10 μM for a variety of other physiologically important receptors). In sensitized guinea-pigs, intra-peritoneally administered AVE5883 has been shown to reduce ovalbumin-induced airway hyperreactivity and eosinophil influx in the BAL [data on file]. Similarly, intratracheally administered AVE5883 protected against capsaicin-induced bronchoconstriction in sensitized guinea-pigs and aerosolised AVE5883 inhibited the NKA-induced increase in airway resistance in dogs [data on file].

We tested the safety and tolerability, in combination with the pharmacological and biological efficacy and the pharmacokinetics, of inhaled AVE5883 in clinically stable patients with mild to moderate persistent asthma not on maintenance anti-inflammatory therapy. In the first study, we tested the pharmacological efficacy of a single inhaled dose of AVE5883 against NKA-induced bronchoconstriction. Subsequently, in another study in patients with similar asthma characteristics, we tested the biological efficacy of multiple inhaled doses of AVE5883 against allergen-induced airway responses and markers of airway inflammation.

Some of the results of both studies have previously been reported in the form of abstracts (22,23).

Methods

SUBJECTS

NKA challenge study: 20 non-smoking, patients with clinically stable, mild to moderate persistent asthma participated in the NKA-challenge study (Table 1). All patients had a history of persistent asthma for at least 1 year (according to Global Initiative for Asthma (GINA) criteria (1)), without any other clinically relevant disorders. Except for inhaled short-acting β2-agonists prn, no one was using concomitant anti-asthma or anti-allergy medication for at least 6 weeks prior to and during the study. Patients had no history of viral infections of the lower airways for at least 6 weeks before enrollment. Caffeine-containing beverages and short-acting inhaled β2-agonists were withheld at least 8 h before each visit. Baseline forced expiratory volume in one second (FEV_1) had to be ≥75% of predicted. Patients were hyperresponsive to both inhaled methacholine bromide (MBR) and inhaled NKA, showing a 20% fall in FEV_1 ($PC_{20}FEV_1$(MBR)) of lower than 19.6 mg/mL (=16 mg/mL methacholine chloride, equals 80 μmol/mL) and a $PC_{20}FEV_1$(NKA) of lower than 441.2×10^{-3} μmol/mL (equals 500 μg/mL), respectively, at screening.

Allergen challenge study: 12 patients participated in the allergen challenge study (Table 1). Four of them have previously participated in the NKA challenge study (>6 months ago). All patients met the same aforementioned criteria, with a maximum $PC_{20}FEV_1$(NKA) of $\leq 882.4 \times 10^{-6}$ μmol/mL (equals ≤ 1000 μg/mL) at screening. In addition, patients had a positive skin prick test to house dust mite (HDM, a positive response was defined as a mean wheal diameter $\geq$3mm) and a documented late asthmatic response (LAR) to inhaled HDM extract, i.e. fall in FEV_1 >15% from baseline between 3 and 9 h post-allergen.

Both study protocols were approved by the Leiden University Medical Centre Ethics Committee, and all participants gave written informed consent.

TABLE 1   PATIENTS' BASELINE CHARACTERISTICS.

CHARACTERISTICS		NKA CHALLENGE	ALLERGEN CHALLENGE
Number of patients		20[a]	12[b]
Gender	Male	5	6
	Female	15	6
Atopy status	Yes	18	12
	No	2	0
Age (years)	Mean [Range]	29.6 [19 – 47]	24.4 [20 – 39]
FEV_1 (L)	Mean [Range]	3.7 [2.6 – 5.7]	3.8 [3.1 – 5.9]
FEV_1 (% predicted)	Mean [Range]	102.4 [80.3 – 125.5]	91.6 [73.6 – 121.4[c]]
$PC_{20}FEV_1$ (MBr) (mg/mL)	Mean [Range]	2.2 [0.1 – 8.5]	2.6 [0.1 – 14.4]
$PC_{20}FEV_1$ (NKA) ($\ast 10^{-3}$ μmol/mL)	Mean [Range]	143.2 [5.9 – 593.8[d]]	357.9 [5.9 – 1115.7[e]]

[a] One subject did not complete the NKA challenge study, [b] One subject did not complete the Allergen challenge study, [c] One subject with a lower baseline FEV_1 (73.6% predicted) was included, [d] One subject with a $PC_{20}FEV_1$(NKA) of 593.8 $\ast 10^{-3}$ μmol/mL was included, [e] One subject with a $PC_{20}FEV_1$(NKA) of 1115.7 $\ast 10^{-3}$ μmol/mL was included.

STUDY DESIGN

NKA challenge study: This was a single-center, randomized, double-blind, placebo-controlled, single-dose, cross-over study. Before entering the study, selection criteria were examined on 2 screening visits, 24 h apart. Fourteen to 28 days after screening, eligible patients were randomized into the study.

Each treatment period consisted of two study days, separated by a washout period of 12-16 days. Clinically stable subjects inhaled either AVE5883 (cumulative dose of 4.8 mg) or placebo, 30 minutes before an NKA-challenge. Blood samples for pharmacokinetics were collected from a venous cannula inserted in a forearm vein (pre-dose, 10, 30 and 45 minutes and 1, 2, 4, 6 and 8 h post-dose). A standardized NKA challenge was performed 30 minutes after the last study drug inhalation. In principle, pre-challenge FEV_1 had to return within 10% of baseline. However, in order to keep the time-interval between dosing and NKA challenge constant in all patients and to allow also more hyperresponsive asthmatics in the study, in the case of an unexpected drop in FEV_1 following study medication, decreases in pre-NKA FEV_1 were accepted up to 20% from baseline, provided within a safe range ($FEV_1 > 2.3$ L, allowing a safe $PC_{20}FEV_1(NKA)$). Ten to 14 days post-study, there was a follow-up visit. Allergen challenge study: This was a single-center, randomized, multiple-dose, placebo-controlled, double-blind, cross-over study. At least 3 weeks before the study, selection criteria were examined. Eligible patients with a documented $PC_{20}FEV_1(NKA)$ and a demonstrated LAR to inhaled HDM at screening were randomized into the study. On Day 1, exhaled NO (eNO) measurement, followed by a $PC_{20}FEV_1(MBR)$, and 1 h later, a sputum induction, were performed. To ensure asthma stability, mean baseline FEV_1 had to be within 10% and the $PC_{20}FEV_1(MBR)$ had to remain within 1 doubling dose on Day 1 of both treatment periods. Clinically stable patients inhaled the first dose of study medication at 2:00 pm (± 2 h) followed by an evening dose at approx. 8:00 pm, followed by FEV_1 measurements. On Days 2-7, study medication was inhaled three times daily at 8:00 am, 2:00 pm and 8:00 pm (± 2 h). Subjects were discharged from the unit 1 h after the morning dose on Day 2 and returned on Day 7 (intake was monitored by telephone contact). On Day 8, the afternoon dose was skipped and on Day 9 patients only inhaled the morning dose. On Day 8, a standardized allergen bronchoprovocation test was conducted approximately 45 min post-dosing (provided pre-allergen FEV_1 returned within 10% of the predose value), and the airway response was recorded by FEV_1 until 9 h post-allergen. Blood samples were collected for pharmacokinetics from a venous cannula inserted in a forearm vein on Day 8 (pre-dose, 15 and 30 minutes and 1, 1.5, 2, 3, 4, 5, 6, 7, 8 and 9 h post-dose) and on Day 7 and 9 (pre-dose, and 1, 2, 4, and 6 h post-dose). The pro-inflammatory effects of inhaled allergen were monitored by eNO, sputum cell differentials and $PC_{20}FEV_1(MBR)$ on Days 7 and 9, 24 h pre- and post-allergen, respectively. Ten to 14 days post-study, a follow-up visit was scheduled (Figure 1). Throughout the study, patients recorded any symptoms and signs of asthma, medication use, and adverse events into a diary.

All bronchoprovocation tests were performed at the same time of the day (±2 h). After all bronchoprovocation tests, patients received salbutamol ≥2x100 μg through Volumatic (GlaxoSmithKline, Zeist, The Netherlands), until the FEV$_1$ returned within 10% from baseline.

Screening → [21-28 days] → Placebo → [21-28 days] → AVE5883 4.8mg → [10-14 days] → Follow-up

Screening → [21-28 days] → AVE5883 4.8mg → [21-28 days] → Placebo → [10-14 days] → Follow-up

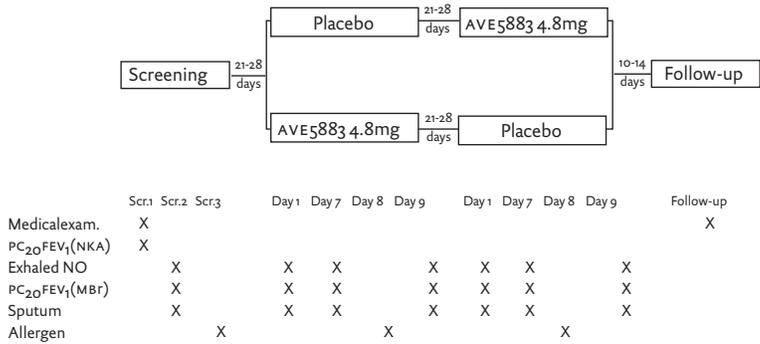|	Scr.1	Scr.2	Scr.3	Day 1	Day 7	Day 8	Day 9	Day 1	Day 7	Day 8	Day 9	Follow-up
Medical exam.	X											X
PC$_{20}$FEV$_1$(NKA)	X											
Exhaled NO		X		X	X		X	X	X		X	
PC$_{20}$FEV$_1$(MBr)		X		X	X		X	X	X		X	
Sputum		X		X	X		X	X	X		X	
Allergen			X			X				X		

FIGURE 1 Study flowchart - allergen challenge study. there was at least a one-week washout between screening visit 1 and 2. SCR = screening, PC$_{20}$FEV$_1$(NKA) = provocative concentration of neurokinin a causing a 20% fall in FEV$_1$, PC$_{20}$FEV$_1$(MBR) = provocative concentration of methacholine bromide causing a 20% fall in FEV$_1$, sputum = sputum induction, allergen = allergen challenge.

STUDY MEDICATION

For both study protocols AVE5883 ([4-(1-{2-3-(3,4-Dichloro-phenyl)-1-(3,4,5-trimethoxy-benzoyl)-pyrrolidin-3-yl]-ethyl}-4-phenyl-piperidine-4-carbonyl)-piperazin-1-yl]-acetic acid) was supplied by sanofi-aventis pharmaceuticals Inc. (Cheshire, UK) as a sterile solution or matching placebo in an alcohol/propellant (HFA-227) mixture, both delivered in a pressurized metered dose inhaler (pMDI; 300 μg/actuation). Each administration consisted of 16 actuations in 8 min yielding a cumulative dose of 4.8 mg AVE5883. Post-dosing subjects were required to rinse their mouth.

INHALATION CHALLENGES & RESPONSE MEASUREMENTS

The airway response to the inhaled aerosols was measured by FEV$_1$ according to standardized lung function techniques and recorded by a spirometer connected to a personal computer (Vmax Spectra, Sensor Medics, Bilthoven, The Netherlands) (24). MBR, NKA and allergen bronchoprovocation challenge tests were performed by tidal breathing method according to validated techniques as described previously (25-27). A detailed description of methods used is presented in the online supplement.

SPUTUM INDUCTION AND ANALYSIS

Sputum induction and analysis were performed according to the entire expectorate ('full sample') method, that has been previously validated (26). Hypertonic saline aerosols (NaCl 4.5%) were generated at room temperature by a DeVilbiss Ultraneb 2000 ultrasonic nebulizer (Tefa Portanje, Woerden, The Netherlands) and inhaled for 3x5 min at 15 min intervals (26). Sputum samples were processed according to a previously validated method after adding DTT 0.1% (28). Subsequently, cytospins were made (50 µL/cytospin; Shandon Cytospin 4, Thermon Electron Corporation, Runcorn, UK) and differential cell counts performed on May-Grünwald-Giemsa-stained cytospins; cells were expressed as a percentage of 500 nucleated cells excluding squamous cells as described previously (26). Samples with > 80% squamous cells were excluded from analysis. A detailed description of methods used is presented in the online supplement.

EXHALED NITRIC OXIDE

Exhaled NO measurements were performed in triplicate within 10% at a exhalation flow of 50 mL/s according to current ATS recommendations (29), by a chemoluminescence analyzer (Ecomedics CLD88sp, Duernten, Switzerland). A detailed description of methods used is presented in the online supplement.

PLASMA CONCENTRATION OF AVE5883

Blood samples (7 mL) were collected into sodium heparinate vacutainers and centrifuged at 3000 rpm for 10 minutes at 4 °C. AVE5883 was determined in plasma samples of subjects treated with AVE5883 by Bioanalytics, DMPK, sanofi-aventis pharmaceuticals Inc, Bridgewater, NJ, using a validated LC/MS/MS method with a lower limit of quantification of 0.1 ng/mL.

ANALYSIS

NKA challenge study: The protective effect of AVE5883 against NKA-induced bronchoconstriction was assessed by comparison of the $PC_{20}FEV_1(NKA)$ (log10 transformed) after AVE5883 and placebo pre-treatment. $PC_{20}FEV_1(NKA)$ was calculated by linear interpolation of the inhaled dose of NKA below and above a 20% fall in FEV_1 (27). If no fall in $FEV_1 \geq 20\%$ was reached after inhalation of the highest NKA-dose, $PC_{20}FEV_1$ (NKA) was set at 1.76 µmol/mL (=2000 µg/mL; i.e. one doubling dose higher than the highest dose tested) (27). The difference in $PC_{20}FEV_1$ (NKA) between AVE5883 and placebo was tested using an analysis of variance with sequence, subject (within sequence), and treatment as factors. The safety and tolerability was primarily assessed through examination of treatment-emergent adverse

events. Treatment-emergent adverse events consisted of all on-treatment adverse events and any pre-treatment adverse events that worsened in intensity (severity or frequency) after the start of study medication. Descriptive statistics were provided for plasma concentration-time data and plasma pharmacokinetic parameters.

Allergen challenge study: The effect of AVE5883 on the allergen-induced airway responses was determined by comparing the absolute corresponding area under the time-response curve (AUC) for both the EAR (0-3 h post-allergen), and the LAR (3-9 h post-allergen) between AVE5883 and placebo. The trapezoidal rule was applied for the calculation of the AUCs (30). Similarly, the differences in the maximal percent fall in FEV_1 during the EAR and LAR were compared between the two treatments.

In addition, the differences in allergen-induced changes in eNO, $PC_{20}FEV_1$ (MBR) and the sputum differential cell counts (mast cells, eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells) were assessed by comparing the (changes of the) corresponding values 24 h before and 24 h after allergen challenge between the two treatments. $PC_{20}FEV_1$ (MBR) was calculated by linear interpolation of the airway responses below and above a 20% fall in FEV_1 (27). The airway responses to inhaled allergen were expressed as percentage fall in FEV_1 from post-diluent baseline and plotted as time-response curves during both treatment periods. For the assessment of treatment differences (AVE5883 vs placebo) in these outcome parameters, an analysis of variance (ANOVA) appropriate to the 2-period, 2-sequence, 2-treatment cross-over design was used. The ANOVA model contained factors of treatment, sequence, and subject within sequence. Carry-over effects were examined for the LAR AUC analysis. P-values <0.05 were considered statistically significant.

The sample size of 12 evaluable patients for this study was based upon the simplifying assumption for a comparison of the two treatments using a paired t-test. Given this assumption, the calculated sample size required to detect a 30% mean difference in the LAR was 10 subjects (α = 0.05 (two-tailed), β = 0.10 (one-tailed), power = 90%; within subject standard deviation = 25%) (31).

Results

SAFETY & TOLERABILITY

NKA challenge study: A total of 20 patients were randomized and 19 patients completed the study. One subject was withdrawn after the first study day because of a moderate bronchoconstriction (i.e. a fall in FEV_1 of 34% from

baseline) within 5 min of inhalation of AVE5883. Overall, the study medication was well-tolerated, although all patients on AVE5883 vs. none on placebo (P) reported bad taste. The most commonly occurring adverse event was transient, self-limiting bronchospasm starting within 12 minutes after study drug inhalation reported by 8 patients on AVE5883 and 4 on P. Other reported adverse events were headache (5 patients on AVE5883 and 3 on P) and self-limiting dyspnoea (2 patients on AVE5883 and 5 on P).

Allergen challenge study: A total of 12 patients were randomized and 11 patients completed the study. One subject was withdrawn because of a viral exacerbation requiring oral prednisone between treatment period I and II. Similar to the NKA challenge study, AVE5883 was generally well-tolerated although all patients reported a bad taste after AVE5883 inhalation. The most common adverse event was self-limiting dyspnoea occuring within 30 minutes of study drug inhalation (in 5 patients on AVE5883 vs. 2 on P). However, in all patients pre-allergen FEV_1 was within 10% of predose value (see table E2 of individual pre-dose and pre-allergen FEV_1 in online supplement). In addition, headache was reported by 3 patients on AVE5883 vs. 3 on P. Based on observations in the unit, diaries and canisters' weight, all patients were compliant with the dosing regime. There was no difference in asthma control, including short-acting $\beta2$-agonist usage and PEF-measurements between the two treatment periods.

In both studies, no serious adverse events occurred and there were no clinically significant changes in physical examination, vital signs, laboratory parameters and baseline spirometry values.

PHARMACOKINETICS

The pharmacokinetics of AVE5883 after single dose (NKA challenge study) and multiple dose (allergen challenge study) administration are shown in Table 2. AVE5883 plasma concentrations showed considerable inter-subject variability and were similar in the two studies.

EFFECT OF AVE5883 ON AIRWAY CALIBER

In the NKA challenge study, pre-NKA FEV_1 was between 80% and 90% of baseline in 5 out of 19 patients. However, in the allergen challenge study multiple drug dosings did not significantly affect the pre-allergen airway caliber. In both studies, pre-challenge FEV_1 was not significantly different between the two treatments (NKA study: (mean ± SEM) 3.40 ± 0.23 L (AVE5883); 3.46 ± 0.20 L (P); p=0.40) and allergen challenge study: (mean ± SEM): 3.82 ± 0.3 L (AVE5883); 3.76 ± 0.3 L (P); p=0.52). Individual pre-dose and pre-challenge FEV_1 data for both studies are provided in the online supplement (Table E1 and E2).

TABLE 2   MAIN PHARMACOKINETIC PARAMETERS OF INHALED AVE5883

PARAMETER	NKA CHALLENGE STUDY	ALLERGEN CHALLENGE STUDY
Dose (mg)	4.8 single dose	4.8 three times a day
Number of patients	19	11
AUClast (ng x h/mL)	11.1 ± 6.0*	9.8 ± 4.9*
Cmax (ng/mL)	5.4 ± 3.7*	3.6 ± 1.8*
Tmax (h)	0.30 [0.28 – 1.12]**	0.35 [0.25 - 1.00]**
T 1/2 (h)	ND	6.93 ± 2.11 *

ND = Not determined, auclast = AUC from time zero to time of last measured concentration, 8 h in the NKA challenge study and 9 h in the allergen challenge study (*mean ±SD, **median [range])

EFFECT OF AVE5883 ON NKA-CHALLENGE

A single inhaled dose of AVE5883 reduced the NKA-induced bronchoconstriction in 16 out of 19 patients. In 5 of these 16 patients there was an increase in $PC_{20}FEV_1(NKA)$ of at least 2 doubling doses. In addition, 7 patients did not reach a $PC_{20}FEV_1$ (NKA) following inhalation of the highest dose of NKA versus 2 subjects in the placebo group.

On average, AVE5883 caused a rightward shift of the dose-response curve to inhaled NKA of at least 1.2 doubling doses as compared to P pretreatment (mean difference in $log10$ $PC_{20}FEV_1$ (NKA) ± SD: 0.35 ± 0.10; 90% CI: 0.17-0.53; p=0.004). Excluding those 5 subjects with pre-NKA FEV_1 FEV_1 between 80-90% of baseline, a subgroup analysis still showed a significant effect of AVE5883 compared to P (see online supplement, Table E3).

EFFECT OF AVE5883 ON ALLERGEN-INDUCED AIRWAY RESPONSES

In all patients, inhaled HDM induced an EAR and a LAR at screening. Pretreatment with AVE5883 (4.8 mg TID, 9 days) did not protect against the allergen-induced airway responses (Figure 2). Conversely, as compared with P, there was a slightly greater fall in FEV_1 from baseline following AVE5883 inhalations in terms of area under the curve (AUC) during both the EAR: mean AUC (0-3 h) ± SEM (%fall*h): 23.7 ± 3.0 (AVE5883) and 18.0 ± 3.0 (P) (p=0.02); and the LAR: mean AUC (3-9 h) ± SEM (%fall*h): 145.5 ± 11.7 (AVE5883) and 116.2 ± 11.7 (P), (p=0.01). Although the maximal percentage fall (max%fall) from baseline FEV_1 during EAR was comparable between the two treatments: max%fall ± SEM: -19.9 ± 2.2 (AVE5883) and -18.0 ± 2.2 (P), (p=0.29), during LAR it was more pronounced following AVE5883: (max %fall ± SEM) -38.7 ± 2.9 as compared to -33.6 ± 2.9 (P); (p<0.01). At 24 h post-allergen (Day 9), allergen challenge caused a significant decrease in baseline FEV_1 in both treatment groups. However, the changes in FEV_1 were not significantly different between the two treatments (p=0.77).
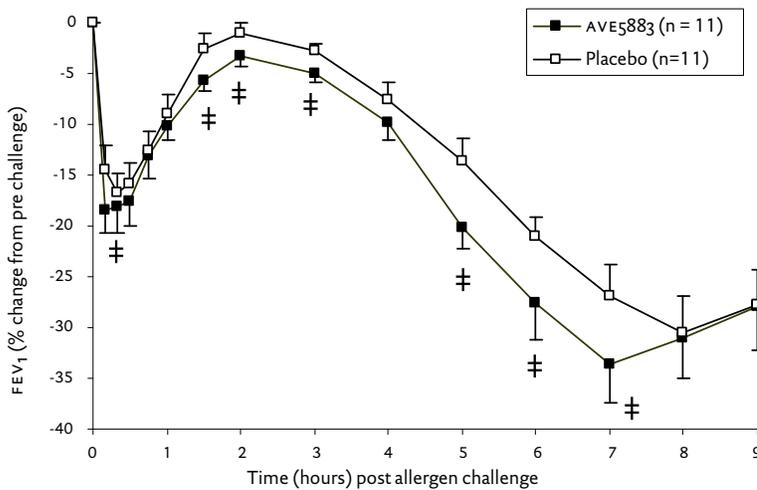
FIGURE 2   Allergen-induced airway responses in percentage change from pre-allergen FEV_1 (mean ± sem) 0-9 h following allergen challenge during AVE5883 (closed circles) and P (open squares) treatment. There was a significant difference in both the EAR (AUC 0-3 h) and LAR (AUC 3-9 h) between the two treatments, ‡ = P<0.05.

EFFECT OF AVE5883 ON ALLERGEN-INDUCED CHANGES IN AIRWAY RESPONSIVENESS TO METHACHOLINE

Allergen challenge caused a significant increase in AHR to methacholine (24 h pre- vs. 24 h post-challenge) during AVE5883 treatment (mean change in $PC_{20}FEV_1$(MBR) ± SEM: -0.23 ± 0.08 mg/mL; doubling concentrations; p=0.02). Similarly, there was an allergen-induced decrease in $PC_{20}FEV_1$ (MBR) following placebo (mean change in $PC_{20}FEV_1$ (MBR) ± SEM: -0.32 ± 0.08 mg/mL; doubling concentrations; p=0.003). However, these allergen-induced changes in airway responsiveness to MBR were not significantly different between the two treatments (p=0.21) (Figure 3).

EFFECT OF AVE5883 ON ALLERGEN-INDUCED CHANGES IN eNO

Multiple doses of AVE5883 did not affect baseline eNO values as compared to placebo treatment (Day 1 vs Day 7, p=0.28). The allergen challenge induced a significant increase in eNO (Day 7 vs Day 9) in both treatment periods (p <0.001). However, the changes in eNO were not statistically different between the two treatments (mean change ± SEM (Day 7 vs Day 9) 37.64 ± 6.40 ppb (AVE5883) and 43.44 ± 6.57 ppb (P), p=0.32) (Figure 4).
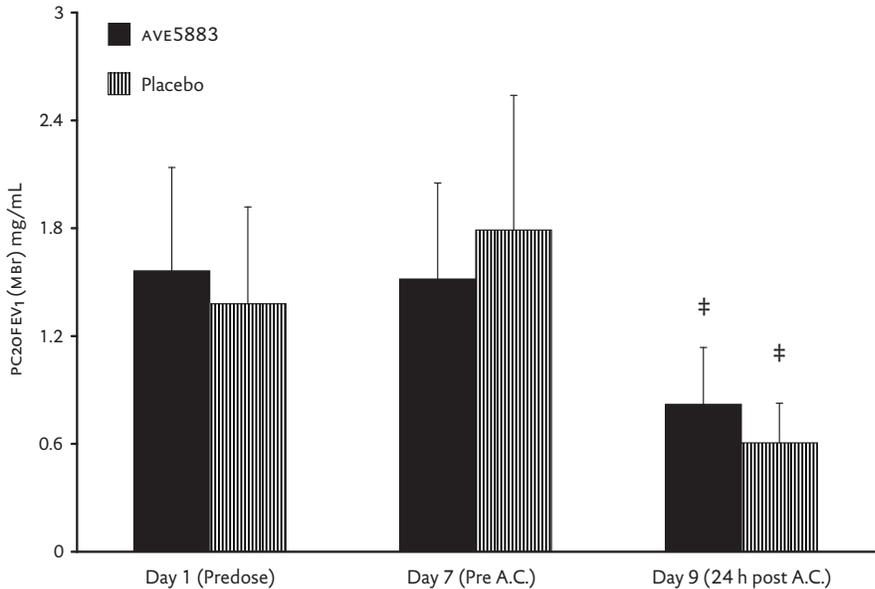
FIGURE 3   Airway responsiveness to mbr expressed as $PC_{20}FEV_1(MBR)$ on days 1, 7 and 9 during both AVE5883 (filled bars) and P (striped bars) treatment period. Allergen challenge decreased $PC_{20}FEV_1(MBR)$ during both treatment periods (day 7 vs. 9), ‡ = P<0.05. However, there was no significant difference in the changes in allergen-induced airway responsiveness to MBR between the two treatments (p=0.21).

EFFECT OF AVE5883 ON ALLERGEN-INDUCED CHANGES IN SPUTUM CELL DIFFERENTIALS

On pre-allergen Day 7, there was no difference in the % sputum eosinophils between both treatment periods (mean ± SEM: 4.86 ± 1.75 % (AVE5883) and 3.33 ± 1.58 % (P)). The allergen challenge induced a rise in sputum eosinophils during both treatment periods (Day 7 vs. Day 9; mean change ± SEM 8.09 ± 3.018; (AVE5883)) and mean change ± SEM 7.08 ± 2.972; (P)). Since only 3 patients managed to expectorate evaluable sputum samples on both Days 7 and 9 of the two treatment periods, no adequate power analysis could be performed on the allergen-induced changes in sputum cell count between the two treatments. However, based on evaluable samples, there was a clear trend towards increase in sputum eosinophils following allergen challenge. This is in agreement with the allergen-induced changes in eNO and $PC_{20}FEV_1(MBR)$.
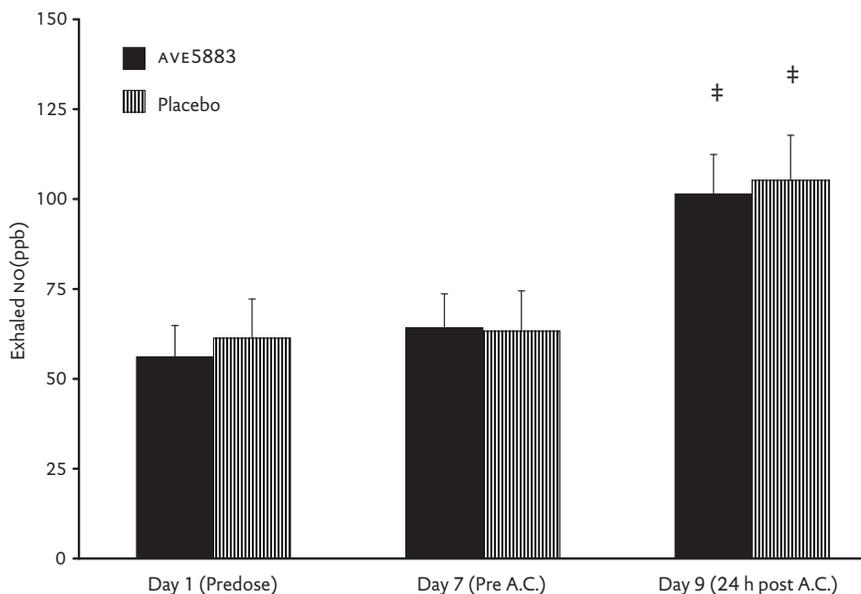
FIGURE 4   Exhaled NO as mean (± SEM) on days 1, 7 and 9 during both AVE5883 (filled bars) and P (striped bars) treatment period. As compared with pre-allergen (day 7), there was a significant increase in eNO 24 h post-allergen (day 9) during both treatments, ‡ = (P<0.05). However, the changes in eNO were not significantly different between the two treatments (P=0.32).

Discussion

We report combined study data on the safety, tolerability, pharmacological and biological efficacy and pharmacokinetics of a single and multiple inhaled doses of AVE5883, a novel dual tachykinin NK1/NK2 receptor antagonist, in patients with mild to moderate persistent asthma. In all patients, both dosing regimens of inhaled AVE5883 were safe and generally well-tolerated. and no clinically relevant adverse effects occurred. However, the substantial number of 16 actuations in combination with repeated deep inhalations from the pMDI device may have induced self-limiting dyspnoea accompanied by a transient drop in FEV_1 in some patients. As dyspnoea and FEV_1 were recorded at 12 minutes post-dosing in the single dose study and at 30 minutes in the multiple dose study, respectively, this may explain the higher occurrence of dyspnoea and/or drop in FEV_1 in the single dose (NKA-challenge) study. Despite pharmacological activity of a single inhaled dose (4.8 mg) against NKA-induced bronchoconstriction, multiple inhaled doses of AVE5883 (4.8

mg TID for 7 days) increased the allergen-induced airway responses and failed to reduce allergen-induced markers of airway inflammation and airway hyperresponsiveness in patients with similar asthma characteristics.

In a comparable proof of concept study in patients with similar asthma characteristics, a single inhaled dose of a less specific tachykinin NK1/NK2 receptor antagonist, FK224, failed to protect against NKA-induced bronchoconstriction (32). In contrast, a single oral dose of another dual tachykinin NK1/NK2 receptor antagonist, DNK333, provided significant protection against NKA-induced bronchoconstriction in patients with mild persistent asthma, causing a rightward shift of the dose-response curve to inhaled NKA by on mean 4.08 doubling doses (18). Moreover, in patients with similar asthma characteristics, an oral triple tachykinin receptor antagonist, CS-003, has been shown to produce a potent and long-lasting rightward shift of the NKA-dose-response curve (21). Our study results confirm and extend previous findings, showing that an inhaled dual tachykinin NK1/NK2 receptor antagonist is also capable of inhibiting NKA-induced bronchoconstriction in asthma, albeit to a lesser extent than the more potent oral compounds (18;21). Alternatively, an inhaled formula may offer the benefit of targeted therapy with possibly fewer systemic side effects.

In correspondence with several animal studies with other tachykinin NK1/NK2 receptor antagonists (33-35), aerosolized AVE5883 not only provided protection against both NKA-induced bronchoconstriction, but also against other (tachykinin-driven) bronchoconstrictor stimuli, including capsaicin and ovalbumin in sensitized guinea-pigs and dogs [data on file]. To our knowledge, this is the first study reporting on the effects of a dual tachykinin NK1/NK2 receptor antagonist on allergen-induced airway responses and markers of AHR/inflammation in asthmatic subjects *in vivo*. Despite a partial antagonistic effect of a single inhaled dose (4.8 mg) against exogenous NKA in patients with asthma, unexpectedly, pretreatment with multiple inhaled doses of AVE5883 (4.8 mg TID for 9 days) enhanced the allergen-induced airway responses without affecting the markers of airway AHR/inflammation. Furthermore, since AVE5883 did not affect baseline FEV_1 throughout the treatment period and since pre-allergen FEV_1 was not different between both treatments, this argues against a clear-cut mechanistic explanation for this phenomenon. In conclusion, while animal studies have produced a large body of evidence warranting efficacy of dual tachykinin NK1/NK2 receptor antagonists in asthmatic patients in vivo, we were unable to substantiate this hypothesis in the present study.

We do not believe that the lack of efficacy of AVE5883 against allergen-induced airway and inflammatory responses has been caused by methodological or dosing errors. First, we applied previously validated methods

and all participating patients had both an airway responsiveness to inhaled NKA and an allergen-induced late asthmatic response (25;26). In addition, pre-allergen FEV_1, recorded at approx. 1 h post-dosing, was not affected by inhalation of the study medication, nor were there any significant differences in baseline data between both treatment groups. Second, we based our dosing regimen on the same dose and mode of administration that effectively reduced the NKA-induced bronchoconstriction in the first part of the study in patients with similar asthma characteristics. Since a steady state plasma concentration was expected within three days, 7 days treatment with AVE5883 pre-allergen was deemed sufficient to demonstrate biological efficacy. In a similar study protocol, inhaled corticosteroids have been shown to provide a significant reduction of allergen-induced airway responses and markers of airway inflammation following 7 to 8 days pretreatment (36;37).

What could possibly account for the lack of effect of AVE5883 against allergen challenge? First, it may be possible that while NKA and SP play an important role in the allergen-driven airway inflammation in several animal models of asthma (38), this may not similarly apply to asthmatic patients due to species-related differences. For instance, Bowden *et al* (39) reported that in guinea-pigs, the most commonly applied laboratory species, approximately 60% of intra-epithelial fibres within the trachea constitute of SP nerve fibres, while in humans this is only 1% (40). Furthermore, in asthmatic airways the number of the SP fibres was not found to be increased as compared to nonasthmatic controls (41). Correspondingly, up to now reported (single) tachykinin NK1 or NK2 receptor antagonists have shown little if any efficacy against (tachykinin-driven) bronchoconstrictor stimuli in asthmatic subjects, despite previously shown pharmacological efficacy against the respective agonist (NKA or SP) (20;42). In the first study, multiple oral doses of the specific NK2 receptor antagonist, SR 48968, failed to protect against adenosine-induced bronchoconstriction in subjects with allergic asthma (20). In another study, CP-99,994, an NK1 receptor antagonist, did not inhibit hypertonic saline-induced bronchoconstriction and cough in patients with mild persistent asthma (42). Still another possibility could be that SP, being a major pro-inflammatory tachykinin (8), is likely to play a more important role in allergen-induced airway inflammation than NKA, which appears to possess more direct bronchoconstrictor properties (13). And although AVE5883, being a dual NK1/NK2 antagonist was expected to inhibit the effects of both tachykinins, we only tested its protective properties against NKA, and are hence not fully informed about its pharmacological efficacy against inhaled SP in asthma in vivo. In line with this and based on its modest antagonistic properties against inhaled NKA, it may be that AVE5883 is not potent enough to offer protection against allergen-induced airway response and inflammation. Comparable findings

were reported in clinical trials with early leukotriene (LT) receptor antagonists in asthma. Despite a 3.8-fold rightward shift in the dose response curve to inhaled LTD4 in mild asthmatics, the oral LT receptor antagonist L-649,923 failed to protect against allergen-induced bronchoconstriction. However, the more potent LT antagonist zafirlukast, causing an approx. 10-fold shift in the dose-response curve to inhaled LTD4, significantly protected against allergen-induced airway responses and the associated AHR (43-46). Finally, although similar plasma concentrations of AVE5883 were observed in both studies, higher and longer lasting plasma exposure of AVE5883 may have been required to warrant adequate drug concentrations within the airways, before and during the allergen-induced late asthmatic response. Therefore, considering the relatively short terminal elimination half-life of the drug ($T_{1/2} = 6.93$ h), higher doses and/or a more frequent dosing of AVE5883 may have been required to achieve any protective effect.

In conclusion, a single inhaled dose of AVE5883 provided a modest protection against NKA-induced bronchoconstriction in patients with mild to moderate persistent asthma, whereas 7 days pretreatment with multiple daily doses of this dual tachykinin NK1/NK2 receptor antagonist paradoxically enhanced the allergen-induced airway responses without affecting the markers of airway inflammation/hyperrresponsiveness in a patient population with similar asthma characteristics. Therefore, these findings question the prominent role of neurogenic inflammation in asthma and consequently, the therapeutic potential of dual tachykinin antagonists. Hence, more research is required to determine the precise role of tachykinins and their receptors in the allergic airway inflammation that will help to establish the position of potent combined tachykinin receptor antagonists in the treatment of asthma.

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NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA AND ALLERGIC RHINITIS

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NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

Reversal of the late asthmatic response increases exhaled nitric oxide

J.D. Boot[1], S. Tarasevych[1], P.J. Sterk [2], R.C. Schoemaker[1], L. Wang [3], D. Amin [3], A.F. Cohen1 and Z. Diamant[1].

1   Centre for Human Drug Research, Leiden, Netherlands
2   Leiden University Medical Center, Leiden, Netherlands
3   Aventis Pharma Inc, Bridgewater, NJ, United States

Introduction

Exhaled nitric oxide (eNO) is indicative of the severity of the airway inflammation in asthma (1,2). Consequently, this non-invasive, patient-friendly methodology has recently been introduced into clinical practice as a diagnostic and monitoring tool especially for children (3-5). However, despite standardization of the eNO protocol (6), there are still some issues that need to be addressed. For example, whether there does or does not exist a relationship between the airway calibre and the eNO levels. A clear-cut relationship of eNO and the airway calibre may have implications for the measurements (7-10).

Late asthmatic airway response (LAR) to inhaled allergen, defined as a fall in forced expiratory volume in 1 second (FEV_1) of at least 15% from pre-allergen baseline (11), have been shown to be associated with airway inflammation, including increased levels of eNO (12). We hypothesized that eNO levels during LAR are related to the degree of airway narrowing. Therefore, we studied the effect of vigorous bronchodilation with inhaled salbutamol during LAR on eNO levels.

Subjects and methods

SUBJECTS

Data from 12 asthmatics (6M/6F, 21-40 y) $PC_{20}FEV_1$ methacholine<8 mg/mL) with mild to moderate persistent asthma (FEV_1 73.6-121.4 % predicted) and dual responses to inhaled house-dust mite (HDM) extract participating in an intervention study were used. All subjects had a history of persistent asthma for at least 1 year (according to criteria by GINA 2002), without any other clinically relevant disorders. None of the subjects had smoked tobacco during the past year. None was using concomitant anti-asthma or anti-allergy medication for at least 6 weeks prior to and during the study, except for inhaled short-acting β2-agonists *prn*. There was no history of viral infections of the lower airways for at least 4 weeks. The study was approved by the Ethics Committee of the Leiden University Medical Center and all participants gave written consent.

STUDY DESIGN

The allergen-induced airway response during the LAR was measured at 1-hourly intervals until 9-h post-allergen. To reverse the LAR, 600 μg salbutamol was administered through an aerochamber (Volumatic, GlaxoSmithKline, Zeist, The Netherlands), and the FEV_1 was repeated 15 min

later. Exhaled NO was measured before and approximately 30 minutes post-salbutamol. For ethical reasons, no placebo-arm was included in this study.

Methods

ALLERGEN BRONCHOPROVOCATION TEST

The allergen bronchoprovocation test was performed using the standardized 2-minutes tidal breathing method according to Cockcroft (11). Purified aqueous allergen extract of Dermatophagoides Pterinyssinus (SQ503, ALK-BPT, ALK-Abelló, Nieuwegein, The Netherlands), with 0.5% phenol as a preservative was used for the bronchoprovocation tests (BPT). Preparation of the HDM extract dilutions was performed according a previously validated protocol (13). The allergen aerosols were generated by a DeVilbiss 646 nebulizer (output 0.13 mL/min) connected to an in-and expiratory valve box with an expiratory aerosol filter (Pall Ultipor BB50T, Medica BV, Den Bosch, The Netherlands). Subjects first inhaled the allergen diluent, and provided the subsequent fall in FEV_1 remained <10% of baseline, they subsequently inhaled a total of 3 doubling concentrations of HDM extract at 12 min intervals that previously caused a LAR. The airway response during the LAR was measured at 1-hourly intervals until 9 h post-allergen or earlier if subjects experienced unbearable discomfort or the FEV_1 fell below 1.4 L.

AIRWAY RESPONSE

The airway response to the inhaled aerosols was measured by FEV_1 according to standardized lung function techniques and recorded by a spirometer connected to a PC (Vmax Spectra, Sensor Medics, Bilthoven, The Netherlands) (14). At each specified timepoint, the FEV_1 was measured in duplicate, and the highest, technically satisfactory FEV_1 was implicated in the analysis. The airway response was quantified as percentage change from pre-allergen baseline FEV_1.

EXHALED NO MEASUREMENTS

Exhaled NO levels were measured in triplicate (within 10%) by a chemoluminescence analyzer (Ecomedics CLD88sp, Duernten, Switzerland) at the specified timepoints. The mean ppb-value was implicated in the analysis; the response was quantified as percentage change from pre- to post-salbutamol value.

ANALYSIS

FEV_1 and eNO responses were correlated using a Spearman Rank Order Correlation Coefficient.

Results

All subjects had a LAR. As compared to pre-allergen baseline, the mean fall in FEV_1 at the time of reversal was 33.3% (range 15.1-57.7%). Salbutamol increased FEV_1 on average by 43% (SD:16%) as compared to pre-salbutamol FEV_1. At the end of the allergen challenge, the mean eNO pre-salbutamol was 60.2 ppb (30.6-108.1 ppb). Salbutamol increased eNO on average by 30% (range: 39.06 – 140.6 ppb; SD:17%). The Spearman Rank Order Correlation between the % change in FEV_1 and the % change in eNO (pre- versus post-salbutamol) was 0.51 (p<0.02) (Figure).
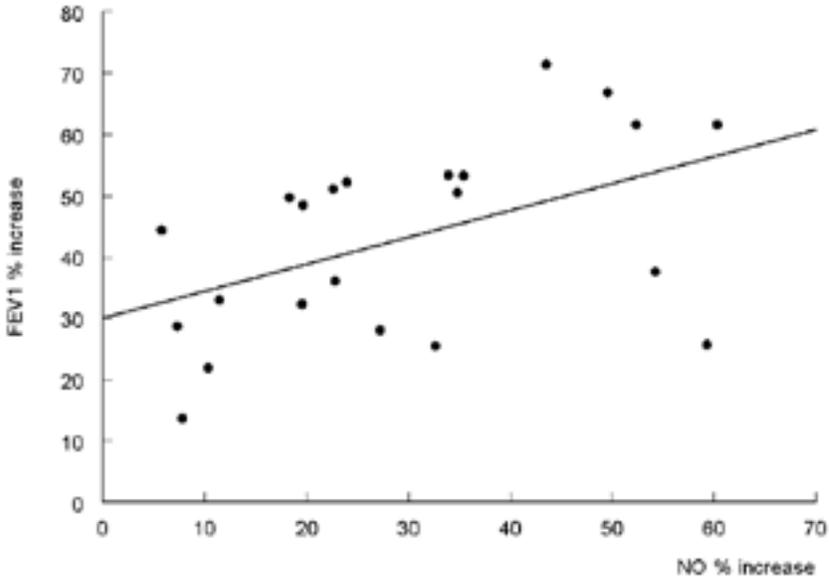
FIGURE 1   There was a significant correlation between the % change in FEV_1 and the % change in eNO (pre- versus post-salbutamol); the spearman rank order correlation was 0.51 (P<0.02).

Discussion

We found a raised eNO level following reversal of airways obstruction with inhaled salbutamol during the LAR at 9 hours post-allergen. Our data underscore and extend earlier findings.

First, late asthmatic responses (LAR) are associated with increased airway inflammation and accordingly, Kharitonov *et al* found increased eNO levels 10 hours post-allergen in asthmatic subjects, corresponding with the magnitude

of the LAR at 9 h post-allergen (12). Second, eNO levels have been found to relate to the airway diameter. Various studies in asthma showed decreases in eNO levels following bronchoconstrictor stimuli including methacholine, histamine, hypertonic saline, adenosine monophosphate (AMP) and exercise-induced bronchoconstriction (7,9,10). Alternatively, following inhalation of salbutamol, Silkoff *et al* found increased levels of eNO corresponding with increases in FEV_1 in asthmatic subjects in absence of allergen challenge (8). The present study combines all abovementioned observations: in agreement with Kharitonov's data (12), we found similar pre-bronchodilator eNO values during the LAR at 9 h post-allergen. And, according to the observations of Silkoff *et al* in the absence of allergen, there was a 30% raise in eNO levels following bronchodilation (8).

Our data imply that as a result of allergen-induced bronchoconstriction, the eNO level during the LAR is usually underestimated. Although eNO has been shown to reflect the degree of airway inflammation in asthma (15,16), Ricciardolo *et al* have demonstrated that the allergen-induced eNO may also act as an endogenous bronchoprotective mechanism (17). Therefore, using a bronchodilator following allergen challenge may not only relieve the bronchoconstriction, but may also support this endogenous bronchoprotective mechanism. Another implication of our study may be that for a correct non-invasive assessment of airway inflammation in asthma, eNO should be measured after (appropriate) bronchodilatation. To enable comparison with other studies or measurements in individuals, we suggest that both the bronchodilator dose and timepoint of post-bronchodilator eNO measurements should be standardized.

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Comparison of exhaled nitric oxide measurements between NIOX MINO® electrochemical and Ecomedics chemoluminescence analyzer

J.D. Boot[1], L. de Ridder[1], M.L. de Kam[1], C. Calderon[2], M.A. Mascelli[2], Z. Diamant[1]

1   Centre for Human Drug Research Leiden, the Netherlands
2   Centocor Inc., Malvern, PA, USA

Abstract

Background: Exhaled nitric oxide (eNO) is an established, noninvasive bio-marker of active airway inflammation in (atopic) asthma. Treatment with anti-inflammatory therapy, such as inhaled corticosteroids, effectively decreases eNO levels. The NIOX MINO® (MINO) is a hand-held, relatively inexpensive, electrochemical device that has been shown to yield comparable eNO measurements to the NIOX stationary unit.

Aim: To compare measurements of MINO with another widely used and vali-dated stationary chemoluminescence analyzer, the Ecomedics (ECO).

Methods: We performed subsequent eNO measurements on ECO and MINO in 50 subjects (19 healthy volunteers, 18 healthy smokers and 13 non-smoking, atopic asthmatics, not on controller therapy) on two visits 4-10 days apart. The mean of three acceptable measurements by ECO and the first acceptable measurement with the MINO were used for analysis.

Results: Both devices yielded reproducible eNO values for all subjects on both visits, with an overall CV of 22.7% (ECO) and 18.3% (MINO). A significant cor-relation was found between both devices (r = 0.97, p < 0.0001). Bland Altman plots showed a high degree of agreement for the entire study population (mean difference MINO vs. ECO = -10%; 95% limit of agreement = -36% and +28%) and in the three individual subgroups.

Conclusions: Exhaled NO values measured with the MINO are reproducible and in agreement with the ECO. Our results add further evidence to the reli-ability of the MINO and warrant its applicability in research and clinical prac-tice.

Introduction

Asthma is a chronic inflammatory disease presenting with variable symptoms and mostly reversible airway obstruction within the lower airways. According to international guidelines, modern asthma management is aimed at the suppression of airway inflammation by avoidance of allergens and anti-inflammatory, 'controller' therapy (1). For optimal guidance of disease control, measuring biomarkers of airway inflammation has become increasingly important (1). To this aim, several non- and semi-invasive sampling techniques have been developed and validated (2).

Exhaled nitric oxide (NO) is an established biomarker of airway inflammation that may serve to monitor the response to (novel) controller therapy. In asthma, elevated levels of eNO have been shown to correlate with disease severity, showing increases prior and during an asthma exacerbation and decreases following anti-inflammatory treatment (2). In addition, using eNO measurements to guide individual doses of inhaled corticosteroids resulted in reduced airway hyperresponsiveness along with an overall lower dose of inhaled corticosteroids without compromising asthma control (3,4). Stationary chemoluminescence NO analyzers are validated devices for online measurement of NO levels in exhaled air (5). However, their usage is largely hampered by their bulkiness and high costs. More recently, MINO has been marketed for portable, online eNO measurements. This hand-held and relatively inexpensive device is simple to use and yields reproducible measurements, even when performed by children at home (6). In addition, a recent economic evaluation revealed that the use of MINO in the treatment of asthma offers cost savings compared to asthma management based on standard guidelines, while both methods result in comparable health benefits (7). These properties warrant its potential applicability in both primary health care setting and in clinical trials. However, its reliability needs to be fully assessed. So far, MINO has been compared with the NIOX stationary unit but, to our knowledge, not with the other validated and widely used chemoluminescence analyzer, ECO (8-10). In this study we compared eNO measurements by MINO with the previously validated ECO in healthy volunteers, healthy smokers and atopic asthmatics.

Methods

SUBJECTS

The study population consisted of three subgroups: 19 healthy volunteers, 18 healthy smokers and 13 non-smoking, atopic asthmatics (Table 1).

The healthy volunteers were non-smokers for at least 12 months with less than 5 pack years (1 pack year = 20 cigarettes or equivalent smoked per day for 1 year). The healthy smokers were current smokers (last cigarette was smoked 1-2 hours before the study procedures) with a smoking history of at least 10 pack years. The asthmatic-subgroup only used inhaled short-acting β2-agonists as needed and had no controller medication for at least 1 month prior to the study. All had intermittent to mild persistent asthma and clinical stability was assessed by stable lung function (FEV_1 within 10% on both study visits), absence of symptoms and stable, infrequent use of rescue medication in the last 3 months. Atopy was demonstrated by a positive skin prick test for at least 1 of 10 airborne allergens. None of the participants had a history of airway infection in the previous 4 weeks prior and during the study. All subjects gave written informed consent and the study was approved by the Ethics Committee of Leiden University Medical Centre.

STUDY DESIGN

Exhaled NO measurements were performed in all subjects on ECO and MINO on 2 study visits, 4-10 days apart. All the measurements were performed during the same time of the day (±2 hours).

EXHALED NO MEASUREMENTS

All eNO measurements were performed according to current guidelines (11). Briefly, subjects were sitting in upright position and wearing a nose clip during the eNO measurements with both devices. They inhaled NO-free air through the device and subsequently exhaled at 50 ml/s for approximately 10 seconds. The mean of the first three technically acceptable measurements within 10% performed with the ECO (Ecomedics CLD88sp; Ecomedics, Duernten, Switzerland) were implicated into analysis. For measurements by the MINO (Aerocrine AB, Solna, Sweden), the first technically acceptable measurement was used for analysis (10).

SPIROMETRY

Following eNO measurements, spirometry (forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) and peak expiratory flow (PEF)) was performed according to standardized lung function techniques by a calibrated spirometer connected to a personal computer (Vmax Spectra Sensor Medics; Cardinal Health, Houten, The Netherlands) (12).

As eNO values were not normally distributed, data were log transformed prior to analysis. Comparisons between healthy volunteers and the other subgroups were made using an unpaired t-test. The reproducibility of both devices was assessed by the within subject variation between visits and expressed as a coefficient of variation (CV = the standard deviation expressed as percentage of the mean). In order to compare both devices, data were plotted in a scattergraph and the Pearson correlation was calculated on log-transformed data. Bland Altman plots were made with the difference (ECO - MINO) of measurements of both methods on the y-axis and the mean of the two methods on the x-axis, along with an estimation of the upper and lower limit of agreement, being 1.96 times the standard deviation (SD). The presented Bland-Altman plots incorporated all data and were constructed as if every pair of measurements was independent. Analysis of the first and second visit independently yielded similar results.

All calculations were performed using SAS for windows V9.1.2 (SAS Institute, Inc., Cary, NC, USA).

TABLE 1   PATIENTS' CHARACTERISTICS AT BASELINE

	HEALTHY VOLUNTEERS	HEALTHY SMOKERS	ATOPIC ASTHMATICS	P-VALUE
Total number	19	18	13	
Female : Male	10 : 9	9 : 9	8 : 5	
Age (years)	41 (30-53)	40 (32-52)	29 (21-54)	
FEV$_1$ (% predicted)	104.4 (87.5-119.5)	103.6 (82.1-121.1)	91.8 (61.0-122.0)	
Exhaled NO Ecomedics (ppb)	18.0 (7.4-35.5)	11.1 (4.7-20.5)	60.8 (10.9-184.6)	<0.01*
Exhaled NO NIOX MINO (ppb)	20.3 (8.0-39.0)	12.2 (5-23)	63.8 (13-172)	<0.01*

All values are given as mean (range). *Healthy volunteers compared to healthy smokers and compared to asthmatics.

Results

One subject from the healthy non-smokers' group produced eNO values <5 ppb on both occasions. Two subjects (one healthy volunteer and one atopic asthmatic) failed to perform acceptable eNO measurements on the ECO. These measures were excluded from analysis. All other subjects completed the study and performed technically acceptable manoeuvres on both study visits. Overall, healthy smokers had significantly lower and asthmatics significantly higher eNO values compared to healthy volunteers (Table 1). Exhaled NO values by MINO were slightly, but not significantly, higher than the ECO values in all three subgroups (Table 1).

Both devices yielded reproducible eNO values for all subjects on both visits, with an overall CV of 22.7% (ECO) and 18.3% (MINO). The Pearson correlation analysis yielded an r of 0.975 (p<0.0001) between eNO values measured by MINO and ECO (Figure 1).

In addition, Bland-Altman plots demonstrate agreement between both devices in the entire study population and the three subgroups (Figure 2) for both low and high values of eNO.

Discussion

In recent years, eNO has become widely accepted as a biomarker of airway inflammation in asthma. The availability of simple and reliable eNO-measurements is of major importance in the diagnosis and monitoring of day-to-day asthma. Hence, we compared the hand-held MINO to the widely used stationary ECO analyzer in a study population consisting of 3 subgroups: healthy volunteers, healthy smokers and atopic asthmatics not on controller therapy. Apart from a good reproducibility of eNO values on both study visits, a significant correlation and a high degree of agreement was observed between eNO measurements yielded by both devices. Subgroup analysis revealed a superior agreement in the high eNO ranges (atopic asthmatics) compared to the low eNO ranges (healthy smokers). However, with respect to the latter the group sizes were too small to draw definitive conclusions. Our results are in agreement with previous data comparing the MINO to the stationary NIOX unit and extend the findings to another widely used and validated chemoluminescence analyzer, (Ecomedics) (8-10).

Correlation= 0.975, P -value=<.0001
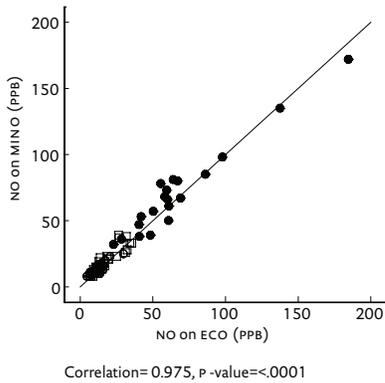
FIGURE 1   Pearson correlation between exhaled NO levels measured with the Ecomedics (x-axis) and the NIOX MINO (y-axis) analyzer. Healthy volunteers (=open squares), healthy smokers (=open circles) and atopic asthmatics (closed circles).

——— = Mean difference (-10%)
--- = Lower (-36%) and Upper limit of agreement (28%)

——— = Mean difference (-10%)
--- = Lower (-38%) and Upper limit of agreement (31%)

——— = Mean difference (-10%)
--- = Lower (-38%) and Upper limit of agreement (31%)

——— = Mean difference (-9%)
--- = Lower (-34%) and Upper limit of agreement (25%)

FIGURE 2   Bland Altman plot of exhaled NO measurements using the Ecomedics vs NIOX MINO for the entire study population, healthy volunteers (=open squares), healthy smokers (=open circles) and atopic asthmatics (closed circles).

Exhaled NO values are not normally distributed and require log-transformation prior to analysis (10). Therefore, in the current paper the upper and lower limits of agreement are presented in percentages difference which may not be suitable for clinical interpretation. However, if we use the mean value of eNO for the entire study population (26.4 ppb for the ECO) and back-translate to an arithmetic scale the upper and lower limit of agreement are +7.4 ppb and -9.5 ppb, respectively. In our study, both devices yielded comparable eNO measurements in individual subjects, while MINO systematically produced slightly higher values. Although similar with other studies comparing MINO with the NIOX stationary unit, this may impact clinical interpretation if patients are assessed on both devices alternat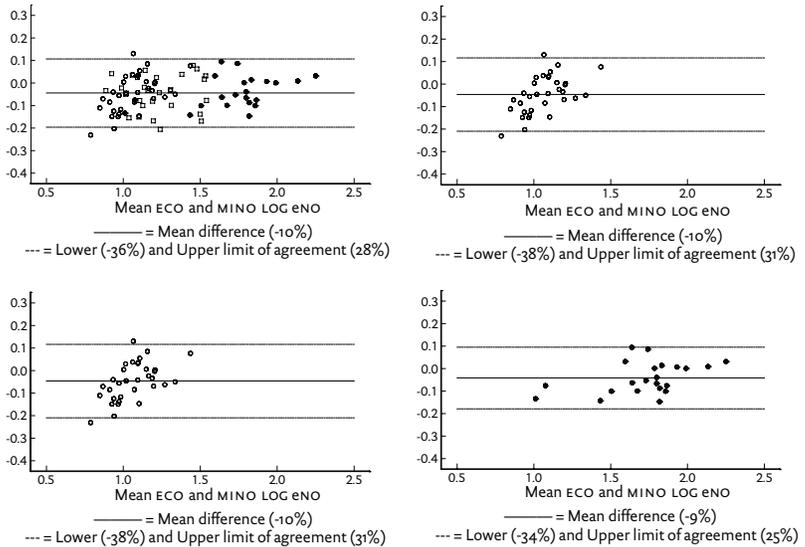ely (3,4,10,13). Hence, the cut-off eNO values should be adjusted for MINO. In a previously conducted study comparing chemoluminescence analyzers, the ECO produced overall lower eNO values than the other stationary analyzers (14,15). In conclusion, it is clear that eNO analyzers yield comparable, but not interchangeable eNO values. This implicates that, ideally, in an individual patient, all eNO measurements should be performed on the same analyzer.

A potential issue may be the difference in lower detection limits between the ECO (0.1 ppb) and the MINO (5 ppb). Indeed, in this study one subject (smoker) had to be excluded from analysis because his eNO values on the ECO were under the detection limit of the MINO. From a clinical perspective, this may not have consequences since very low eNO values are not clinically relevant in asthma. However, should the device need to be employed in the lower detection range, this issue will have to be resolved. This may explain the larger variance between both devices in the smoker group, although the subject numbers were too small to show a significant difference in variances in the lower detection ranges between study groups. Other studies comparing the MINO device with chemoluminescence analyzers did not find any difference between the lower and higher eNO values (8,10).

In conclusion, eNO values measured with MINO are reproducible and generally in agreement with the ECO. Its simplicity, relatively low costs and small size make the MINO device more suitable than the stationary chemoluminescence analyzers for primary healthcare and large clinical trials. Conversely, it cannot be used in research settings requiring more sophisticated measurements including nasal NO, very high or low eNO values or samplings at different flow rates. Our results add further evidence to the reliability of MINO and warrant its applicability in clinical practice and research.

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NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

Combining alternative sputum processing methods & sensitive detection techniques for biomarker analysis: a feasibility study

J.D. Boot[1], K. van Dyck[2], M. Veselic[3], Arnaldo Pica-Mendez[4], M. Tanen[4], M. Ruddy[4], A.F. Cohen[1], Z. Diamant[1]

1   Centre for Human Drug Research Leiden, The Netherlands;
2   Merck Sharp and Dohme (Europe) Inc., Brussels, Belgium;
3   Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
4   Merck Research Laboratories, Rahway, USA

Abstract

Rationale Traditional biomarker quantification methods in exhaled breath condensate (EBC) and sputum have yielded conflicting data due to the lack of standardization of the collection technique (EBC), denaturant effects of sputum processing (dithiothreitol (DTT)), and the limited sensitivity of most detection assays.

Aim We aimed to improve the recovery of inflammatory biomarkers from sputum and EBC from atopic asthmatics. To this end, we applied two alternative processing methods to sputum and sensitive detection techniques in both sputum and EBC.

Methods On two separate visits, EBC and NaCl 4.5%-induced sputum was obtained from 10 non-smoking, clinically stable, atopic asthmatics (4M/6F, 21-54 y, FEV_1 70-122% predicted, $PC_{20}FEV_1$ methacholine<8 mg/mL, SABA *prn* only). EBC was analyzed with antibody based flow-cytometry using Luminex Multi-Analyte Profiling beads. The sputum was processed conventionally, by treatment with DTT to solubilize mucins followed by dialysis to remove DTT (DTT-dialysis) and analyzed with antibody based electrochemiluminescence using Mesoscale multi-array microplates. Subsequently, sputum was obtained from 4 of these patients on two extra occasions: the sample was split manually and half of each sample was processed conventionally and the cell pellet was used as a quality check; the other half was ultracentrifuged (35,000rpm, Ultra) and analyzed with Mesoscale and Luminex.

Results All subjects expectorated analyzable sputum. In DTT-dialysis-processed sputum, spiking experiments with exogenously added cytokines and chemokines showed a poor recovery and almost all biomarkers remained under detection level. After Ultra-processing, several biomarkers were measurable with Mesoscale yielding similar levels on two separate occasions. Hence, Luminex platform analysis was applied allowing detection of a large array of biomarkers. Applying Luminex in EBC, many biomarkers remained below detection limit. In the sputum samples we were able to isolate RNA and perform RNA profiling in the majority of collected samples. Gene expression profiling appeared similar on both visits within subjects.

Conclusion In this exploratory pilot study, we found that recovery of biomarkers was superior in Ultra-processed sputum as compared with DTT-dialysis treated samples, when measured with Mesoscale. Luminex allowed the detection of similar and other biomarkers. In EBC most biomarkers remained below detection limit despite a sensitive detection technology (Luminex). RNA isolation from sputum cells was highly successful and appeared repeatable.

Introduction

Non-invasive sampling methods, including the collection of exhaled breath and sputum analysis, are increasingly applied for assessments of the airway inflammation in clinical monitoring and research in asthma and COPD (1). However, traditional biomarker quantification methods in exhaled breath condensate (EBC) and sputum have yielded conflicting data, due to the lack of standardization of the collection technique (EBC) or to denaturant effects of sputum processing with dithiothreitol (DTT), or to limited sensitivity of most detection assays (2-4).

Quantification of inflammatory mediators in sputum supernatant requires homogenization of the sputum sample with subsequent release of cells and mediators from the mucous bonds. Therefore, DTT is added to the sample to reduce disulphide bonds which cross-link mucinous glycoproteins (5). However, this activity also interferes with the essential disulphide bonds in cytokines and chemokines and hampers the immunological detection of these mediators (5) Recently, Erin *et al,* introduced a dialysis technique to DTT-processed sputum samples, removing DTT after homogenisation and restoring the disulphide bonds, and hence substantially increasing the recovery of cytokines and chemokines (6). Significant differences in cytokine and chemokine levels in supernatant were detected between healthy volunteers and asthmatics, whilst these differences could not be established with the standardized processing.

Ultrasonification or ultracentrifugation is another technique for releasing cells and mediators by physical homogenization which does not require the addition of DTT (7,8). Ultracentrifugation of the entire sample, immediately after collection, yields a homogenous supernatant, devoid of cell debris and mucous. Hadjicharalambous *et al* showed that eotaxin could readily be measured by enzyme linked immunosorbent assay (ELISA) in induced sputum samples from both asthmatics and healthy volunteers following ultracentrifugation whilst this was not feasible in samples (from the same subjects) treated with dithioerythritol (DTE; i.e. an isomer of DTT) (9). A drawback of this technique concerns the disruption of cells and spilling of intracellular content in the homogenate – which of course, can partly account for higher biomarker concentrations (8).

Following adequate sample processing, mediators are mostly measured with immunoassays (3,8). However, several authors failed to demonstrate most of the soluble inflammatory components due to limited sensitivity of these assays (10). Multiplex platforms may offer an advantage due to their capability of simultaneous measurements of up to 100 analytes in small and diluted sample volumes (25-50 ml), such as sputum supernatant and EBC.

The Luminex platforms combine a sandwich immunoassay with fluorescent bead–based detection increasing the specificity and sensitivity of a measurement (11). Gessner *et al* were able to detect a wide range of cytokines in the exhaled breath condensate of COPD patients using a Luminex immunoassay (12). So far, this has been difficult using traditional immunoassays. Mesoscale is another multi-array technology based on chemiluminesence detection for increased sensitivity.

Inflammatory mediators can also be detected at the RNA level in induced sputum after the cells have been lysed (13). Reverse transcriptase polymerase chain reaction (RT-PCR) is a sensitive and specific method for the detection of messenger RNA (mRNA) concentrations even in samples with a low total cell count (14).

In this pilot study we have collected EBC and sputum from a limited number of atopic asthmatic subjects. We aimed to recover RNA from the sputum cells and using microarray analysis conduct gene expression profiling and investigate, via 2-dimensional clustering, the reproducibility of this technique in sputum samples collected on two separate visits in the same subject. The collected sputum supernatant underwent new and optimized processing methods (dialysis and ultracentrifugation) and was analyzed with more sensitive detection techniques (Mesoscale platform, Luminex immunoassay) on two visits to investigate which inflammatory mediators can be measured and assess the reproducibility. In addition, EBC collected in these same subjects also underwent a Luminex immunoassay analysis. With these results the most suitable processing and detection techniques will be selected for a subsequent allergen challenge study in a similar patient population.

Methods

SUBJECTS

Ten non-smoking subjects with clinically stable, mild to moderate persistent allergic asthma were recruited. All patients had a history of persistent asthma for at least 1 year (according to Global Initiative for Asthma (GINA) criteria 15), without any other clinically relevant disorders. Atopy was established by a positive skin prick test to Dermatophagoides Pteronyssinus (ALK-Abello, Nieuwegein, The Netherlands) at screening. Except for stable, infrequent use of inhaled short-acting β2-agonists as required, no one was using concomitant anti-asthma or anti-allergy medication for at least 6 weeks prior to and during the study. Patients had no history of viral infections of the lower airways for at least 3 weeks before enrollment. Caffeine-containing beverages and short-acting inhaled β2-agonists were withheld at least 8 h before

each visit. The study protocol was approved by the Leiden University Medical Centre Ethics Committee, and all participants gave written informed consent.

DESIGN

The study was performed in a single-center setting in two parts (figure 1). In the first part, EBC and subsequently, sputum samples were obtained from 10 eligible, asthmatic subjects on two separate study visits. The sputum was processed according to a novel dialysis technique described by Erin *et al* (6). Following analysis of the sputum samples from the first part, the second part of the study was initiated. Four asthmatic subjects (from the initial 10) returned for two extra sputum inductions (visit 3 and 4). These sputum samples were split in two equal parts by a forceps: one part was processed according to standard procedures and cell differentials were counted as a quality check, while the other part was ultracentrifuged and analyzed, as described below. All the samplings were performed during the same time of the day (±2 hours) on all study visits – and all study visits were separated by 4-10 days.
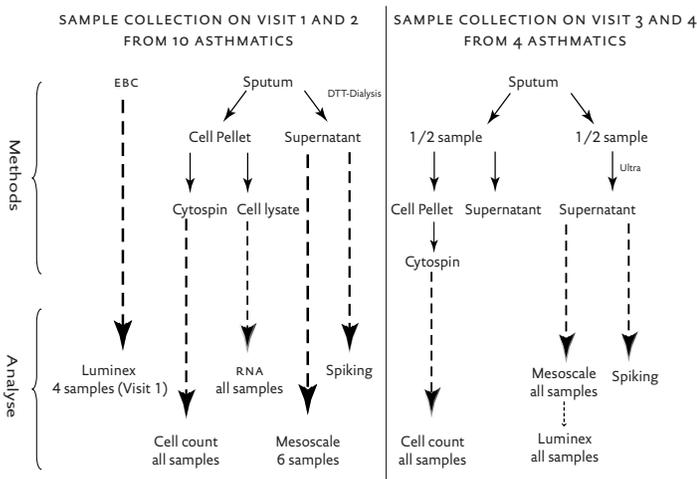
FIGURE 1   Study flowchart

Collection techniques

EXHALED BREATH CONDENSATE COLLECTION

After an acclimatization period of approximately 30 minutes and before sputum induction, each subject was asked to inhale and exhale calmly, while wearing a nose clip, through a mouthpiece and two-way nonrebreathing

valve connected with the EcoScreen condenser (Jaeger, Cardinal Healthcare, Houten, The Netherlands) during a fixed period of 20 minutes (3). Immediately after collection, condensate samples were aliquoted and stored at -80°C pending analysis.

SPUTUM INDUCTION

Sputum induction was performed according to a validated method. Hypertonic saline aerosols (NaCl 4.5%) were generated at room temperature by a DeVilbiss Ultraneb 2000 ultrasonic nebulizer (Tefa Portanje, Woerden, The Netherlands) and inhaled for 3x5 min at 15 min intervals (16). After each inhalation, or as soon as the subjects experienced cough, they were asked to blow their nose, to rinse their mouth and throat with water, and to expectorate sputum into a clean plastic container by coughing.

Sputum processing techniques

DIALYSIS TECHNIQUE (DTT DIALYSIS)

On study visits 1 and 2 whole sputum samples were processed according to a previously validated protocol by Fahy *et al* (17), with modifications (18,19). Briefly, the volume of the induced sputum samples was determined and mixed with an equal volume of 0.1% sputolysin (Dithiothreitol, Calbiochem, La Jolla, CA, USA). Prior to addition to the sputum, a protease inhibitor tablet (Complete Protease Inhibitor Cocktail tablets, Roche Applied Science #11 697 498 001) was added to 0.1% DTT solution (1 tablet per 50 mL of solution). To ensure adequate homogenization, the samples were placed in a shaking water bath at 37°C for 15 min, once interrupted by gently mixing the sample with a plastic pipette. The homogenized sputum was centrifuged at 1400 r.p.m. at 4°C for 10 min. The sputum supernatant was collected and stored at -80°C until further processing.

After thawing the sputum supernatant 2 μL of 0.5M EDTA and 50 μL of 20% BSA was added per 1 mL of sputum supernatant. Prepared samples were transferred into equilibrated 3,500 MWCO dialysis cassettes (Thermo Scientific cat. no. 66330; 0.5-3.0 mL capacity) with the use of a syringe (Pierce cat. no. 66494). The dialysis cassettes containing prepared samples were placed in a 4 L beaker filled with prechilled Dialysis Buffer 1 and were incubated stirring overnight at 40°C. On the next day, the dialysis cassettes were transferred into a 4 L beaker containing prechilled Dialysis Buffer 2 and were incubated stirring overnight at 40°C. After the double dialysis the sputum supernatants were collected using a syringe and stored at -80°C until Mesoscale and Luminex analysis.

RNA ANALYSIS

After centrifugation and removing the supernatant, the sputum cell pellets were resuspended in PBS to a final volume of 2 mL, followed by filtration through a gauze (pore size 1 mm) to remove clumps. Subsequently, 360 μl was taken for differential cell count. The remaining cell suspension was centrifuged at 4°C for 10 minutes at 390 G (1500 rpm). The supernatant was carefully collected to prevent disrupting of the cells and discarded. The cell pellet was immediately re-suspended in 1ml of cold TRIzol reagent (Invitrogen, Cat. #15596-018). 1.5 mL of TRIzol was added if the total cell count is > 5×10^6 cells to ensure complete lysis. The solution was mixed by pipetting up and down with a 1 ml pipette, and vortexing. The resulting lysate was stored at -80°C pending RNA analysis.

ULTRACENTRIFUGE (ULTRA)

Four subjects were asked to expectorate sputum on two additional study visits. The sputum samples were carefully divided manually into two portions of equal weight. One part was processed using DTT (as described above) and only the cell pellet was used for cell differentials (as quality control). The other part of the sample was weighed into an ultracentrifuge container (Beckman Coulter 1.5 mL polyallomer microfuge tube; cat. no. 357448 or 3.2 mL glass tube; cat. no. 362333) and 50 μL of a protease inhibitor cocktail was added per 200 mg of weighed sputum. The protease inhibitor cocktail was prepared by dissolving one protease cocktail tablet into 50 mL of 1X PBS (Invitrogen cat. no. 14040). Prepared samples were subsequently ultracentrifuged for 90 minutes in a high-speed ultracentrifuge (Beckman Coulter Inc. Optima Max Ultracentrifuge 130,000 rpm; Fullerton, California) at 35,000rpm (53,500 x g) at 4°C. Sputum supernatants were then collected without disrupting the pellet and stored at -80°C until Mesoscale and Luminex analysis. The pellet was discarded.

Analysis techniques

EXHALED BREATH CONDENSATE ANALYSIS

Biomarkers were measured using the Luminex Human Multi-Analyte Profiles (MAP) immunoassay platform of Rules Based Medicine (Austin, TX, USA) according to manufacturer's directions.

SPUTUM ANALYSIS – SOLUBLE PHASE

To determine the effects of both processing methods on sputum mediators, a recovery analysis was performed by spiking 500 pg of two cytokines, IL-13

(MSD IL-13 Calibrator for Ultrasensitive kit (MSD cat. no. k111ACS-2)) and TNF-alpha (R&D Systems cat. no. 210-TA-050) per 1 mL of sputum prior to incubation at 37°C in the procedure in three randomly selected samples. The spiked analyte levels were within the detection range of the assays. The Mesoscale assays (TNF-α, IL-13, TARC and eotaxin) were performed by electrochemiluminescence (MSD Clinical Development Laboratory, NJ, USA) according to manufacturer's directions.

In samples where biomarkers could be measured using Mesoscale, additional analyses were performed using the Luminex HumanMAP antigen platform of Rules Based Medicine according to manufacturer's directions.

SPUTUM ANALYSIS – CELLULAR PHASE

Cell counts were performed in a haemacytometer (Novex Microscope, The Netherlands), to obtain an estimate of the total number of cells per sample. Subsequently, the samples were diluted with PBS to a final concentration of ± 0.4 x 10^6 cells/mL which was used for preparation of cytocentrifuge slides (50 µL/cytospin; Shandon Cytospin 4, Thermon Electron Corporation, Runcorn, UK). Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells were performed on May-Grünwald-Giemsa-stained cytospins by a certified cytopathologist. In addition, mast cells were counted on Toluidine blue-stained cytospins. In each sputum sample, at least 500 nucleated cells, excluding squamous cells, were counted twice and the average percentage of each cell type was calculated and expressed as percentage nonsquamous cells. If > 80% of the cell count consisted of squamous cells, the quality of the sputum sample was judged unsatisfactory and the entire sample (including the supernatant) was excluded from analysis.

SPUTUM ANALYSIS - GENE EXPRESSION

RNA was isolated from the cell lysate collected on visit 1 and 2 following standard RNA extraction procedures. Total RNA quality (sample integrity) was evaluated utilizing 28S/18S ratio and RNA Integrity Number (RIN) scores obtained from the Agilent Bioanalyzer capillary electrophoresis system. Sample concentration was assessed utilizing the RiboGreen® (Invitrogen) fluorescent dye system. Established pass/fail criteria were then used to identify samples of sufficient quality and concentration for expression profiling. Only specimens that passed the two QC steps were amplified and profiled. These samples were amplified using the NUGEN Ovation amplification technology and profiled on Affymetrix arrays. After scanning, the array data were normalized by multiarray average (RMA) of background-adjusted, normalized, and log-transformed perfect match (PM) values. A profiling QC step was

performed, assessing in particular the percentage of present calls, scale factor, GAPDH and Actin mRNA 5' to 3' ratios for each hybridization, in order to identify potential outliers. Based on this quality check outliers were excluded from subsequent data analysis. The remaining samples were analyzed by 2D unsupervised hierarchical clustering.

ANALYSIS

Only sputum samples with an adequate quality (<80% squamous cells and 500 nucleated cells counted) were included into analysis. The least detectable dose (LDD) was determined as the mean + 3 standard deviations of 20 blank readings. Group sizes in this pilot study were too small for statistical analysis, hence, descriptive statistics was used to describe the results.

Results

EBC ANDSPUTUM QUALITY ASSESSMENT

EBC was obtained from all subjects. All ten subjects were able to expectorate adequate (<80% squamous cells and 500 nucleated cells counted) sputum samples on all visits.

SPUTUM SUPERNATANT AND EBC

Spiking showed a poor recovery for the sputum samples obtained on the first two study visits and processed with the DTT-Dialysis technique (Table 1). Therefore, Mesoscale analysis was randomly performed in 6 samples only. Almost all cytokines and chemokines appeared below the least detectable dose (LDD) (Table 2) and hence, the remaining samples were not analyzed. In the second part of the study, we collected sputum samples from 4 of the 10 asthmatics on two extra visits. Following processing with the Ultra technique, these samples showed a much higher recovery in the spiking experiments (Table 1). Subsequently, these samples were analyzed with the Mesoscale platform (Table 2). The positive results provided support to additional analysis with Luminex. The relevant detectable mediators for inflammatory airway disease are summarized in Table 3. In the same subjects EBC (collected on Visit 1) was also analyzed with Luminex (Table 3).

RNA RECOVERY FROM SPUTUM CELLS

In part 1 of the study, from 17 out of 20 collected sputum samples (10 subjects; 2 visits) sufficient RNA could be extracted from the cell pellet lysate. Specimens collected from the same patient at different times were co-clustered in a dendogram as presented in Figure 2. These data show that total

RNA extraction and profiling of samples obtained on both study visits (visit 1 and 2) were reproducible with variation in the gene expression profiles being smallest within the individual patient.

TABLE 1   PERCENTAGE RECOVERY OF SPIKED IL-13 AND TNF-α MEASURED BY MESOSCALE

PROCEDURE	IL-13 (PG/ML)	TNF-α (PG/ML)
DTT-Dialysis technique (n=3)	27.9 % (± 11.6)	51.6 % (± 4.3)
Ultra technique (n=3)	110.6 % (± 11.3)	62.4 % (± 6.2)

Values are presented as mean (± SD)

TABLE 2   LEVELS OF INFLAMMATORY MEDIATORS IN SPUTUM SUPERNATANT FOLLOWING DTT-DIALYSIS AND ULTRA-PROCESSING MEASURED BY MESOSCALE

PROCEDURE	SUBJECT	EOTAXIN-3 (PG/ML)	TARC (PG/ML)	IL-13 (PG/ML)	TNF-α (PG/ML)
DTT-Dialysis technique	Nr 1 - Visit 1	<LDD	<LDD	<LDD	<LDD
	Nr 1 - Visit 2	<LDD	<LDD	<LDD	<LDD
	Nr 4 - Visit 1	147	<LDD	<LDD	<LDD
	Nr 5 - Visit 1	<LDD	<LDD	<LDD	<LDD
	Nr 5 - Visit 2	<LDD	<LDD	<LDD	<LDD
	Nr 8 -Visit 1	129	12.2	<LDD	<LDD
Sputum Ultra technique	Nr 7 - Visit 3	14.5	<LDD	<LDD	1.74
	Nr 8 - Visit 3	216	20.5	<LDD	5.89
	Nr 9- Visit 3	641	14.3	0.79	25.8
	Nr 10 - Visit 3	37.9	<LDD	<LDD	<LDD
Sputum Ultra technique	Nr 7 - Visit 4	<LDD	<LDD	<LDD	1.57
	Nr 8 - Visit 4	24.4	10.5	<LDD	1.91
	Nr 9 - Visit 4	4892	59.5	3.2	40
	Nr 10 - Visit 4	71.2	<LDD	<LDD	<LDD
<LDD		9.6	9.6	.59	1.52

Mesoscale analysis was randomly performed in 6 samples only on Visit 1 and 2. Four asthmatics returned for Visit 3 and 4 for an extra sputum induction. LDD = Least detectable dose

TABLE 3   LEVELS OF INFLAMMATORY MEDIATORS IN SPUTUM SUPERNATANT AND EXHALED BREATH CONDENSATE MEASURED BY LUMINEX.

PROCEDURE	SUBJECT	α1 ANTI (UG/ML)	α2 MACRO (UG/ML)	ENA-78 (NG/ML)	ENDO-1 (PG/ML)	EOTAXIN (PG/ML)	GLUT-S-TRANS (NG/ML)	IL-13 (PG/ML)	IL-16 (PG/ML)	IL-6 (PG/ML)	IL-8 (PG/ML)	MCP-1 (PG/ML)	MMP-2 (NG/ML)	MPO (NG/ML)	TIMP-1 (NG/ML)	TNF-α (PG/ML)
Sputum Ultra technique	Nr7 - Visit 3	1.04	1.98	0.60	7.63	17.50	0.14	<LDD	87.20	4.96	1350.00	21.60	44.90	84.90	182.00	1.18
	Nr 8 - Visit 3	0.83	11.50	2.89	7.63	26.80	0.14	<LDD	1900.00	50.70	7720.00	230.00	60.10	150.00	316.00	3.65
	Nr9 - Visit 3	1.00	3.20	2.43	9.13	26.70	0.19	<LDD	2200.00	151.00	16400.00	206.00	59.40	98.30	271.00	12.40
	Nr10 - Visit 3	0.65	12.90	0.79	20.80	26.30	0.13	<LDD	64.50	3.11	507.00	39.90	48.80	42.20	109.00	0.46
Sputum Ultra technique	Nr7 - Visit 4	2.16	1.96	0.55	3.81	44.50	0.14	<LDD	126.00	8.12	2730.00	71.70	14.80	731.00	267.00	2.18
	Nr 8 - Visit 4	2.78	10.70	2.08	1.57	56.10	0.17	<LDD	734.00	30.70	3990.00	347.00	19.60	2830.00	415.00	2.74
	Nr9 - Visit 4	2.36	<LDD	2.03	5.14	199.00	0.20	<LDD	3880.00	298.00	33200.00	471.00	30.60	6260.00	>533	49.70
	Nr10 - Visit 4	2.99	3.75	1.57	20.20	154.00	0.16	<LDD	348.00	11.20	2030.00	200.00	31.50	855.00	351.00	1.45
EBC	Nr 7 - Visit 1	<LDD	<LDD	0.01	6.75	26.60	0.18	<LDD	<LDD	<LDD	1.59	4.31	9.72	<LDD	<LDD	1.81
	Nr 8 - Visit 1	<LDD	<LDD	0.01	8.26	22.50	0.18	<LDD	<LDD	<LDD	2.62	4.66	12.30	<LDD	0.13	1.96
	Nr9 - Visit 1	<LDD	<LDD	0.02	5.78	22.50	0.20	<LDD	<LDD	<LDD	2.10	6.67	8.25	<LDD	<LDD	1.19
	Nr10 - Visit 1	<LDD	<LDD	<LDD	6.03	22.50	0.15	<LDD	<LDD	<LDD	<LDD	3.61	6.78	<LDD	<LDD	1.35
<LDD		5.3E-05	0.30	0.02	1.44	8.20	0.08	11.38	13.24	2.44	0.70	10.40	30.00	1.36	0.04	0.80

Four asthmatics returned for Visit 3 and 4 for an extra sputum induction. In the same subjects EBC (collected on Visit 1) was also analyzed. LDD = Least detectable dose
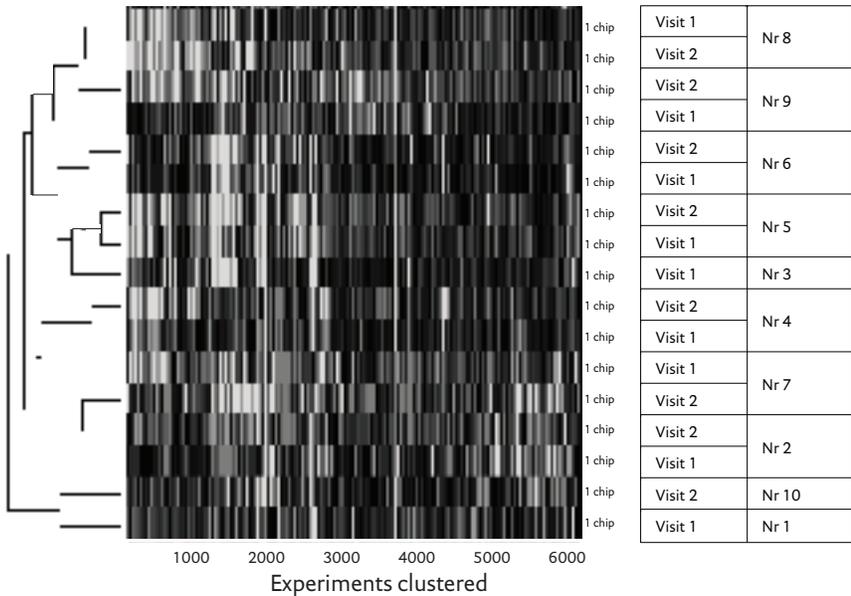
FIGURE 2   Two-dimensional clustering of MRNA collected from asthmatic subjects on study visit 1 and 2.

Discussion

This pilot study was aimed to improve the recovery of inflammatory bio-markers obtained with non-invasive airway sampling techniques, EBC and induced sputum – both from the soluble and the solid phase. To this end we tested two alternative sputum processing methods and applied novel sensitive detection techniques to both the sputum and EBC samples. Applying Mesoscale analysis on sputum samples, processed by the dialysis technique previously introduced by Erin *et al,* we were unable to measure relevant cytokines and chemokines (6). Therefore, this processing technique was not pursued any further, and we processed sputum supernatant with the DTT-free Ultracentrifuge technique described previously (9). In these samples we found a good recovery of spiked cytokines and chemokines, and we were able to quantify several cytokines and chemokines. In addition, in these samples we were able to measure multiple inflammatory mediators with the

NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA AND ALLERGIC RHINITIS

Luminex analysis. In contrast, applying Luminex analysis to the EBC samples of these subjects, most mediators remained under LDD. Regarding the solid phase-biomarkers: RNA isolation from sputum cells was highly successful. Seventeen out of 20 collected samples passed the quality control tests and the gene expression profiling appeared similar on both visits within subjects.

In contrast to the DTT-Dialysis, applying the Ultra technique, we were able to quantify several inflammatory mediators in sputum samples. We decided to specifically measure eotaxin-3 with the Mesoscale, since a study by Berkman *et al* reported that eotaxin-3 - and not eotaxin - was upregulated in bronchial biopsies and bronchoalveolar lavage fluid in atopic asthmatics following inhaled allergen challenge (20). We found similar levels of eotaxin-3 measured with Mesoscale and eotaxin measured with Luminex as previously reported by Hadjicharalambous following ultracentrifugation of sputum samples of patients with mild to moderate persistent asthma (9). The authors applied this technique because in their study spiked sputum samples showed low recovery following treatment with DTE without any increase following ultrafiltration. These findings are comparable to our results applying the DTT-Dialysis technique. Interestingly, in the same study, eotaxin levels were not higher following ultracentrifugation (13,000g) compared to regular speed centrifugation (400 g). However, it is unlikely that the Ultra technique yields similar data as the DTT processing technique, since ultracentrifugation causes intracellular contents to spill into the supernatant and consequently may increase the levels of the inflammatory markers. In addition, applying this technique, cell counts cannot be performed due to the cellular disruption by the high speed centrifugation. This can be overcome by splitting the samples, so in one part cell counts can be performed, for the purpose of quality control of the entire sample, while the remaining part is used to investigate the presence of DTT-sensitive biomarkers. Although the numbers were small in the second part of the study, the techniques appeared to yield reproducible results. Patients with the highest biomarker levels on visit 3, presented with the highest levels on visit 4 and in the same range for several detectable mediators (Table 2). A similar pattern was seen in the Luminex analysis (Table 3). Obviously, more precise data on the reproducibility of these detection techniques need to be obtained in larger patient and sample numbers in a more standardized manner. In this setting, the Ultra technique should also be compared to the standardized sputum processing methods (including DTT), and not only to the DTT-Dialysis technique.
Despite the sensitive detection technique employed, a wide range of biomarkers remained below the LDD in the Ultra samples and even more so in the EBC. In agreement with the results by Bayley *et al* in a population with COPD, we were also unable to measure α-antitrypsin, IL-8 and myeloper-

oxidase in EBC (21). Possibly, these biomarkers are present in higher concentrations in other asthma-phenotypes or under different conditions, e.g. in a more severe disease or following an exacerbation, rather than in stable disease. For example, Csoma *et al* found higher levels of leukotrienes in EBC in patients with severe asthma than in patients with moderate persistent asthma, while levels in mild asthmatics were similar to healthy controls (22). In addition, several studies reported increased levels of leukotrienes following an asthma exacerbation as compared to stable disease (22,23).

The large array of mediators in the Luminex platform is ideal to provide an impression of which inflammatory mediators are present in a sample and hence, can help to decide on what biomarkers to focus. Subsequently, the Mesoscale platform that was specifically developed at a collaborating laboratory to measure mediators in sputum can reliably quantify these mediators.

Most studies have employed RT-PCR to focus on the expression of several genes of interest (13,24). In this pilot study, we are amongst the first to perform global genes expression profiling in sputum cells. We were able to recover suitable RNA in the majority of samples and the profiles were fairly similar on visit 1 and 2 in the same subjects. Again, RNA expression profiling is probably more valuable in an intervention study whereby the profiles before and after the intervention (e.g. allergen challenge or drug administration) can be compared. For example, a recent study reported a clear enhancement of expression patterns involved in airway inflammation following an endotoxin challenge in allergic individuals compared to pre-challenge expression levels (25).

In conclusion, we compared two alternative processing techniques of induced sputum in combination with two sensitive biomarker detection techniques to improve biomarker quantification from non-invasive sampling techniques. The DTT-dialysis technique did not yield measurable levels of inflammatory mediators in sputum samples. Applying the Ultra technique, we were able to measure several cytokines and chemokines in sputum and also in EBC employing the broad Luminex platform and sensitive Mesoscale analysis. However, many mediators still remained below the LDD. In the same sputum samples we were able to isolate RNA and perform expression profiling. This technology provides a viable platform for identification of specific signatures between patients. These techniques will be applied and further validated in an interventional allergen challenge study in our institute. These findings may open a pathway to identify a magnitude of potential biomarkers even in small volume-samples.

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NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

Applicability and Reproducibility of Biomarkers for the Evaluation of Anti-Inflammatory Therapy in Allergic Rhinitis

J.D. Boot[1], P. Chandoesing[1], M.L. de Kam[1], M.A. Mascelli[2], A.M. Das[2], R. Gerth van Wijk[3], H. de Groot[3], R. Verhoosel[4], P.S. Hiemstra[4], Z. Diamant[1]

1   Centre for Human Drug Research, Leiden, The Netherlands
2   Centocor Inc., Malvern, Pennsylvania, USA
3   Department of Internal Medicine, Allergology Section, Erasmus Medical Centre, Rotterdam, The Netherlands
4   Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands

Abstract

Background: We aimed to study the reproducibility of several biomarkers of allergic rhinitis to investigate their potential as outcome measures in clinical intervention trials. Furthermore, we investigated the kinetics of the biomarkers studied in nasal lavage and brush material following a placebo-controlled nasal allergen challenge.

Methods: We performed a skin prick test and measured serum specific immunoglobulin (Ig) E levels and inflammatory biomarkers in nasal lavage and brush material in 20 patients with allergic rhinitis on 2 separate days (washout, 14-21 days). The patients were then randomly assigned to undergo an intranasal challenge with a relevant allergen (n = 10) or diluent (n = 10) in order to assess the kinetics of several biomarkers of allergic airway inflammation in nasal lavage and brush samples.

Results: Baseline serum IgE levels and skin wheal sizes were highly reproducible measurements, with a coefficient of variation (CV) of 13.4% and 18.2%, respectively. This was not the case with the majority of inflammatory biomarkers, whose CV varied considerably (range, 6.1%-224.1%). The nasal allergen challenge induced an increase in composite symptom scores in all patients. Compared to placebo, tryptase (p = .004), eosinophilic cationic protein (ECP) (p = .03) and α2-macroglobulin (p = .002) were increased in nasal lavage at 20 minutes post-allergen. Nasal lavage ECP levels and nasal brush eosinophils were still significantly increased at 7 hours (p = .03 and p = .04), but all statistical significance had been lost at 24 hours post challenge.

Conclusion: Serum specific IgE assays and skin prick tests exhibited good reproducibility in patients with clinically stable allergic rhinitis. We were also able to investigate the kinetics of allergen-induced upper airway inflammatory markers in nasal lavage and brush material. Hence, nasal allergen challenge, when used in combination with nasal lavage and brush sampling, is a suitable research tool for early drug development.

Introduction

Allergic rhinitis is a chronic inflammatory disorder of the upper airways that produces characteristic signs and symptoms in sensitized individuals exposed to relevant allergens (1). Allergic airway inflammation is characterized by immunoglobulin (Ig) E-triggered mast cells and activated eosinophils, which release proinflammatory mediators such as histamine and leukotrienes (2). Furthermore, allergic rhinitis is associated with elevated serum IgE levels and a positive skin prick test (SPT) to corresponding allergens (1, 3). In addition to their diagnostic value, serum specific IgE levels and SPT results may serve as biomarkers to monitor disease activity in response to antiallergic therapy (4). In allergic rhinitis, interventional studies with antihistamines and immunotherapy have shown decreases in allergic symptoms and reduced wheal and flare responses to intradermal allergens (5, 6). Similarly, in patients with allergic rhinitis and asthma, treatment with the anti-IgE antibody omalizumab substantially lowered free serum IgE levels with subsequent improvements in symptoms (7, 8).

Nasal allergen challenge is a validated, reproducible clinical model for investigating the pathophysiology of allergic rhinitis that also permits the study of the kinetics of nasal inflammatory responses (9). As such, it may serve as a tool to study the effects of anti-allergic interventions targeting specific inflammatory mechanisms in relationship to upper airway response (10). Although nasal biopsies are the golden standard for studying cellular inflammatory response, there are some limitations to this invasive technique: it can only be performed by an experienced ear, nose, and throat physician; it provides information on just a limited part of the upper airways; and it does not allow repeated samplings within short time intervals (11, 12). Nasal brushing, in contrast, is a less invasive method that has emerged as a possible viable alternative for interventional trials requiring repeated sampling. In patients with allergic rhinitis, Jacobson *et al* showed that seasonal changes in the number of mast cells and eosinophils in nasal brush samples correlated well with those found in nasal biopsies (12). Likewise, intranasal fluticasone produced a similar degree of reduction in these inflammatory cells in both brush and biopsy samples. Nasal lavage is another relatively noninvasive sampling technique which allows serial assessments of the effects of anti-inflammatory therapy on soluble components of upper airway inflammation (13).

The aim of this study was to analyze the reproducibility of various biomarkers associated with allergic rhinitis to assess their potential use as outcome measures in clinical trials testing novel anti-inflammatory therapies. To this end, in a group of clinically stable patients with untreated allergic rhinitis, we combined several semi-invasive (sampling) techniques and analyzed serum

specific IgE levels, SPT results, and various cellular and soluble biomarkers of upper airway inflammation identified in nasal lavage and brush material. In addition, we tested the effect of nasal allergen challenge on the kinetics of these inflammatory biomarkers in a randomized study design.

Methods

Twenty multisensitized subjects (10 females, 10 males; age, 19-51 years) with nonsymptomatic allergic rhinitis participated in the study. All the subjects had a history of intermittent or persistent allergic rhinitis for at least 1 year prior to enrollment (1). Maintenance anti-allergy or anti-asthma medication was discontinued at least 6 weeks prior to the study. Atopy was confirmed by a positive SPT for at least 2 of 6 common airborne allergens (grass pollen, tree pollen, Dermatophagoides pteronyssinus [HDM], Dermatophagoides farinae, and cat and dog dander) (ALK Abelló, Nieuwegein, The Netherlands). Subjects with any other clinically relevant disorders were excluded. Eligible subjects were sensitized to at least 1 of the allergens used for nasal provocation (grass pollen, HDM, or cat dander extracts) (ALK Abelló). Symptomatic subjects with mite or pollen allergy were tested outside the relevant season (October-February for HDM and May-August for pollen in the Netherlands). In the case of subjects with concomitant allergy to pets, only those with no close contact with pets during the study were included. Allergic rhinitis symptoms were monitored throughout the study using a composite symptom score validated by Lebel *et al* and subjects with a baseline score of more than 2 on the allergen challenge day (visit 2) were excluded (14). Respiratory tract infections were excluded by confirming the absence of relevant symptoms in the 3 weeks prior to and during the study and each nostril was inspected for accessibility using a nasal speculum. All subjects were non-smokers or ex-smokers who had quit at least 12 months prior to the study with a smoking history of under 10 pack years. The study protocol was approved by the Leiden University Medical Centre Ethics Committee and all the subjects gave their written informed consent prior to enrollment in the study.

STUDY DESIGN

We used a combined study design (Figure 1). In the first part of the study, we tested the reproducibility of serum specific IgE measurements, SPT results, and baseline inflammatory marker measurements using nasal lavage and brush material. In the second part of the study, we tested the kinetics of the inflammatory markers analyzed in the first part of the study following a nasal

challenge with an allergen or diluent. Eligible subjects were included in the study and specific serum IgE levels, SPT, and nasal lavage and brush inflammatory markers were measured during visit 1 and repeated 14 to 21 days later during visit 2. Subsequently, subjects were allocated to an allergen group (n = 10) or a placebo (diluent) group (n = 10) in a randomized, double-blind, parallel fashion. Nasal lavage measurements were repeated 20 minutes and 7 hours post challenge (i.e., after the last placebo/allergen dose), and nasal brush measurements were repeated 7 hours post challenge. Symptom scores were recorded before the challenge, after the administration of the diluent, 10 minutes after each challenge dose, and hourly until 7 hours post challenge (14). Clinically stable subjects were dismissed from the unit 8 to 9 hours post challenge and asked to return 24 hours later (visit 3) for recording of symptom scores and nasal lavage and brush measurements and again 7 days later (visit 4) for the final lavage and brush measurements. The occurrence of adverse events was monitored throughout the study. As a safety check, airway response was measured in all patients by forced expiratory volume in 1 second (FEV_1) (Vmax Spectra; Sensor Medics, Bilthoven, The Netherlands) in accordance with standardized lung function techniques. This check was performed during screening, pre allergen, and 7 hours post allergen (15).
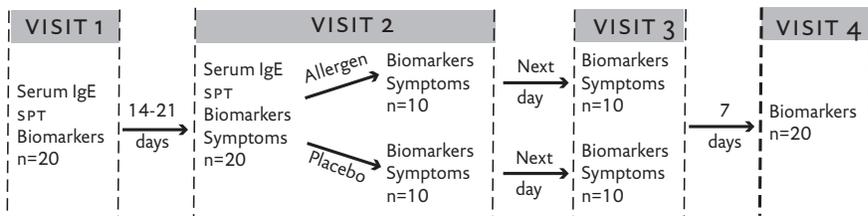
FIGURE 1   Study flowchart. The biomarkers studied were inflammatory markers in nasal brush and nasal lavage material. SPT indicates skin prick test; IgE, immunoglobulin E; n, number of subjects.

SERUM IGE LEVELS

Five mL of venous blood were collected and stored at −80°C for subsequent analysis of total and specific serum IgE levels. Total and specific IgE levels for HDM, grass pollen, tree pollen, and cat and dog dander were determined by fluorescence enzyme immunoassay (FEIA) using the Immuno CAP system (Phadia AB, Uppsala, Sweden). For the reproducibility analysis, we compared baseline specific serum IgE levels from visit 1 versus visit 2 for the allergen with the highest specific IgE level during visit 1.

SKIN PRICK TEST

SPTs were performed in duplicate at the volar site of each forearm by the same experienced technician by application of 1 drop of each allergen extract to the skin, at least 3 cm apart, according to a previously validated protocol (3). Histamine chloride (10 mg/mL) was used as a positive control and a diluent of each allergen (ALK Abelló) as a negative control. Subsequently, the dermis was punctured once with an ALK lancet (ALK Abelló); a clean lancet was used for each solution. Fifteen minutes later, the results were read by the same technician, who marked the edges of the wheal responses (excluding pseudopods) with a special thin-tipped pen. The mark was transferred to transparent tape and attached to the result form. The mean value of the 2 largest perpendicular diameters of wheal responses was included in the analysis. For the reproducibility analysis, we compared wheal sizes (visit 1 vs visit 2) for the allergen yielding the largest wheal response during visit 1.

NASAL ALLERGEN CHALLENGE

Nasal allergen challenges were performed according to a previously validated protocol (13). The procedure is briefly described as follows: to select the intranasal allergen (HDM, grass pollen, or cat dander) for each patient, account was taken of the allergen that had caused the largest wheal in the SPT in combination with the patient's history of clinical symptoms and the season. Pre challenge, subjects were acclimatized for 30 minutes in the test room and then administered 1 puff of xylometazoline 0.1% in each nostril. Approximately 20 minutes later, a puff of allergen diluent (phosphate buffered saline [PBS] containing human serum albumin 0.03% in benzoalkonium chloride 0.05%) (ALK Abelló) was sprayed into each nostril; this was followed by the administration of 1 puff of intranasal allergen (at 3 increasing concentrations of 100, 1000 and 10 000 biological units [BU]/mL) into each nostril at 10-minute intervals. Subjects in the placebo group received the allergen diluent intranasally 4 times using the same time intervals. The puffs were delivered as distally as possible into the nasal cavity using a nasal pump that delivered a fixed dose of 0.125 mL solution per puff (nasal pump 1013463; ALK Abelló). Subjects were instructed to inhale gently to prevent the solution from entering the lower airways. The nasal response at the respective time points was quantified by a validated composite symptom score system (14). Symptoms were recorded using the following scoring system: sneezes ≤ 2 = 0, sneezes 3-4 = 1 point, sneezes ≥ 5 = 3 points, anterior rhinorrhoea = 1 point, posterior rhinorrhoea = 1 point, difficult breathing = 1 point, 1 blocked nostril = 2 points, 2 blocked nostrils = 3 points, pruritus in the nose = 1 point, pruritus of palate or ear = 1 point, conjunctivitis = 1 point (total possible score, 0-11 points). Composite symptom scores were recorded 10 minutes after each dose and dosing was discontinued

when a total score of 6 or more was reached or after the administration of the highest dose (10 000 BU/mL or diluent).

NASAL LAVAGE

The nasal lavage was performed in the same nostril throughout the study using a modified protocol of the technique described by De Graaf-In 't Veld *et al* (13). Briefly, the subjects, in a seated position, were instructed to flex their neck approximately 30° from the vertical and to not breathe through their nose. A nasal olive was inserted into the corresponding nostril and 8 mL of PBS (37°C) was gently instilled using a 10 mL preweighed syringe with a dwelling time of approximately 10 seconds. This procedure was performed twice. The lavage fluid was then collected, kept on ice, weighed, and processed within 1 hour (centrifugation for 10 minutes at 400 g and storage of supernatant at −80°C pending analysis). The following soluble inflammatory markers, listed below with their respective assay kits and detection limits, were determined in the nasal lavage samples according to the manufacturers' directions: interleukin (IL)-8, enzyme-linked immunossorbent assay (ELISA) (CLB, Amsterdam, The Netherlands), 30 pg/mL; IL-13, ELISA (R&D systems, Minneapolis, Minnesota, USA), 600 pg/mL; eotaxin, ELISA (R&D systems), 10 pg/mL; thymus and activation-regulated chemokine (TARC), ELISA (R&D systems), 75 pg/mL; eosinophilic cationic protein (ECP), FEIA (Phadia AB), 2 µg/L; tryptase, FEIA (Phadia AB), 1 µg/L; IgE, FEIA (Phadia AB), 1 kU/L; α2-macroglobulin, ELISA (R&D systems), 10 ng/mL; mucin 5AC (MUC5AC), immunoblotting using apical secretions obtained from bronchial epithelial cells cultured at the air-liquid interface as standard (Neomarkers antibody; Lab Vision Corporation, Fremont, California, USA), 1 arbitrary unit (AU)/mL. Samples were not concentrated before analysis.

NASAL BRUSHINGS

All nasal brushings were performed in the nostril not used for the lavage throughout the study according to a modified version of the original technique described by Jacobson *et al* (12). Briefly, the brush (Buccal Swab Brush; BIOzymTC, Landgraaf, The Netherlands) was introduced between the nasal septum and the inferior turbinate and gently rotated 90° to 180°. Immediately after sampling, the brush was placed in a 3 mL plastic tube containing 2 mL of PBS at room temperature, carefully shaken, and brushed off gently against the wall of the tube. Aliquots (100 µL) containing the cell suspension were loaded into a cytocentrifuge for the preparation of slides, spun for 7 minutes at 400 g (Shandon Cytospin 4; Thermon Electron Corporation, Runcorn, UK), and dried prior to storage at 7°C. Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, nasal epithelial cells, and squamous cells

were performed on coded May-Grünwald-Giemsa-stained cytospins. The cell differentials were expressed as a percentage of 250 nucleated nonsquamous cells; cytospins containing fewer cells were deemed unsuitable for analysis.

Reproducibility of serum IgE results (highest level), SPT measurements (largest diameter), and inflammatory biomarker measurements was assessed by analyzing intrasubject variation between visits, expressed as a coefficient of variation (CV, standard deviation expressed as percentage of the mean). The necessary change in these measurements for the detection of an intervention effect in future studies was calculated using a power calculation program (nQuery advisor 3.0; Dixon Statistical Associates, Los Angeles, California, USA). The within-subject CV was used to estimate the standard deviation of the difference and the calculation was based on a crossover study of 12 patients, a power of 80%, and an α-risk of 5% in a 2-tailed comparison. The effect of each challenge was analyzed with a mixed-model analysis of covariance (ANCOVA) using intervention, time, and intervention by time as fixed factors, the subject as a random factor, and the prechallenge measurement as a covariate. Where necessary, variables were log-transformed to meet analysis requirements. Contrasts between the placebo and allergen challenge were calculated at various time points. When results fell below the detection limit, levels were set at 50% of this limit. When ANCOVA was not possible (in the placebo group, for example, due to a lack of variance), the first postchallenge measurements were analysed using the t test (null hypothesis: mean = 50% of detection limit). An intervention effect with a P value of less than .05 was considered statistically significant. Sample size estimation was based on previous work examining the reproducibility of most of the biomarkers we aimed to evaluate and their response following allergen challenge (16, 17). All the calculations were performed using SAS for Windows, version 9.1.2 (SAS Institute, Cary, North Carolina, USA).

Results

PATIENT CHARACTERISTICS AT BASELINE

None of the subjects had symptomatic allergic rhinitis on either of the 2 study visits; the maximum baseline symptom scores were 2.

REPRODUCIBILITY OF SERUM SPECIFIC IGE LEVELS AND SPT WHEAL SIZES

Both specific serum IgE levels and SPT wheal sizes were found to be reproducible parameters in patients with allergic rhinitis over an observational

period of 14 to 21 days. In all cases, total and maximal serum specific IgE levels and histamine-induced and maximal wheal sizes remained fairly constant between visits 1 and 2 (Figure 1 and Table 2).
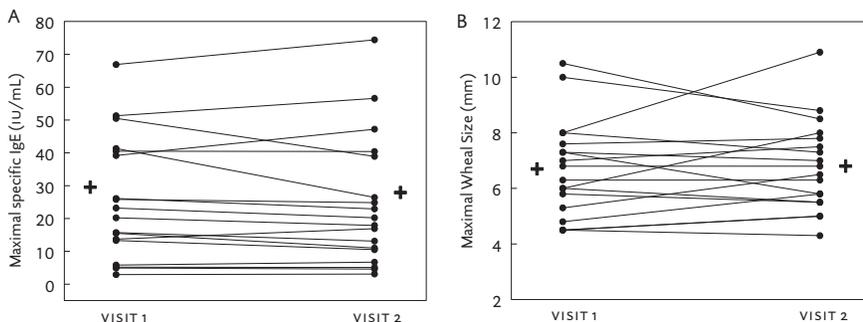
REPRODUCIBILITY OF BASELINE INFLAMMATORY MARKERS IN NASAL LAVAGE AND BRUSH MATERIAL

The reproducibility of inflammatory markers in nasal lavage and brush material varied considerably. While nasal IgE remained fairly constant between visit 1 and 2, ECP and α2-macroglobulin levels differed greatly (Table 1). In addition, levels of IL-8, IL-13, eotaxin, TARC, and tryptase in the nasal lavage fluid were below the detection limit in most samples and hence could not be included in the reproducibility analysis. The changes in serum IgE levels, wheal size, and inflammatory marker measurements needed to detect a significant intervention effect (eg, drug treatment) was calculated using the within-subject CV in a crossover study design with 12 patients (Table 1).

TABLE 1   INFLAMMATORY MARKER MEASUREMENTS ON VISIT 1 AND 2 WITH CORRESPONDING COEFFICIENT OF VARIATION (CV) AND REQUIRED EFFECT SIZE[*]

	VISIT 1	VISIT 2	CV WITHIN SUBJECTS, %	REQUIRED EFFECT SIZE
Serum Immunoglobulin (Ig) E Levels				
Maximal specific IgE, IU/mL	29.3 ± 5.7	27.5 ± 5.3	13.4	18.3%[**]
Total IgE, IU/mL	266.2 ± 54.3	249.5 ± 51.4	18.4	25.9%
Skin Prick Test				
Histamine wheal size, mm	5.7 ± 0.2	6.3 ± 0.2	11.7	0.9 mm
Maximal wheal size for allergen, mm	6.9 ± 0.5	7.1 ± 0.4	18.2	1.6 mm
Nasal Lavage				
Nasal IgE, IU/mL	5.4 ± 0.2	5.5 ± 0.1	6.1	7.9%
Mucin 5AC, AU/mL	44.2 ± 6.5	37.9 ± 7.7	224.1	436%
α2-macroglobulin, ng/mL	377.5 ± 115.5	296.1 ± 91.2	219.7	436%
ECP, ng/mL	3.9 ± 0.9	8.1 ± 4.2	123.3	234%
Nasal Brushing				
Eosinophil count, %	12.4 ± 5.5	12.7 ± 3.7	99.5	183.8%
Neutrophil count, %	76.6 ± 6.7	79.8 ± 5.2	39.9	63.3%

Abbreviations: AU, arbitraty units; ECP, eosinophil cationic protein. * Results are expressed as mean (± SEM) unless otherwise indicated. ** For example, in a crossover study with 12 patients, a real change in serum specific IgE of 18.3% or more can be related to the treatment effect and not the natural variation of this biomarker in 80% of experiments.

FIGURE 2   A, individual maximal serum specific immunoglobulin (IGE) levels at visits 1 and 2. B, individual maximal wheal sizes at visits 1 and 2. + shows mean levels.

NASAL ALLERGEN CHALLENGE

Intranasal interventions (allergen and diluent) were well tolerated by all subjects and no clinically significant adverse events were recorded. Furthermore, there was no significant fall in FEV_1 from baseline in any of the patients at 7 hours post challenge. The number of patients with concomitant allergic rhinitis and asthma was equal in the allergen and diluent group. As compared with placebo (P), intranasal allergen (A) induced a nasal early allergic response (EAR) in all subjects with a mean symptom score (mean ± SEM, 7.25 ± 0.56 [A] vs 0.70 ± 0.26 [P]) at 10 minutes post challenge (Figure 3). The allergen-induced nasal EAR was accompanied by significantly increased levels of tryptase, α2-macroglobulin and ECP (4.39 ± 2.0 [A] vs 0.5 ± 0.0 ng/mL [P]; 6630.7 ± 4240.23 [A] vs 51.7 ± 28.58 ng/mL [P]; 24.19 ± 18.33 [A] vs 1.17 ± 0.17 ng/mL [P], respectively), but not nasal IgE or MUC5AC (5.20 ± 0.14 [A] vs 5.33 ± 0.25 IU/mL [P] and 72.60 ± 23.35 [A] vs 33.8 ± 6.69 AU/mL [P], respectively) in the nasal lavage fluid at 20 minutes post challenge (Figure 3). In addition, all subjects had a nasal late allergic response (LAR), defined as a composite symptom score above baseline on 2 consecutive time points between 3 to 7 hours post allergen (1.3 ± 0.31 [A] vs 0.24 ± 0.21 [P] (Figure 3) in combination with a significant increase in nasal eosinophil count and its degranulation marker ECP (20.9 ± 4.75 [A] vs 12.1 ± 3.79 % [P] and 25.19 ± 19.18 [A] vs 1.72 ± 0.39 ng/mL [P], respectively) (Figure 3) (13). Contrasts between allergen and placebo challenges were calculated at selected time points as shown in Table 2. The inflammatory markers described above had all returned to baseline levels by visit 4 (7 days post challenge). Levels of the other inflammatory markers (IL-8, IL-13, eotaxin, and TARC) remained below detection limits in most nasal lavage samples

and were not affected by either challenge, and hence could not be included in the analysis. The overall sample recovery rate for nasal lavage samples during the study was 72% (mean ± SEM, 5.7 ± 0.9 mL). Eighty seven percent of all the nasal brush samples were suitable for analysis.
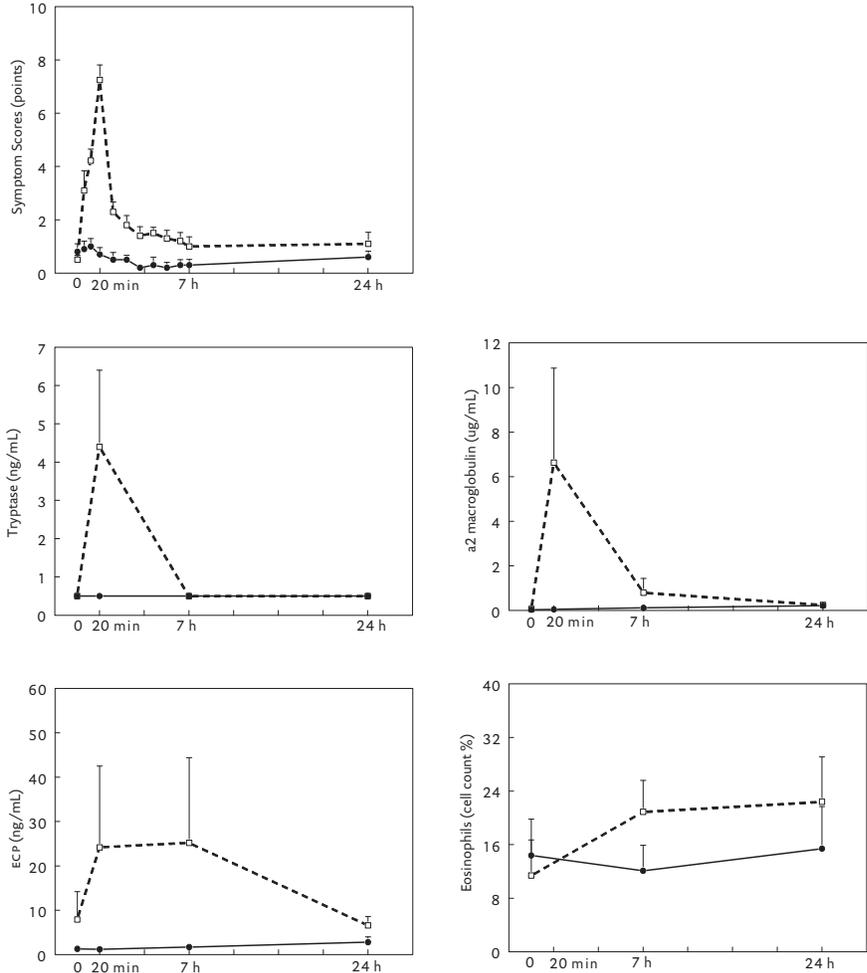

FIGURE 3   Mean change (± SEM) in symptom scores, tryptase, α2-macro-globulin, and eosinophilic cationic protein (ECP) levels in nasal lavage and eosinophil count in nasal brush material following allergen challenge (open square) and placebo challenge (filled circle).

PARAMETER (TIME POINT)	LSM - ALLERGEN	LSM - PLACEBO	LSM DIFFERENCE, % (95% CI)	P VALUE
Overall symptom score, points	2.21	0.28	1.9 (2.4-1.4)	< .001
IgE, IU/mL (20 min post challenge)	5.25	5.22	0.5 (−12.5-15.5)	.93
Mucin 5AC, AU/mL (20 min post challenge)	33.52	39.58	−36.2 (−82.4-131.9)	.64
Tryptase, ng/mL (20 min post challenge)	NA*	NA*	2.21 (0.9-5.3)*	.004*
α2-macroglobulin, ng/mL (20 min post challenge)	815.24	28.75	2735.2 (334-18425)	.002
α2-macroglobulin, ng/mL (7 h post challenge)	121.4	50.1	142.2 (−30.1-741.1)	.15
ECP, ng/mL (20 min post challenge)	4.30	1.11	287.8 (11.3-1251.4)	.03
ECP, ng/mL (7 h post challenge)	5.69	1.44	295.4 (24.9-1151.9)	.02
Eosinophil count, % (7 h post challenge)	25.25	10.45	144.4 (13.8-424.8)	.04
Eosinophil count, % (24 h post challenge)	21.98	9.73	125.8 (-31.0-639.1)	.16

Abbreviations: CI, confidence interval; ECP, eosinophilic cationic protein; LSM, least square means; NA, not applicable. * Tryptase under challenge was tested against the null hypothesis mean = 50% of detection limit.

Discussion

In the present study of patients with allergic rhinitis, we tested the reproducibility of several inflammatory biomarkers obtained by semi-invasive sampling techniques in combination with their kinetics in nasal lavage and brush material following a nasal challenge involving allergens and placebo. We found that, in clinically stable patients with untreated allergic rhinitis, both specific serum IgE levels and SPT wheal sizes proved to be reproducible measures over a period of 14 to 21 days, indicating that these parameters may be reliable outcome measures for interventional trials on anti-allergic therapy. In agreement with previously published data, measurements of soluble and cellular inflammatory biomarkers in nasal lavage and brush samples showed marked variability at baseline (11, 13, 16). Nonetheless, on analyzing these

measurements in a nasal allergen challenge, we observed a clear increase in several upper airway inflammation biomarkers up to 24 hours post allergen, together with an increase in nasal symptoms following the allergen challenge but not the placebo challenge. Our data confirm and extend previous observations. Taken together, repeated nasal lavage and brush samplings are a useful extension of the nasal allergen challenge model and offer additional information in interventional trials with therapy targeting allergic mechanisms. Serum IgE levels showed a low CV in patients with clinically stable allergic rhinitis. Similarly, low intrasubject variation has been observed in other trials involving patients with allergic disease (7, 18). Indeed, due to their correlation with symptoms and other allergic markers (nasal eosinophils) serum IgE levels may be used as outcome parameters for anti-allergic medication (7, 19). However, because there is a substantial variation in precision and accuracy between commercial IgE assays, similar assay techniques should be employed throughout a study (20). In the present study, SPT was performed according to recommendations issued by the Global Allergy and Asthma European Network (GA2LEN) with an ALK lancet (21). Employing a similar SPT technique in a study in allergic children, the CV over 1 week was found to be 24.3% for HDM and 17.3% for histamine (22). Our data thus extend earlier observations as we found a CV over 2 to 3 weeks of under 20% for the largest dermal wheal size and of 11.7% for the histamine-induced wheal size. When applying SPT serially, it is essential to use the same standardized techniques and allergen extracts and to limit confounding factors such as drug intake, changes in circadian rhythm, and an excessive number of investigators (23). Similarly to IgE levels, a decreased SPT response following anti-allergic treatment has been shown to accompany a reduction in disease-related features both in patients with allergic rhinitis and allergic asthma (5, 6, 24).

Even though the patients in our study were clinically stable, defined by an absence of symptomatic disease, all of the nasal inflammatory markers, with the exception of IgE levels, showed marked baseline variability over time. Our findings therefore, call into question the applicability of nasal lavage and brushing in longitudinal studies with a limited number of patients. In the present study, we applied a slightly modified version of the nasal lavage technique previously reported by Naclerio *et al*; nasal lavage fluid recovery was similar to that reported in other studies (65% to 82%) (13, 16, 25). Based on the low variability of nasal IgE levels throughout the study, it may be concluded that nasal lavage, in combination with other semi-invasive sampling techniques such as nasal brushing, can yield representative serial measurements in a relatively short time interval (eg, during the EAR). However, the limited dwelling time used in the present study could explain why certain soluble mediators varied while others remained below the detection limit.

Employing a similar washing technique, other researchers also found low or undetectable levels of several mediators (13, 16, 26).

How could these issues be resolved? A more sensitive assay technique, such as Luminex technology, with a lower limit of detection for a variety of inflammatory mediators, could be used (27). Alternatively, the method described by Greiff and Grundberg, which involves applying an inflated balloon into either nostril, allows an increased dwelling time, which is associated with the recovery of higher levels of soluble markers from nasal lavage fluid (28). However, we anticipated that the allergen-induced swelling in combination with multiple other procedures conducted in the present study would not be compatible with this technique. Although nasal biopsies are currently considered the most suitable tool for investigating the kinetics of inflammatory cells in nasal mucosa, nasal brushing, which is a less invasive sampling method, has been shown to yield comparable results in terms of inflammatory cell differentials (11, 12). However, comparing changes in eosinophil counts in nasal brush and biopsy samples from patients with allergic rhinitis following exposure to grass pollen for 2 weeks out of season, Godthelp *et al* found similar variability in brush cellular counts whereas biopsies yielded less variable data (11). Our overall findings show that simpler, less invasive sampling techniques such as nasal lavage and brushing allow greater flexibility and repeatability, but at the expense of increased variability, especially over longer time intervals.

In a nasal allergen challenge study by Braga *et al,* allergen-induced composite symptom scores were shown to be associated with an increase in total nasal resistance, measured by anterior active rhinomanometry (29). Based on the increase in composite symptoms scores, the nasal allergen challenge produced both an early- and late-phase response in all patients with allergic rhinitis. These events corresponded to increased levels of several upper airway inflammatory markers. In contrast to placebo, the allergen caused a significant increase in nasal lavage tryptase levels, an indicator of mast cell degranulation, at 20 minutes post challenge; these levels were undetectable during later samplings. Measuring tryptase in nasal lavage fluid without a relevant stimulus, therefore, does not seem useful. A similar, short-lived increase in nasal lavage tryptase levels has been observed in other nasal allergen challenge studies (13, 26). α2-macroglobulin was also increased in nasal lavage fluid at 20 minutes post allergen challenge but not at 7 hours. Another nasal allergen study, however, reported increased concentrations of albumin, also a leakage marker, at both 20 minutes and 7 hours post allergen challenge (17). This different finding could be the result of a more pronounced LAR in the latter study or because the level of albumin, being a smaller molecule than α2-macroglobulin, may also be determined by passive diffusion and secre-

tion. We specifically measured α_2-macroglobulin since it is one of the largest plasma solutes and may be more specific for the exudation process (30). In agreement with previous studies, intranasal allergen challenge also induced a rise in ECP, which reached significant levels at 20 minutes and 7 hours post challenge (13, 26). This corresponds to an increase in eosinophilic degranulation, also observed in another study following an allergic stimulus (31). In addition, we were able to demonstrate the presence of MUC5AC, the principal airway mucin, in nasal lavage fluid both at baseline and throughout the challenges (32). However, due to the large variation within and between subjects, no difference in MUC5AC levels could be found between the allergen challenge and the placebo group at any of the time points analyzed. Whether this large variation (high SD) is caused by interindividual differences in the disease process and whether levels are higher than in healthy controls remain to be established. In contrast to the placebo challenge, the allergen challenge induced a significant increase in nasal brush eosinophil counts at 7 hours; these counts were still increased at 24 hours, albeit not significantly. Hence, our data confirm and extend the results reported by Juliusson et al, who investigated the kinetics of the allergen-induced changes in nasal brush eosinophils at 2-hourly intervals until 12 hours post allergen (33). Overall, our data support the validity of repeated nasal brush samplings as a method for analyzing allergen-induced changes in nasal eosinophils. In addition, eosinophils have been shown to be responsive to anti-inflammatory therapy (12). Nonetheless, based on the large variation in eosinophil count within and between subjects, large numbers of patients need to be included in a preferentially crossover study design to ensure optimal statistical power.

In conclusion, in untreated patients with clinically stable allergic rhinitis, we demonstrated that serum specific IgE levels and SPT wheal responses are reproducible parameters, characterized by low intrasubject variation (=CV) over a period of 14 to 21 days. Compared to well established nasal biopsies, nasal lavage and brushing are less invasive techniques that allow greater flexibility and repeatability but at the expense of greater intrasubject variation. On implementing these sampling techniques in a standardized nasal allergen challenge study, we found significantly increased levels of several inflammatory biomarkers following the allergen challenge but not the placebo challenge. We believe that nasal challenge with a relevant allergen conducted in conjunction with semi-invasive sampling techniques such as nasal lavage or brushing is a suitable method for exploring novel anti-allergic therapies. The drawbacks of large intrasubject variation could be resolved by using a crossover design and more sensitive detection techniques.

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NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

Nasal nitric oxide: longitudinal reproducibility and the effects of a nasal allergen challenge in patients with allergic rhinitis

J.D. Boot[1], M.L. de Kam[1], M.A. Mascelli[2], B. Miller[2], R. Gerth van Wijk[3], H. de Groot[3], A.F. Cohen[1], Z. Diamant[1]

1   Centre for Human Drug Research, Leiden, The Netherlands
2   Centocor Inc., Malvern, PA, USA
3   Erasmus MC, Dept of Allergology, Rotterdam, The Netherlands

Abstract

Background: Exhaled NO (eNO) is a validated non-invasive marker of airway inflammation in asthma. In patients with allergic rhinitis (AR), increased levels of nasal NO (nNO) have also been measured. However, the applicability of nNO as a marker of upper airway inflammation awaits validation.

Aim: To test the longitudinal reproducibility of standardized nNO measurements in patients with AR and the effects of nasal allergen challenge.

Methods: 20 patients with clinically stable, untreated AR participated in a combined study design. First, reproducibility of nNO was tested over 1, 7, and 14-21 days. Subsequently, the effect of nasal allergen challenge on nNO was studied in a placebo-controlled, parallel design. nNO was measured with a chemoluminescence analyzer. Ten subjects randomly underwent a standardized nasal allergen challenge; 10 subjects received placebo. Response to nasal challenge was monitored by composite symptom scores.

Results: There was a good reproducibility of nNO up to 7 days (Coefficient of Variation (CV) over 1 (16.45%) and 7 days (21.5%)), decreasing over time (CV (14-21 days): 38.3%). As compared with placebo, allergen challenge caused a significant increase in symptom scores ($p < 0.001$), accompanied by a decrease in nNO at 20 min post-challenge ($p = 0.001$). Furthermore, there was a gradual increase in nNO at 7 h, reaching significance at 24 h post-allergen ($p = 0.04$).

Conclusions: Similarly to eNO in asthma, nNO is a non-invasive marker, potentially suitable to monitor upper airway inflammation following allergen-induced late response. Present data show a good reproducibility of nNO measurements, decreasing over time, probably due to subclinical seasonal influences.

Introduction

Allergic rhinitis is an IgE-triggered chronic inflammatory disorder of the upper airways with pathophysiological and immunological links to allergic asthma (1). Recent studies providing evidence of systemic cross-talk between upper and lower airway compartments, have resulted in the concepts of "allergic airway disease" or 'combined allergic rhinitis and asthma syndrome' (CARAS) (2). The hallmark of CARAS is chronic airway inflammation, mainly characterized by mast cells, eosinophils and their pro-inflammatory products (3). Historically, airway biopsies have been regarded as the gold standard for the sampling of the allergic airway inflammation. However, the applicability of invasive methods is limited for repeated sampling, such as in clinical monitoring or intervention trials. In addition, biopsies are limited to a very small part of the airways. Therefore, several less or non-invasive methodologies are being developed, some of which have been validated (4). Nitric oxide (NO) is a gaseous molecule synthesized in the respiratory compartment by NO-synthases and can be detected in exhaled air of various species (5,6). Increased levels of eNO – originating from the lower airways - have been measured in asthmatic patients (7), with overall higher levels in allergic asthma (4). In patients with untreated asthma, levels of eNO appeared to correlate with the numbers of eosinophils within the airways (8,9). Likewise, both airway eosinophils and eNO can be reduced by anti-inflammatory therapy (10,11). Alternatively, increased eNO levels following tapering off inhaled corticosteroids (ICS) have been shown to predict asthma exacerbations (12). Hence, eNO measurement is presently a validated tool for non-invasive repeated assessment of the airway inflammation in asthma, both for intervention trials and clinical practice (4,13,14). In patients with AR, nasal eosinophilia and increased nNO levels have been demonstrated (15,16). These increased nNO levels may be the result of enhanced iNOS expression within the nasal epithelium due to persistent mucosal inflammation (17). In the upper airways, paranasal sinuses are major contributors to NO production: direct samplings from paranasal sinuses show substantially higher nNO-levels than nasal samplings (18). The applicability of nNO as a marker of upper airway inflammation, however awaits validation (17). With this study, we wished to test the applicability of nNO as a potential outcome parameter for clinical intervention trials. First, we studied the longitudinal reproducibility of standardized nNO measurements in clinically stable patients with untreated AR (5). Second, the effect of intranasal xylometazoline, a commonly used decongestant, on nNO was assessed. And finally, we performed a nasal allergen challenge in a randomized, placebo-controlled, parallel design, in the same group of patients. Some results have previously been reported in the form of an abstract (19).

Methods

Twenty multi- sensitized subjects with clinically stable allergic rhinitis participated in the study (Table 1). All subjects had a history of AR (intermittent or persistent) for at least 1 year prior to enrollment (1). Subjects with concomitant stable, intermittent or mild persistent asthma, using inhaled short-acting β_2-agonists on demand only, were allowed to participate. During the study, they were asked to withhold the β_2-agonists at least 6 hours before each visit. Concomitant maintenance anti-asthma or anti-allergy medication was discontinued at least 6 weeks prior to the study. Atopy was confirmed by a positive skin prick test for at least 2 of 6 common airborne allergens (grass, trees, Dermatophagoides pteronyssinus (HDM), Dermatophagoides farinae, cat-, and dog-dander, ALK Abelló, Nieuwegein, The Netherlands). Potential subjects with any other clinically relevant chronic or acute disorders were excluded. All eligible subjects were sensitized to at least one of the allergens used for nasal provocation, i.e. grasspollen, HDM- or cat-dander extracts. Symptomatic subjects with pollen allergy were tested outside the relevant pollen season (in the Netherlands outside the period May – August) and those with concomitant allergy to pets were only included, provided they had no close contact with pets (e.g. in their homes) during the study. AR symptoms were monitored throughout the study by a composite symptom score validated by Lebel, and subjects with a baseline symptom score of more than 2 on the allergen challenge day (Visit 2) were excluded (20). Furthermore, respiratory tract infections were excluded by patients' history at least 3 weeks prior and during the study. All subjects were non-smokers or ex-smokers (stopped at least 12 months prior to the study with less than 10 pack years). Starting the day before a study visit, all participants had to refrain from strenuous exercise and nitrate rich foods. The study protocol was approved by the Leiden University Medical Centre Ethics Committee and all subjects gave their written informed consent prior to enrolment into the study.

The study consisted of two parts (Figure 1). First we examined the reproducibility of nNO measurements over 1, 7, and 14-21 days. Second, we studied the effect of intranasal xylometazoline and allergen versus placebo on nNO levels. Before entering the study, the inclusion criteria were examined. Eligible subjects were included into the study and baseline nNO levels were measured (Visit 1). Fourteen to 21 days later (Visit 2), baseline nNO levels were repeated, and all subjects received intranasal xylometazoline (0.1%; Pharmachemie, Haarlem, The Netherlands). Nasal NO levels were recorded 30 min post-xylo-

metazoline. Subsequently, subjects randomly underwent a nasal challenge with either a relevant allergen (n=10) or its diluent (n=10) in a double-blind, parallel fashion. nNO measurements were repeated 20 min and 7 hours post-challenge. Symptom scores were recorded pre-challenge, post-diluent, 10 min after each subsequent challenge dose and hourly, until 7 hours post-challenge (20). Clinically stable subjects were dismissed from the unit 8-9 h post-challenge and returned 24 hours later (Visit 3) for nNO measurements and recording of symptom scores and 7 days later (Visit 4) for a final nNO measurement (Figure 1). The occurrence of adverse events was monitored throughout the study. In order to exclude an allergen-induced late phase bronchoconstriction, the airway response was measured by FEV_1 according to standardized lung function techniques at screening, pre- and 7 h post-allergen (Vmax Spectra, Sensor Medics, Bilthoven, The Netherlands) (21).
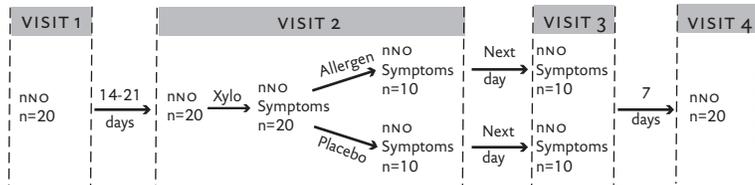
FIGURE 1   Study flowchart. nNO = nasal NO, N = number of subjects, Xylo = xylometazoline administration.

NASAL NO MEASUREMENTS

Nasal NO measurements were performed according to current ATS recommendations (5), using a chemoluminescence analyzer (Ecomedics CLD88sp, Duernten, Switzerland) with a nasal sampling tube. Briefly, subjects seated in an upright position were instructed to inhale "NO free" air (<1 ppb) through their mouth to total lung capacity and subsequently exhale through their mouth with a constant expiratory flow of 50 mL/s using online visual monitoring. An expiratory resistance was applied to ensure soft palate closure to exclude NO originating from the lower airways (eNO). During exhalation a Teflon tube connected to the NO analyzer aspirated nasal air with a constant flow rate of 0.3 L/min. During the study, nNO was measured in the same nostril, which was completely occluded with a foam plug (containing the Teflon tube) to prevent leakage of air. The contralateral nostril remained unobstructed. To minimize diurnal variation, all measurements were performed at the same time of the day (±2 hours). During each measurement, plateau nNO levels were determined and expressed as parts per billion (ppb). nNO levels were measured until three acceptable measures were obtained (within 10%) and the mean ppb-value was implicated into the analysis.

TABLE 1   SUBJECTS' CHARACTERISTICS

SUBJECT NR	AGE (YRS)	GENDER (M/F)	ATOPY	ASTHMA (+/-)
1	21	M	Cat, HDM, grass, trees, dog, D. Far	+
2	21	M	HDM, D. Far, dog	+
3	24	F	Grass, trees, Dog	-
4	24	F	Cat, HDM, trees, grass, D. Far	+
5	21	M	HDM, D. Far, dog	-
6	22	M	Grass, cat, dog, D. Far., trees, HDM	+
7	19	F	HDM, D. Far, trees, cat, dog	-
8	27	F	Cat, HDM, dog, D. Far	+
9	27	M	Cat, HDM, D. Far, grass, dog	+
10	50	F	Cat, dog, grass, trees	+
11	35	F	HDM, D. Far	-
12	32	M	HDM, D. Far	-
13	20	M	D. Far, HDM, grass, trees, cat, dog	+
14	22	F	Cat, HDM, D. Far, grass, dog	+
15	24	M	Grass, HDM, D. Far, cat, dog	+
16	21	M	HDM, D. Far, grass, trees, cat, dog	-
17	21	F	Grass, dog	-
18	19	F	Cat, HDM, D. Far, dog	-
19	43	F	D. Far, HDM, cat, dog	-
20	23	M	Grass, trees, dog	-

HDM = Dermatophagoides pteronyssinus, D. Far = Dermatophagoides farinae.

NASAL ALLERGEN CHALLENGE

Nasal allergen challenges were performed according to the previously validated protocol by de Graaf-in't Veld *et al* (22). Briefly, the procedure can be described as follows: selection of the relevant allergen was based on whichever allergen caused the largest wheal in the SPT in combination with clinical symptoms and depending on the season. Pre-challenge, subjects acclimatized for 30 minutes in the 'challenge' room and each nostril was inspected for accessibility using a nasal speculum. Subsequently, subjects received 1

puff of xylometazoline 0.1% in each nostril. Approximately 20 min later, the allergen's diluent (phosphate-buffered saline containing HSA 0.03% in benzoalkonium chloride 0.05%, ALK Abelló, Nieuwegein, The Netherlands) was sprayed in both nostrils (1 puff/nostril), followed by the allergen in 3 increasing concentrations of 100, 1,000 and 10,000 BU/mL (1 puff/nostril) each at 10 minutes intervals. Subjects were challenged with HDM, grasspollen or cat dander (ALK Abelló, Nieuwegein, The Netherlands). During placebo challenge, subjects received 4 times the allergen's diluent intranasally. The puffs were delivered as distally as possible into the nasal cavity using a nasal pump, delivering a fixed dose of 0.125 mL solution per puff (ALK Abelló, Nieuwegein, The Netherlands). Subjects were instructed not to inhale too deeply to prevent the solution to enter the lower airways. The nasal response at the respective time points was quantified by composite symptom scores validated by Lebel *et al* (20). Symptoms were recorded using the following scoring system: sneezes ≤ 2 = 0, sneezes 3-4 = 1 point, sneezes ≥ 5 = 3 points, anterior rhinorrhoea = 1 point, posterior rhinorrhoea = 1 point, difficult breathing = 1 point, one blocked nostril = 2 point, two blocked nostrils = 3 points, pruritus in the nose = 1 point, pruritus of palate or ear = 1 point, conjunctivitis = 1 point (total score 0-11). Symptom scores were recorded 10 min after each allergen dose and further dosing was discontinued at a total score of 6 or more, or after the highest dose was given (10,000 BU/mL) (22).

ANALYSIS

Prior to analysis, nNO data were tested for normal distribution using several tests, including the Kolmogorov-Smirnov test. These tests showed no significant deviation of normal distribution; hence, data were not log transformed. To assess the reproducibility of nNO measurements the within subject variation between visits was calculated and expressed as a CV (= the standard deviation expressed as percentage of the mean). The necessary change in nNO for the detection of an intervention effect was calculated with a power calculation program nQuery (nQuery advisor 3.0) using the within subject CV to estimate the standard deviation of the difference and based on a crossover study with power = 80% and α=0.05, two-sided. The data were analyzed with a mixed model analysis of covariance (ANCOVA) with intervention, time, and intervention by time as fixed factors, the subject as a random factor and the measurement before challenge/placebo but after xylometazoline as covariate. Contrasts between placebo and allergen challenge were calculated at various timepoints. A treatment effect or contrast with a p-value less than 0.05 was considered statistically significant. All calculations were performed using SAS for windows V9.1.2 (SAS Institute Inc., Cary, NC, USA).

Results

All subjects were able to perform the nNO maneuvers. On average (mean ± SD) 6.1 ± 2.4 maneuvers during maximally 7 minutes were needed to obtain three acceptable measurements (good plateau response and within 10%). Although comparable mean nNO levels were measured on visits 1 and 2 (mean ± SD 1017 ± 477 ppb and 1104 ± 496 ppb, respectively), there was a substantial within subject variability (CV = 38.3%; Figure 2, Table 2). Better reproducibility was found over shorter time intervals: 1 day and up to 7 days later, i.e. between Visit 2 and 3; and between Visit 2 and 4 (Table 2). To exclude potential influence of nasal allergen challenge, the reproducibility of nNO over 1 and 7 days was analyzed in the placebo group only.

The necessary change in nNO for the detection of a significant intervention effect (e.g. following drug treatment) was calculated using the within subject CV for several sample sizes in a cross-over study design (Table 3).

TABLE 2   REPRODUCIBILITY OF NASAL NO OVER VARIOUS TIME POINTS (MEAN ± SD).

	NUMBER OF SUBJECTS	FIRST MEASUREMENT	SECOND MEASUREMENT	CV (%) WITHIN SUBJECTS
Δ 14-21 days (Visit 1 - 2)	20	1017 ± 477 ppb	1104 ± 496 ppb	38.3
Δ 14-21 days (Visit 1 - 2)	10*	874 ± 310 ppb	977 ± 531 ppb	33.5
Δ 7 days (Visit 2 - 4)	10*	977 ± 531 ppb	1038 ± 285 ppb	21.5
Δ 1 day (Visit 2 −3)	10*	977 ± 531 ppb	968 ± 373 ppb	16.5

*n=10 subjects in the placebo challenge group only. CV = coefficient of variation.

TABLE 3   CHANGE IN NASAL NO FOR DETECTION OF A SIGNIFICANT INTERVENTION EFFECT

NO. OF SUBJECTS	Δ 7 DAYS	Δ 14-21 DAYS
12 subjects	261 ppb	510 ppb
16 subjects	220 ppb	430 ppb
20 subjects	194 ppb	379 ppb

Calculations based on a cross-over study with power = 80% and α=0.05, two-sided.
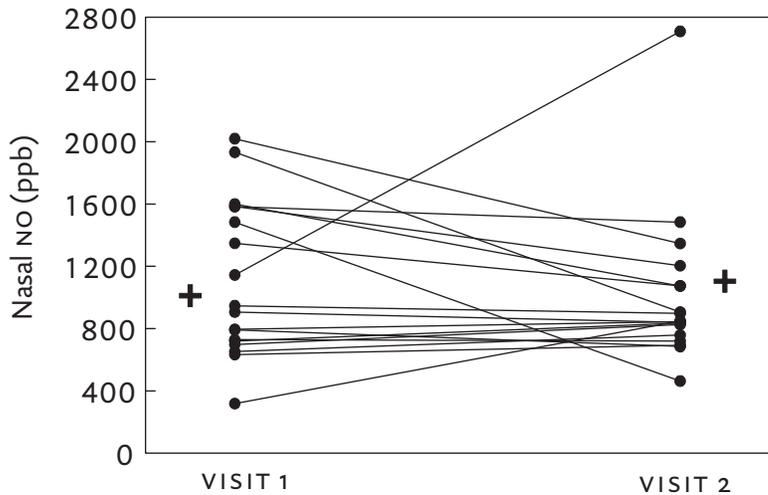
FIGURE 2   Individual baseline values of nasal no on visit 1 and 2 (∆ 14-21 days) for all subjects (n=20).
+ = mean nasal no level.

EFFECTS OF INTRANASAL XYLOMETAZOLINE AND NASAL CHALLENGES

All intranasal interventions (xylometazoline, allergen and the allergen's diluent) were well-tolerated by all subjects and no clinically significant adverse events occurred. Pre-challenge, none of the subjects had lower airway complaints and all FEV_1 values were similar to screening. At 7 h post-challenge, there was no significant fall in baseline FEV_1 following either intervention (mean ± SD: -0.03 ± 0.13 L (Allergen (A)); p=0.43; -0.03 ± 0.09 L (Placebo (P)); p=0.39). Neither were the changes in FEV_1 significantly differ-ent between the two challenges (p=0.88).

As compared with baseline measurements, 30 min post-administration, xylo-metazoline significantly decreased nNO levels in all patients by on average 24% (265 ppb; p=0.001).

For evaluation of the effect of nasal allergen challenge, post-xylometazoline symptom scores and nNO levels were used as baseline measurements in the analysis. Ten patients were randomized to intranasal allergen (2 subjects received cat-allergen; 2 grass-pollen; 6 HDM) and ten to placebo. As com-pared with placebo, intranasal allergen induced a nasal early allergic response (nasal EAR) in all subjects with a mean symptom score ± SD: 0.70 ± 0.82 (P) and 7.25 ± 1.58 (A) at 10 min post-challenge (Figure 3). In addition, all subjects had a nasal late allergic response (nasal LAR), defined as a symptom score above baseline on two consecutive timepoints between 3-7 h post-allergen

(mean ± SD: 0.24 ± 0.66 (P) and 1.3 ± 1.0 (A) (Figure 3) (22). The changes in symptom scores between allergen and placebo were significantly different (p<0.001).

As compared with placebo, nasal allergen challenge induced a significant decrease in nNO at 20 min post-challenge (estimated difference −514 ppb, 95% confidence interval (CI) -780 to -247 ppb; p=0.001). Seven hours post-allergen, both nNO levels and symptom scores were slightly increased as compared to placebo. At 24 hours post-challenge, there was a further increase in nNO levels following allergen as compared with placebo (estimated difference 344 ppb, 95% CI 24 to 664 ppb; p=0.04) (Table 4). Mean changes (± 95% CI) in symptom scores and nNO levels following allergen and placebo are shown in Figures 3 and 4.
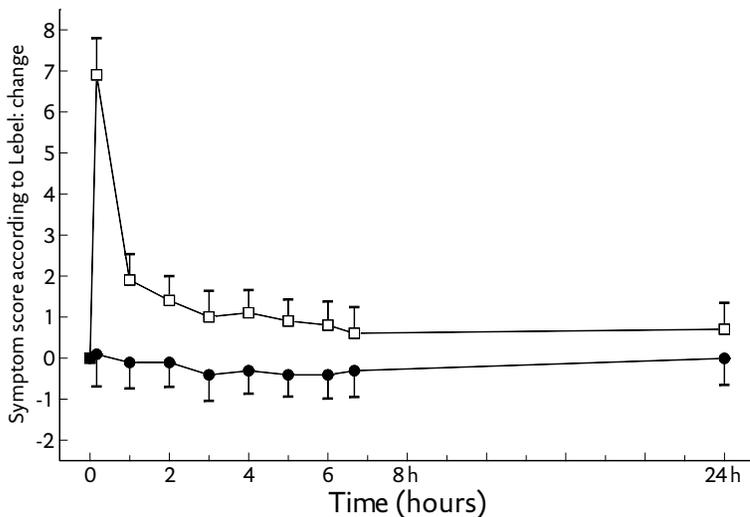
FIGURE 3   Mean change (± 95% confidence interval) in composite symptom scores following nasal allergen versus placebo challenge. Open squares: relevant allergen, closed dots: placebo.
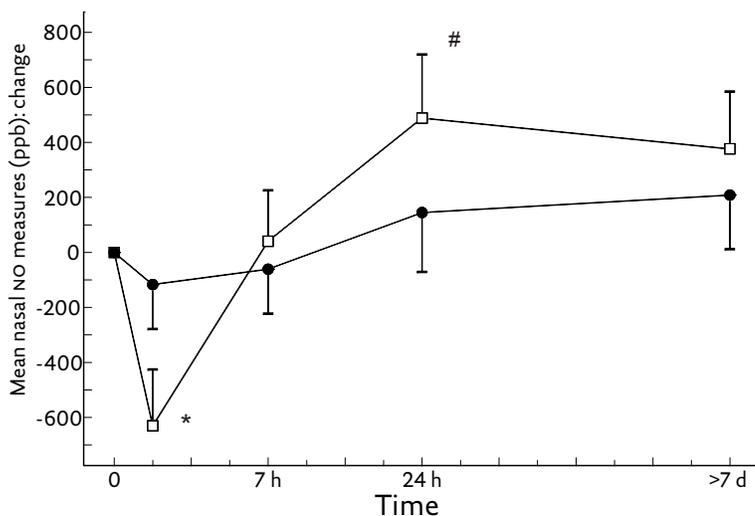
FIGURE 4   Mean change (± 95% confidence interval) in nasal NO concentrations (PPB) following nasal allergen versus placebo challenge. Baseline was set after xylometazoline administration in all subjects. Open squares: relevant allergen, closed dots: placebo. *Indicates a significant difference between allergen and placebo at 20 min post-challenge (P=0.001), #indicates a significant difference between allergen and placebo at 24 hours post-challenge (P=0.04).

TABLE 4   EFFECT OF NASAL ALLERGEN/PLACEBO CHALLENGE ON NASAL NO LEVELS (MEAN ± SD).

	PRE-CHALLENGE	20 MIN POST-CHALLENGE	7 H POST-CHALLENGE	24 H POST-CHALLENGE	7 DAYS POST-CHALLENGE
Allergen					
Nasal NO	980 ± 345 ppb	336 ± 287 ppb	946 ± 267 ppb	1393 ± 307 ppb	1272 ± 294 ppb
Placebo					
Nasal NO	717 ± 358 ppb	682 ± 258 ppb	752 ± 220 ppb	968 ± 373 ppb	1038 ± 285 ppb

Discussion

We have demonstrated that nNO is a reproducible parameter over short time intervals in clinically stable patients with untreated AR. A potential explanation for the increased variability over time in our study could be due to (subclinical) seasonal effects. Moreover, we showed that unlike placebo challenge, intranasal decongestant and allergen challenge significantly affected nNO levels. Overall, our data suggest that nNO may be a potential outcome parameter in clinical intervention trials in patients with AR.

Our findings confirm and extend data from previous studies in both healthy subjects and patients with AR applying different nNO sampling methods (23,24). While in these studies recordings were made during breath holding, we measured nNO according to ATS recommendations (5,23,24). This method holds sampling of nNO during oral exhalation against a fixed resistance for optimal closure of the velum to prevent mixing from other (airway) compartments. Applying this technique, baseline nNO levels on both visits 1 and 2 were higher to those reported in two other studies in patients with untreated AR (25,26). This discrepancy can be ascribed to the higher sampling flow rate applied in our study, as comparable nNO levels have been measured in another study in patients with AR applying a similar flow rate (0.25 L/min compared to our 0.3 L/min) (27).

We found good reproducibility of nNO levels in patients with untreated AR up to 7 days. Comparable data have been reported in healthy subjects over a similar time period (23). The decay in reproducibility over time (14 to 21 days) may be caused by subclinical seasonal or weather influences and hence, should be taken into account when calculating the necessary sample size. Based on our findings, it can be anticipated that in clinical intervention trials outside pollen season, a change in nNO levels beyond baseline variation will be needed to detect a potential drug effect. So far, there are few data on nNO as an outcome parameter in clinical intervention trials. In a comparative study mean nNO levels were approximately 55% lower (mean difference of 846 ppb) in patients with AR treated with nasal corticosteroids as compared to nNO measured in patients with untreated AR (16). Although this was not a placebo-controlled study, it provides further evidence on potential applicability of nNO as a biomarker of upper airway allergic inflammation, even over longer (treatment)-periods. Finally, in accordance with other studies, we found marked inter-individual differences in nNO (Figure 2) (27). In order to minimize the effect of between-subject variability, a cross-over design is recommended.

Since xylometazoline is often incorporated in a nasal allergen challenge protocol when measurements of pro-inflammatory biomarkers (e.g. in nasal

lavage) are performed, we wished to evaluate its effect on pre-allergen nNO levels as part of the analysis (28,29). We found a 24% mean fall in nNO from baseline levels, at 30 min following intranasal xylometazoline. In correspondence with our findings, previous studies in healthy volunteers and patients with AR showed a mean decrease in nNO levels by 12-14% and 20-25%, respectively, 10 minutes following administration of intranasal decongestants with comparable pharmacological properties as xylometazoline (25). Moreover, a comparable fall in nNO has been encountered in subjects with an upper respiratory tract infection following intranasal oxymetazoline (30). The reduction in nNO is possibly caused by the xylometazoline-induced vasoconstriction, decreasing NO-diffusion (25). In addition, evidence from in vitro animal studies suggests additional anti-inflammatory effects through inhibition of the NO-producing enzymes (31). However, prolonged use of nasal decongestants in humans is associated with unwanted side effects, surpassing these potentially beneficial properties (32).

In contrast to sham challenge, nasal allergen challenge (irrespective of the relevant allergen applied) caused similar symptoms and signs of acute rhinoconjunctivitis in all subjects, consistent with a nasal EAR (22). In agreement with previous studies in allergic rhinitis, we found a significant fall in nNO at 20 minutes post-allergen (16). At least one possible explanation for this phenomenon may be the allergen-induced swelling of the nasal mucosa during the nasal EAR – not occurring after placebo – resulting in hampered NO diffusion (33). Our data are consistent with findings by Colantonio *et al* in patients with allergic rhinitis and polyposis nasi, in whom - despite iNOS-upregulation – decreased nNO levels have been found (34). In this study, nNO levels appeared to be inversely correlated to the polyp size and raised following treatment of the polyps; suggestive of a mechanic blockade of NO diffusion from the sinuses. In addition, similar observations have been reported for the lower airways, measuring eNO following bronchial allergen challenge in asthma (35,36). In these studies, there was an (albeit statistically non-significant) decrease in eNO accompanied by allergen-induced bronchial obstruction during the early asthmatic response

In agreement with previous data on eNO following allergen-induced bronchial LAR, we found a (gradual) increase in nNO at 7 h (i.e. during the nasal LAR), reaching statistical significance at 24 h post-allergen (35,37). However, it should be noted that pre-challenge administration of intranasal xylometazoline in the present study protocol, may account for potentially underestimated values of both the symptom scores and nNO up to 7 h post-allergen (38). Indeed, at 24 h post-challenge when the xylometazoline effect had worn off, the increase in nNO became statistically significant. Similarly, Hanazawa *et al* found increased nNO levels in patients with AR at 24 h following intrana-

sal administration of eotaxin (39). In addition, an increase in nasal eosinophils was measured at 8 h post-eotaxin; accompanied by maximal levels of nNO (no pretreatment with nasal decongestant) (39). Likewise, during allergen-induced LAR in asthma, increases in eNO appeared to be accompanied by sputum eosinophilia (37). Based on these data, nNO may be a useful outcome parameter for allergen-induced airway inflammation during the LAR in patients with AR, preferably not pre-treated with nasal decongestant.

Based on the link between the upper and lower airways, it could be anticipated that nasal allergen challenge may affect lung function especially in patients with allergic rhinitis and asthma (40). Although in the present study there were no significant changes in FEV_1, this does not entirely rule out co-existent pro-inflammatory events within the lower airways induced by nasal allergen. However, assessments of the lower airway inflammation were kept outside the scope of this study. Moreover, including a limited number of AR patients in combination with different phenotypes (with and without concomitant asthma) precludes a heterogeneous lower airway response to nasal allergen.

In conclusion, nNO measurement is a quick and non-invasive tool in patients with allergic rhinitis. Based on our data and previous other findings in AR patients, nNO may potentially serve as a biomarker of (allergen-induced) nasal inflammatory response during "the stable phase", such as the nasal LAR (27,39). However, confounding factors such as acute allergen-induced nasal response resulting in massive rhinorrhea and occlusion of the paranasal sinuses or pretreatment with nasal decongestants or corticosteroids may limit the applicability of nNO during the nasal EAR.

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CLINICAL STUDIES –

ALLERGIC ASTHMA & ALLERGIC RHINITIS

NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

Cleaved secretory leukocyte protease inhibitor as a biomarker of chymase activity in allergic airway disease

S.M. Belkowski[1], J.D. Boot[2], M.A. Mascelli[3], Z. Diamant[2], L. de Garavilla[1], B. Hertzog[1], D. Polkovitch[1], M. Towers[1], A. Batheja[1] and M.R. D'Andrea[1]

1   Johnson and Johnson Pharmaceutical Research and Development, Spring House, PA 19477
2   Centre for Human Drug Research, Leiden, Netherlands
3   Centocor, Radnor, PA 19080

Abstract

Background: Secretory leukocyte protease inhibitor (SLPI), which is present in many physiological fluids including saliva, sputum and nasal discharge, is the most effective inhibitor of chymase. Previously, we demonstrated that chymase is able to cleave SLPI and that the cleaved portion, CSLPI, is a biomarker of chymase activity.

Objective: We investigated the potential of CSLPI as a biomarker of chymase activity in subjects with allergic rhinitis and asthmatic airway disease.

Methods: Baseline sputum samples were collected from atopic asthmatics and healthy controls. Nasal lavages (NAL) were performed in subjects with allergic rhinitis (AR) both at baseline and following a nasal challenge with allergen or placebo. Levels of CSLPI and chymase were determined by western analysis and tryptase and α2-macroglobulin were measured by immunoassay.

Results: As compared with healthy controls, asthmatics showed a significant increase in baseline CSLPI/total SLPI ratios and an increase in chymase levels. There was a high correlation of CSLPI/SLPI ratios to chymase levels in normal individuals and untreated asthmatics. In the NAL of patients with AR, as compared with placebo, allergen challenge increased inflammatory biomarkers, including: CSLPI/SLPI ratios, chymase levels, tryptase levels, and α2-macroglobulin levels. Correlations were observed between CSLPI/SLPI ratios and chymase levels and CSLPI/SLPI ratios and α2-macroglobulin levels; no correlation was seen between CSLPI/SLPI ratios and tryptase levels.

Conclusion: Our data indicate that CSLPI reflects chymase activity in AR and asthma. Hence, CSLPI may serve as a biomarker for disease activity and for monitoring the efficacy of novel anti-inflammatory treatments in chymase-mediated diseases.

Introduction

The clinical hallmarks of asthma and allergic rhinitis (AR) are persistent airway inflammation and airway hyperresponsiveness (AHR) (1, 2). The observation that clinical symptoms and function do not fully reflect the severity of the underlying airway inflammation and AHR has initiated the search for more adequate biomarkers of asthma and AR (1, 3). Subsequently, the development and validation of non- and semi-invasive sampling techniques, including exhaled nitric oxide, induced sputum and nasal lavage, helped to identify several potential biomarkers of airway inflammation for both clinical practice and research purposes (4-6). However, there is still an unmet need to discover sensitive biomarkers from easily obtained samples that are closely linked to the inflammatory cascade that could serve to monitor the progress and treatment of airway diseases. The mast cell is an important effector-cell in the pathophysiology of allergic airway inflammation, AHR and airway remodeling (7-9). Degranulation of sensitized mast cells by relevant allergen causes the release of histamine and proteases (tryptase and chymase), and de novo synthesis of other inflammatory mediators (leukotrienes, prostaglandins, platelet activating factor and bradykinin) (10-12). On the basis of proteases content, mast cells can be subdivided into at least two phenotypically different subsets: connective tissue mast cells expressing chymase, tryptase and cathepsin G (MCTC) and mucosal mast cells producing tryptase, but no chymase (MCT) (13). Although not fully elucidated, chymase originating solely from MCTC cells has recently been implicated in the pathophysiology of airway inflammation, AHR and remodeling of allergic airway disease (9, 14). The proteinase inhibitors, α1-antitrypsin, α1-antichymotrypsin, α2-macroglobulin and secretory leukocyte protease inhibitor (SLPI), released from neutrophils, macrophages and epithelial cells within the respiratory tract, have been shown to inactivate chymase, providing local protection against allergic stimuli (15-18). In a previous *in vitro* study we have shown that SLPI, is not only a chymase inhibitor, but also a substrate of chymase and that its cleaved product, CSLPI, is a biomarker of chymase activity in vitro (19).

Based on these data, we hypothesized that CSLPI may be a biomarker of chymase activity in allergic airway disease *in vivo*. To this end, in two studies originally designed to assess allergic end-points in asthma and allergic rhinitis we retrospectively measured baseline levels of SLPI and CSLPI in relation to chymase levels. In the first study we examined SLPI, CSLPI and chymase levels in the sputum of subjects with allergic asthma in comparison with healthy subjects. In the second study, levels of CSLPI, SLPI, chymase, tryptase and α2-macroglobulin were measured in nasal lavage (NAL) of individuals with AR, both under baseline conditions and following nasal allergen challenge.

SECTION 4 –CLEAVED SECRETORY LEUKOCYTE PROTEASE INHIBITOR AS A BIOMARKER OF CHYMASE ACTIVITY IN ALLERGIC AIRWAY DISEASE

Methods

Asthmatic subjects study

This part of the study was conducted at the University of Southampton, UK. On one study occasion, hypertonic saline-induced sputum was collected and analyzed from 12 participants (6M/6F, 18-70 years), subdivided into 4 study groups with the following characteristics:

1. Healthy non-atopic, non-smoking controls with no chronic respiratory symptoms and no evidence of upper or lower respiratory tract infection in the last 6 weeks ($FEV_1 > 80\%$ predicted, normal FEV_1/FVC ratio; group 1).
2. Non-smoking subjects with intermittent, atopic asthma ($FEV_1 > 80\%$ predicted, not on inhaled corticosteroids; group 2).
3. Non-smoking subjects with mild persistent, atopic asthma ($FEV_1 > 80\%$ predicted; regular use of inhaled corticosteroids (Beclomethasone (Qvar®) 100µg BD, Serevent 25µg BD; Fluticasone 100µg BD.; group 3).
4. Non-smoking subjects with moderate-severe persistent atopic asthma (FEV_1 60-80% predicted ; symptomatic despite regular treatment with corticosteroids: Becotide 200µg QDS; Seretide (1000µg fluticasone / 200µg salmeterol) BD; Seretide (125µg fluticasone /25µg salmeterol) BD, Prednisolone 40mg OD; group 4).

All subjects gave written informed consent and ethical approval was obtained from the Joint Ethics Committee of Southampton Hospital and General Hospital.

Subjects with allergic rhinitis

Nasal lavage (NAL) specimens were obtained from 20 multi-sensitized, non-smoking patients (10M/10F; 19-51 years) with clinically stable AR who were not on maintenance therapy, participating in a clinical study at the Centre for Human Drug Research, Leiden, Netherlands (20). All subjects provided written informed consent and the study was approved by the Ethics Committee of Leiden University Medical Center, Leiden, Netherlands.

In the asthma and healthy subject cohorts (n = 3 per subgroup) sputum was induced by hypertonic saline according to European Respiratory Society (ERS) recommendations (4, 21). Shortly, following salbutamol pretreatment, aerosolized hypertonic saline (4.5% NaCl) solution was inhaled at three 5-minute intervals by tidal breathing with the nose clipped. In more severe

asthmatics, a modified ERS protocol was used starting with normal saline and moving slowly to hypertonic saline only if there was no significant fall in FEV_1. At the end of each 5-minute interval, expectorated sputum was collected in a petri-dish and FEV_1 and PEFR were recorded. If FEV_1 fell by ≥20% from the post-salbutamol baseline, the procedure was discontinued. Sputum samples were kept on ice and processed within 1 hour of collection as previously described (4). In summary, sputum was teased from the sample using a forceps and collected into a fresh petri dish, avoiding contamination with saliva and squamous cells. One volume of selected sputum was diluted with four volumes of phosphate buffered saline (PBS). Four volumes of 6.3mM dithioerythreitol (DTE) were added to one volume of selected sputum, to achieve a final concentration of 5mM DTE. Subsequently, sputum samples were incubated at room temperature for 30 minutes on a bench roller. Sputum samples were individually filtered through a 100μm nylon mesh cell strainer in order to remove any mucus and debris, and centrifuged at 4°C, 1500 rpm for 10 minutes. Subsequently, sputum supernatant was transferred to a new 15ml centrifuge tube and mixed well, samples were aliquotted and stored at -80°C pending analysis. For cytospins, an adequate sample of cell suspension was added into a cytospin tube to achieve a total number of 20-30,000 cells per slide, spun for five minutes, fixed and stained with Hemacolor staining to allow differentiation of cell types. Cytospin quality was regarded as adequate when squamous cell contamination was < 20%.

NASAL LAVAGE

The patients with AR randomly underwent a standardized nasal challenge with either a relevant allergen (house dust mite (HDM), cat or grass allergen; n=10) or its diluent (n=10) in a double-blind, parallel fashion (22). Selection of the intranasal allergen (HDM, grass pollen or cat dander) was based on the allergen causing the largest wheal in the skin prick test combined with the patient's history of clinical symptoms and the season. The nasal response to allergen was documented by symptom scores previously validated by Lebel (23). NAL collections were performed according to a modified protocol, sampling the same nostril at pre-challenge, and at 20 minutes post-challenge (22). NAL samples were collected, kept on ice and processed within 1 hour according to a validated protocol (22). Briefly, samples were centrifuged for 10 min at 400g; the supernatant was aliquotted and stored at −80 °C pending analysis. Frozen samples were shipped to site of analysis.

REAGENTS

Recombinant SLPI (rSLPI) was obtained from R&D systems (Minneapolis, MN, USA). Rabbit anti-SLPI antibody was obtained from Alexis (San Diego,

SECTION 4 –CLEAVED SECRETORY LEUKOCYTE PROTEASE INHIBITOR AS A BIOMARKER OF CHYMASE ACTIVITY IN ALLERGIC AIRWAY DISEASE

CA, USA). Mouse anti-human mast-cell chymase was obtained from Serotec (Raleigh, NC, USA). The secondary antibodies goat anti-mouse and Goat anti-rabbit were obtained from Pierce Biotechnology (Rockford, IL, USA). Tryptase and α2-macroglobulin were determined according to the manufacturers directions in the NAL samples with the following assay kits and detection limits: Tryptase by FEIA (Phadia, Uppsala, Sweden), 1 µg/L; and α2-macroglobulin by ELISA (R&D systems, Minneapolis MN, USA), 10 ng/mL.

SAMPLE PREPARATION FOR WESTERN ANALYSIS

During processing all samples were maintained at 4°C or less to insure stability of CSLPI and SLPI as determined previously (19). Ten µl of human sputum supernatant or nasal lavage supernatant was diluted to a total volume of 20 µl in loading buffer containing 50% Nupage LDS sample buffer from Invitrogen (Carlsbad, CA, USA), 30% water and 20% 1M DTT from Sigma-Aldrich (St. Louis, MO, USA) and was maintained on ice. The samples were boiled for 6 min at 95 °C.

SLPI DETECTION BY WESTERN ANALYSIS

Denatured and reduced sputum supernatant and nasal lavage supernatant samples were applied to a 12% NuPage bis-tris gel from Invitrogen. The samples were electrophoresed and transferred to a PVDF membrane (Pierce Biotechnology). The blots were blocked in 5% milk /PBS-tween (PBS/0.4% tween-20) overnight at 4°C. Subsequently, the blots were washed for 10 minutes and incubated in the primary antibody (goat anti-SLPI at 1:1000) diluted in the blocking solution for 1 hour at room temperature. After three washes of PBS-tween the blot was incubated for 45 minutes at room temperature with the secondary antibody (goat anti-rabbit HRP) diluted 1:4000 in blocking solution. The blots were washed 6 times with PBS-tween and incubated for 1 minute at room temperature with Western Lightning chemiluminescence reagents (Perkin Elmer, Boston, MA, USA) and developed on Biomax Light Film (Eastman Kodak, Rochester, NY, USA) in the darkroom. Densitometry was performed on each film to determine the relative amount of intact SLPI (~15kD band) and CSLPI (~10kD). The data was expressed as a ratio of CSLPI/ SLPI for normalization of each sample as done previously (19). The reliability of this assay was confirmed by using mass spectrometry analysis during its development (data not shown).

CHYMASE WESTERN ANALYSIS

Due to the lack of a commercially available chymase ELISA, chymase levels were determined by western analysis. In brief, denatured and reduced sputum supernatant and nasal lavage supernatant samples were applied to a 12%

NuPage bis-tris gel from Invitrogen. The samples were electrophoresed and transferred to a PVDF membrane (Pierce Biotechnology). The blots were blocked in 5% milk /PBS-tween (PBS/0.4% tween-20) overnight at 4°C. Subsequently, the blots were washed for 10 minutes and incubated in the primary antibody (mouse anti-human mast cell chymase at 1:1000) diluted in the blocking solution for 1 hour at room temperature. After three washes of PBS-tween the blot was incubated for 45 minutes at room temperature with the secondary antibody (goat anti-mouse HRP) diluted 1:4000 in blocking solution. The blots were washed 6 times with PBS-tween and incubated for 1 minute at room temperature with Western Lightning chemiluminescence reagents (Perkin Elmer) and developed on Biomax Light Film (Eastman Kodak) in the darkroom. Densitometry was performed on each film to determine the relative amount of chymase.

STATISTICAL ANALYSIS

Data was analyzed using a non-parametric Mann-Whitney and Student's t-test of endpoint data for the asthma trial. The chymase and α2-macroglobulin data from the allergic rhinitis trial was log-transformed in order to stabilize variance. Welch two sample t-test was performed on the slopes of pre-post experimental values. Pearson correlation coefficient was determined to evaluate the relationship between CSLPI/SLPI ratios and other biomarkers.

Results

GENERAL

All sampling methods were well tolerated and no serious adverse events occurred. Analyzable sputum could be obtained from all but 1 asthmatic subject. Due to NAL-sample limitations, chymase levels could only be analyzed in 9 subjects challenged with placebo and 7 in the allergen arm.

ASTHMATIC SUBJECTS

In the sputum samples from asthmatic patients (A) CSLPI/SLPI ratios were determined. One subject from Group3 could not be analyzed due to low volume. As compared with healthy controls (HC) (group 1), CSLPI/SLPI ratios were increased in the sputum of asthmatic subjects (groups 2-4) (0.36 ± 0.02 (HC); 0.45 ± 0.02 (A); $p = 0.04$, student's t test; $p = 0.049$, non-parametric Mann Whitney test). On average sputum from untreated, atopic asthmatics (group 2) exhibited the highest CSLPI/SLPI ratio (0.50 ± 0.03), while treatment with corticosteroids (groups 3 and 4; $n = 5$) had a lower CSLPI/SLPI ratio

(0.41 ± 0.02; p = 0.04). (Figure 1) In addition, we measured chymase levels in the same sputum samples and found that the chymase levels followed a similar trend as the CSLPI/SLPI ratios (929.1 ± 301.0 relative units (HC); 1469.4 ± 141.5 relative units (A); p < 0.17)(Figure 2). Despite the low number of samples, a correlation between CSLPI/SLPI ratios and chymase levels in the normal and untreated atopic asthmatics was noted (r=0.83).
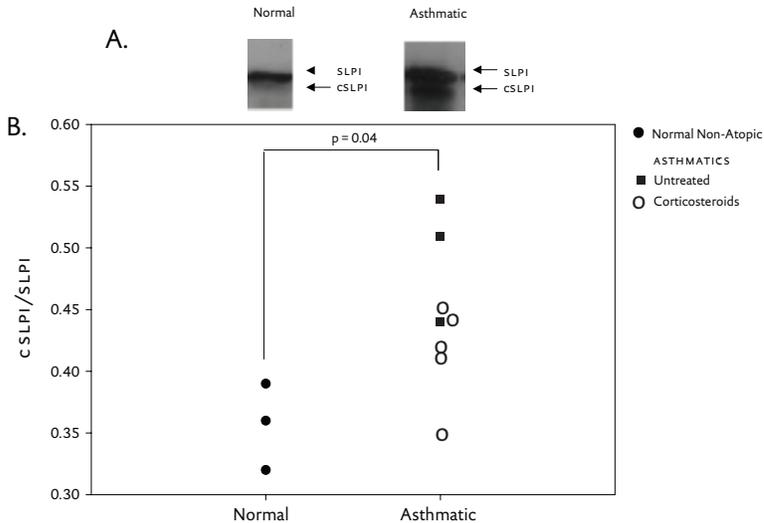

FIGURE 1   SPUTUM CSLPI/SLPI RATIOS. Example of western analysis of lowest and highest CSLPI expression (A.) as compared with healthy controls, sputum CSLPI/total SLPI ratios were significantly increased in allergic asthmatics (P=0.04, student's t test; p=0.049, non-parametric mann whitney test). Corticosteroid use was associated with decreased CSLPI levels (p=0.04, student's t test) (B).

ALLERGIC RHINITIS

Nasal lavages were collected at baseline and 20 min post-challenge. As compared with sham challenge (P), intranasal allergen (A) produced an acute allergic response with a mean symptom score (mean ± SEM: 7.25 ± 0.56 (A); 0.70 ± 0.26 (P); p = 0.001) at 10 min post-challenge. In parallel, there was a significant increase in the CSLPI/SLPI ratio following allergen as compared with placebo challenge (Figure 3). Figure 3a shows the western analysis indicating

the CSLPI band in the nasal lavage from allergen challenged (highest level example shown) (left) and placebo-challenged (lowest level example shown) (right) individuals. Using densitometry, the CSLPI/total SLPI ratio was determined for each time point during both allergen (Figure 3b) and placebo (Figure 3c), respectively. As compared with placebo (0.22 ± 0.02), there was a significant increase in CSLPI/SLPI ratio in the nasal lavage of allergen challenged individuals (0.41 ± 0.05; p =0.007) at 20 min post-challenge. Similarly, there was a significant increase in the NAL-chymase levels in the allergen challenged individuals (1294.85 ± 688.50 relative units; Figure 4b) as compared with placebo-exposed individuals (706.01 ± 206.11 relative units; p=0.038; Figure 4c). Changes in CSLPI/SLPI levels and chymase levels from baseline to 20min post allergen challenge were highly correlated (r = 0.91) (Figure 5). If the highest point is removed the correlation is reduced to r = 0.69, which still shows a strong relationship between chymase levels and the CSLPI/SLPI ratio. Using regression analysis with all points the slope is significant (p < 0.001), with the highest point removed the trend is still significant (p < 0.006).
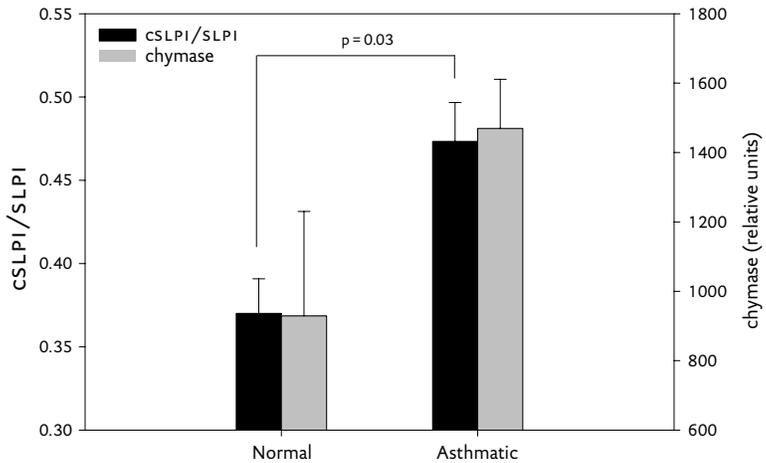

FIGURE 2   SPUTUM CHYMASE LEVELS AND CSLPI. Total slpi ratios. as compared with healthy controls, in patients with untreated allergic asthma sputum CSLPI/total SLPI ratios were significantly increased (P=0.03, student's t test). Chymase levels followed a similar trend with CSLPI/total SLPI ratios in both subject groups.
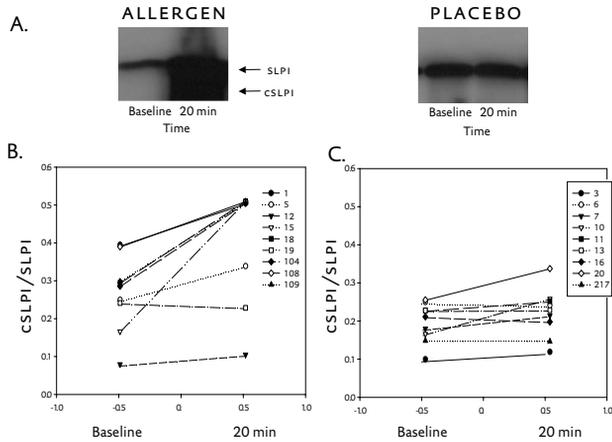
A.

ALLERGEN   PLACEBO

FIGURE 3   NAL CSLPI/SLPI RATIO FOLLOWING NASAL ALLERGEN COMPARED WITH PLACEBO. Example of western analysis of NAL from placebo (lowest level CSLPI) and allergen challenged AR–patients (highest level CSLPI) (A). densitometry analysis of CSLPI/total SLPI ratios in nasal lavage from allergen challenged (B) and placebo challenged human AR– patients (C). Welch T test, P < 0.007, allergen challenged as compared to placebo CSLPI/total SLPI ratio induction from baseline to 20 min post challenge. Legend refers to patient number.
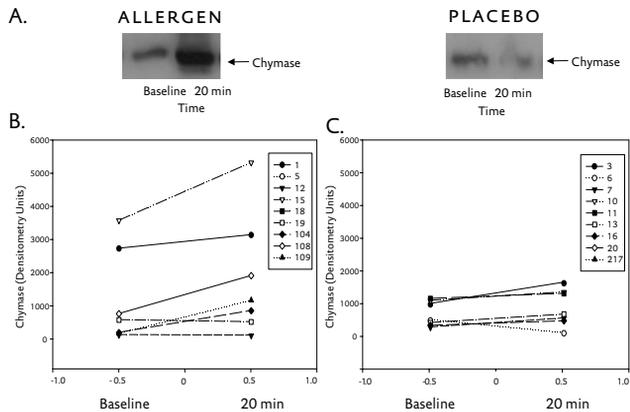

FIGURE 4   NAL CHYMASE LEVELS LEVELS FOLLOWING NASAL ALLERGEN. Example of western analysis of nal from placebo (lowest level chymase) and allergen challenged AR–patients (highest level chymase) (A). Chymase levels (densitometry units) in nasal lavage from allergen challenged (B) and placebo challenged (C) human AR–patients. welch T test, p < 0.038, allergen challenged as compared to placebo from baseline to 20 min post challenge. Legend refers to patient number.
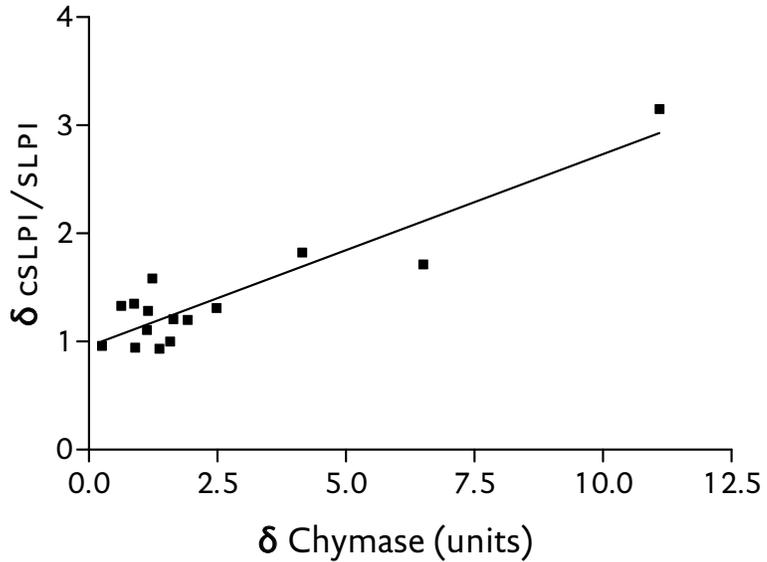
In parallel to the increases in CSLPI and chymase, there was a significant raise in the NAL levels of α2-macroglobulin (a protease inhibitor) (6630 ± 4240 ng/ml (A) vs. 50.75 ± 28.76 ng/ml (P); p = 0.00035) (figure 6a and b) at 20 min post-challenge. Similarly, tryptase (a mast cell protease) also increased in the allergen challenged group (4.3 ng/ml ± 2.01 ng/ml) as compared to placebo (undetectable levels; p< 0.056)(Figure 7a and b). A correlation analysis of CSLPI/SLPI ratios compared to each of the other biomarkers measured in this study shows a much stronger correlation between NAL CSLPI/SLPI ratios and chymase levels (r=0.91) and CSLPI/SLPI and α2-macroglobulin levels (r=0.41) as compared to CSLPI/SLPI ratios and tryptase levels (r=0.07)(Figures 3-7).
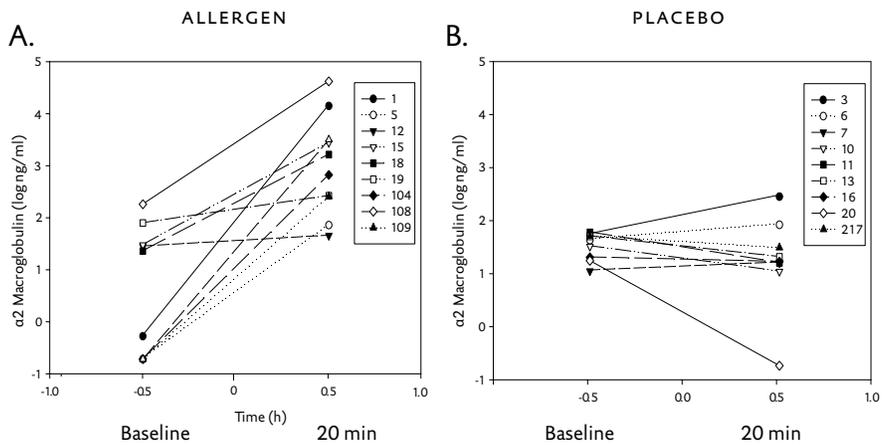
A. ALLERGEN

B. PLACEBO

FIGURE 6   NAL α2–MACROGLOBULIN LEVELS FOLLOWING NASAL ALLERGEN. α2-macroglobulin levels are elevated in NAL following nasal allergen challenge AR–patients (A) versus placebo challenge (B). Welch T test, P = 0.00035, allergen challenged as compared to placebo from baseline to 20 min post challenge. Legend refers to patient number.
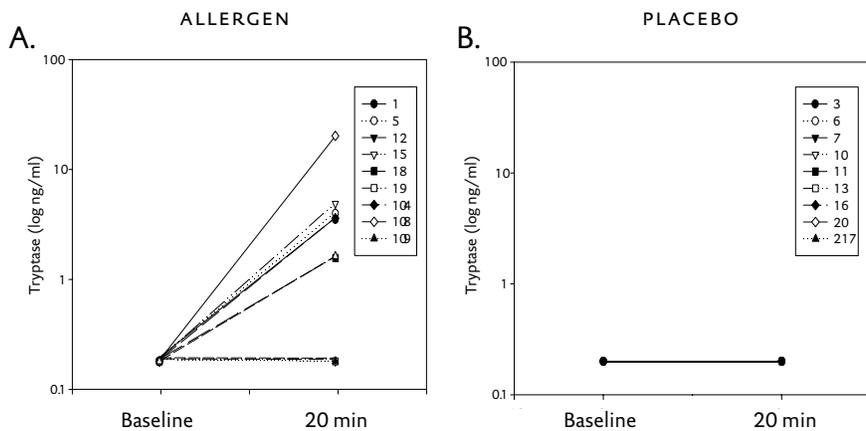
A. ALLERGEN

B. PLACEBO

FIGURE 7   NAL TRYPTASE LEVELS FOLLOWING NASAL ALLERGEN. Tryptase levels are detectable only in NAL following allergen challenge (A) while none is detected following placebo treated AR–patients (B). students T test, p = 0.056, allergen challenged as compared to placebo from baseline to 20 min post challenge. Legend refers to patient number.

Discussion

The function of SLPI is to protect tissues against the harmful effects of environmental irritants through its anti-inflammatory, anti-protease and anti-microbial properties. Within the respiratory tract, SLPI is produced by a variety of cells including neutrophils, macrophages and epithelial cells (15). SLPI is cleaved by chymase at a specific site and the level of this cleavage product, CSLPI, is indicative of chymase activity (15). Here, we report increased baseline CSLPI levels in sputum and nasal lavage of subjects with untreated allergic asthma and allergic rhinitis (AR), respectively, with a further increase following nasal allergen challenge in the AR group. These findings are suggestive of an enhanced chymase activity in allergic airway disease in vivo and hence, extend our previous in vitro observations.

Based on in vitro data of our previous publication (19), CSLPI can be used as a measure of chymase activity in biological samples. We described, that out of a panel of mast cell proteases consisting of proteinase 3, elastase, tryptase, cathepsin G, chymotrypsin and chymase, only chymase is able to cleave SLPI (19). SLPI acts as a first line of inhibition but is also cleaved by the chymase. This allows us to see a record of chymase activity before α-2-macroglobulin and other inhibitors inhibit it irreversibly.

In the present study, we measured CSLPI levels in airway samples from patients with allergic airways disease. Increased CSLPI levels were demonstrated in sputum of patients with untreated allergic asthma, while use of corticosteroids was associated with reduced CSLPI levels. However, this finding is somewhat difficult to interpret since these individuals were also more severe asthmatics. Unfortunately, we are unable to obtain specimens from severe asthmatics who are not receiving either oral or inhaled cortocosteroids. CSLPI was also increased in nasal lavage of subjects with allergic rhinitis at 20min following an intranasal allergen challenge, which elicited an early nasal response. In accordance with previous observations (19), CSLPI appeared closely correlated to chymase levels in both asthma (r=0.83) and AR (r=0.91). Taken together, these data suggest that CSLPI may act as a biomarker of chymase activity in allergic airways disease *in vivo*.

Apart from CSLPI and chymase, other markers of (mast-cell-related) airway inflammation were increased, including tryptase, a marker of mast cell activity, and α2-macroglobulin, a key inhibitor of chymase in AR (24, 25). Following nasal allergen challenge, all these biomarkers were further increased. α2-macroglobulin levels correlated well with the increased levels of CSLPI (r=0.41). This was expected, as α2 macroglobulin is an inhibitor of chymase, and had a high correlation with chymase levels as well (r=0.62) (24). We observed an apparent 10-fold greater amount of tryptase as compared to

chymase and CSLPI in the nasal lavage following allergen challenge. We attribute this to the relatively greater amounts of tryptase stored in mast cells, up to 25% of cellular protein, as compared to chymase (26). Moreover, tryptase can originate from both MCTC and MCT cells, whereas chymase originates only from MCTC cells, and the relative degranulation of each of these mast cell types to the allergic response varies (27). In addition, some of the disparity in relative changes in chymase and tryptase can be related to the differences in methods of measurement – highly quantitative ELISA for tryptase and α-2 macroglobulin, while a qualitative Western was used for chymase and CSLPI. Consequently, it is not surprising to note that the rise in the tryptase levels although indicative of mast cell activity did not correlate with CSLPI/SLPI ratios (0.07) confirming that cleavage of SLPI is not based on presence of tryptase as observed in our previous study (19). Interestingly one third of the subjects in the present nasal challenge study did not experience an increase in NAL tryptase levels. It appears that tryptase is not necessarily elevated in all atopic individuals with airway disease. Bettiol *et al* showed that only 18% of asthmatics exhibited elevated tryptase levels (28).

Until now, no direct measure was available for chymase activity in biological fluids due to its short half-life, mainly caused by the high content of chymase inhibitors (25, 29). The advantages of the use of CSLPI as a biomarker of chymase activity are that SLPI cleavage is easily detectable in readily accessible human samples of saliva, sputum and nasal lavage. Additionally, known chymase inhibitors will inhibit the level of SLPI cleavage (19). Furthermore, disease states such as AR and asthma result in higher amounts of cleaved SLPI. Taken together, the data show that detection of these unique SLPI cleavage products can act as novel biomarkers of chymase activity and chymase-mediated disease.

However we do acknowledge several shortcomings of this particular study that begins with the sample size. Despite obvious relationships between the inflammatory markers, low patient numbers make it difficult to achieve adequate correlation values. Furthermore, in this population some asthma patients were using (inhaled) corticosteroids, whereas others were untreated. Even though significantly lower levels were detected in those on corticosteroids, the effect of these drugs on CSLPI levels needs to be prospectively evaluated in a well-powered, placebo-controlled studies.

In conclusion, our data show that CSLPI may be an in vivo biomarker of chymase activity in allergic airway disease. Our findings need confirmation from larger prospective trials in patients with well-defined disease characteristics and various treatments.

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NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

DISCUSSION

NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

Summary and general discussion

In this thesis, a series of clinical studies have been described, in which we applied, evaluated or modified novel and existing non- or semi-invasive sampling methods and detection techniques for the assessment of biomarkers in allergic airway inflammation.

Clinical studies – allergic asthma

In the first part of this thesis the focus is on drug and method development in allergic asthma. In chapter 3, the pharmacological effect of an inhaled Neurokinin1/Neurokinin2 receptor antagonist (AVE5883) was initially evaluated against exogenous Neurokinin A (NKA) challenge. Subsequently, the efficacy was explored in the allergen challenge model of asthma. Although the drug provided partial protection against NKA-induced bronchoconstriction in asthmatics, a similar dose appeared ineffective in reducing the allergen-induced airway responses. Similarly, the drug failed to inhibit the allergen-induced increases in airway inflammatory biomarkers, such as sputum eosinophils and exhaled NO (eNO) levels.

In the subsequent chapter, the effect of bronchodilation on eNO measurements at 8 h following allergen inhalation causing a late allergic response (LAR) was evaluated. It appeared that levels of eNO further increased following reversal of the allergen-induced airway obstruction by inhaled salbutamol (chapter 4). Since salbutamol administration per se does not affect eNO, widening of the airway diameter caused this effect. Therefore, to enable a correct comparison of eNO values in the individual patient, it should be considered to measure eNO values following appropriate bronchodilation (1). Stationary eNO chemiluminescence analyzers are validated devices but their applicability is largely hampered by their bulkiness and high costs. The NIOX MINO (MINO) is a hand-held, relatively new and inexpensive device for online eNO measurements. In chapter 5 the reliability and repeatability of the MINO was tested by comparing the eNO values with those measured by the Ecomedics chemiluminescence analyzer (ECO). Exhaled NO levels were reproducible and in agreement between both devices, underscoring the validity of eNO samplings by the MINO (chapter 5).

Sampling of biomarkers in sputum and exhaled air, especially if performed by exhaled breath collection and analysis, is hampered by small sample volumes, the denaturant effects of processing and the limited sensitivity of most detection assays. In chapter 6, novel processing (sputum dialysis and ultracentrifugation) and detection techniques (flow-cytometry using Luminex multi-analyte profiling beads, electrochemiluminescence analysis using Mesoscale multi-array microplates and gene-array) were evaluated in allergic asthmatics. In this exploratory study, we demonstrated that the recovery

of biomarkers was improved by ultracentrifuge-processing of sputum supernatant compared with dithiothreitol (DTT)-dialysis treated samples, when measured with Mesoscale. Luminex allowed the detection of similar and other biomarkers. In addition, we were able to isolate RNA and perform expression profiling on the sputum cell pellet. The biomarkers in Exhaled Breath Condensate (EBC) samples were evaluated by Luminex only and most of these markers remained below the detection limit of the assay.

Clinical studies – allergic rhinitis

In the second part of this thesis, the focus was on allergic rhinitis (AR). First the applicability and reproducibility of several commonly used biomarkers in AR was investigated. Serum specific immunoglobulin E (IgE) assays and skin prick tests (SPTs) exhibited good reproducibility in patients with clinically stable allergic rhinitis. Most inflammatory biomarkers measured in nasal lavage (NAL) fluid and nasal brush (NAB) material exhibited a greater variability over 14-21 days. Nonetheless, performing these assessments repeatedly following a nasal allergen challenge, a clear increase in most of these biomarkers was observed at several time-points. Levels remained unchanged following a placebo challenge. Hence, these relatively simple, semi-invasive, sampling techniques are suitable for investigation of the kinetics of allergen-induced upper airway inflammation (chapter 7). In the next chapter the longitudinal reproducibility of nasal NO (nNO) and its response to nasal allergen challenge is described (chapter 8). When measured at the same time of the day, nNO was found to have a good reproducibility over a short time period (CV = 21.4% up to 7 days), but decreased over longer time periods (CV = 38.3% after 14-21 days), possibly due to (subclinical) weather changes or seasonal effects. An acute increase in nasal inflammation elicited by a nasal allergen challenge was accompanied by a paradoxal decrease in nNO at 20 minutes post-allergen, followed by an increase in nNO at 24 hours post-allergen. In contrast, placebo challenge did not affect nNO.

Clinical studies – allergic asthma & allergic rhinitis

Allergic asthma and AR have a similar pathophysiology and in the final clinical chapter (chapter 9) the potential role of chymase (a biologically potent protease released by mast cells) and its cleavage product, cleaved Secretory Leukocyte Protein Inhibitor (SLPI), was studied in both disorders. SLPI is present in many biological fluids where its function is to protect the tissues through its anti-protease properties. The data indicate that levels of cleaved SLPI in sputum are higher in asthmatics compared to non-asthmatic controls,

and increase in patients with AR following nasal allergen challenge. Hence, cleaved SLPI may be a suitable marker to monitor chymase activity in both the lower and upper airways.

The overall goal of the clinical studies described in this thesis was to identify suitable non- or semi-invasive sampling methods for the assessment of biomarkers for early drug development and clinical monitoring of allergic airways disease. The implications of our findings are addressed by the following questions.

IS INDUCED SPUTUM A RECOMMENDABLE SAMPLING TECHNIQUE FOR INFLAMMATORY BIOMARKERS?

Sputum induction is a semi-invasive procedure of collecting secretions from the lower airways to study components of airway inflammation (2). Although not fully interchangeable with bronchoalveolar lavage (BAL) and bronchial biopsy, it has been shown to provide largely similar information on several inflammatory markers whilst being safer, cheaper and generally easier to perform (3,4). Despite these advantages, most subjects experience this procedure as a burden. In addition, the overall percentage of analyzable sputum samples in our trials was approximately 80% - which even in other specialized centers fails to reach 100% (5). Finally, many inflammatory markers in sputum supernatant are affected by the (standard) processing techniques and more sophisticated assays are needed for optimal biomarker detection (6).

One of the major advantages of sputum sample analysis is the possibility of evaluating multiple inflammatory biomarkers. In the solid phase, inflammatory cell differentials can be evaluated. The predominant cell type (eosinophils or neutrophils) helps to characterize the asthma phenotype and monitoring of sputum eosinophilis provides information on the inflammatory status within the airways and can also be used for treatment monitoring as was recently confirmed by a Cochrane review (7,8). More recently, the combination of sputum induction and RT-PCR allows the detection of mRNA in sputum cells (9,10). These studies found an increased expression of several inflammatory cytokines (IL-4, 5 and 13) in asthmatics compared to healthy controls and an increase in inflammatory cytokine expression profile following low dose allergen exposure in asthmatics that was blocked by inhaled corticosteroids (9,10). Adding to these findings, in our study mRNA was isolated from sputum cell pellets from asthmatics with similar expression profiles on two occasions. This opens the possibility to determine expression profiles of multiple inflammatory markers which, given the heterogeneity of asthma, can provide more information than previous sampling techniques, which focus on a limited number of inflammatory markers (11).

In the fluid phase of sputum, several inflammatory mediators are readily

measurable, whilst some measurements are unreliable due to the denaturant effects of sputum processing with DTT and/or limited sensitivity of most detection assays. Erin *et al* developed a dialysis technique protocol, in which the DTT is filtered from the sputum sample and thus increasing the recovery of DTT-sensitive cytokines and chemokines (12). Possibly due to the complexity of this technique or the usage of different antibody detection kits, we were unable to replicate these findings. However, in accordance with previous studies, mechanical homogenization of the samples (by ultra-centrifugation) resulted in a good recovery of spiked cytokines and chemokines, and subsequently, several cytokines and chemokines were quantified in sputum supernatant (6). A drawback of this unrefined technique arises from the disruption of cells and subsequent spilling of intracellular content in the homogenate – which of course, can partly account for higher the biomarker concentrations recovered (13). Similarly to RNA expression profiling in the sputum cell pellet, combining multiple inflammatory mediators to evaluate the cytokine expression patterns in sputum supernatant is a valid method to define several aspects of the heterogeneous nature of the inflammatory airway reaction in asthma. Applying this multi targeted approach, Brasier *et al* were capable to identify distinct asthma phenotypes based on cytokine expression patterns in BAL (11). Comparable studies need to be done in sputum supernatant from asthmatics.

In conclusion, we feel that based on the complexities of sputum induction and processing, this sampling technique is not suitable for routine disease monitoring. However, employing a motivated study population and an experienced laboratory staff in clinical trials of asthma, sputum induction is a useful tool to sample and evaluate multiple biomarkers of the inflammatory airway process. Appropriate inflammatory biomarkers should be selected that are insensitive to the processing techniques and readily detectable or validation of a novel collection and/or processing technique is required prior to implementation.

IS EXHALED NO READY TO BE USED AS A KEY BIOMARKER IN RESEARCH AND MANAGEMENT OF ASTHMA?

Exhaled NO is widely perceived as a potential biomarker of inflammatory airways disease, particularly of allergic asthma. Advantages of standardized eNO samplings are reproducible, non-invasive, online measurements achievable in almost all patients of 4 years and older (14). The drawbacks consist of many (endogenous and exogenous) factors affecting NO measures. One of these factors is the airway diameter - and in line with previous observations at baseline, there was a clear effect of airway diameter on eNO levels in an exacerbation model of asthma (15). Based on these findings, it may be recom-

mended to measure eNO following appropriate bronchodilation for reliable comparison in the individual patient and we suggest that this finding should be incorporated into the list of perturbing factors mentioned in the guidelines (16). Another important disadvantage of eNO measurements is the bulkiness and costs of the equipment. In this respect, the recently introduced hand-held and relatively inexpensive NO electrochemical analyzer (MINO) seems an asset for widespread use of eNO in both the clinic and in research (17). Exhaled NO values measured with the MINO were found to be reproducible and in agreement with the stationary ECO, an ATS-approved chemiluminescence analyzer. These results are in agreement with previous data comparing the MINO to the stationary NIOX unit (17,18). However, similarly to previous studies, the MINO systematically yielded slightly higher values and this may impact clinical interpretation. Therefore, a recent study calculated a conversion factor to correct for this issue (19). Conclusively, most technical issues surrounding eNO measurements appear to be solved or manageable and the remaining question is the clinical relevance (and disease specificity) of this biomarker.

When compared to induced sputum or EBC, the clear disadvantage is that only one component from the airways is sampled even though this single biomarker is related to the underlying airway inflammation. Exhaled NO levels are correlated with sputum eosinophils in steroid naïve asthmatics and levels increase following a spontaneous exacerbation or allergen-induced late response (20-22). In addition, baseline eNO levels have been shown to predict the clinical response to treatment with inhaled corticosteroids and levels decline after initiation of anti-inflammatory treatment (23,24). Baseline levels can also aid in the diagnosis of asthma. A cut-off value of >20 ppb has a sensitivity and specificity of approximately 70% which is superior to spirometry (FEV_1) measurement (25,26).

In day-to-day asthma management the role of eNO is controversial. On one hand, it seems that low levels of eNO can predict a successful dose reduction in inhaled corticosteroids while maintaining asthma control (22). In children, a treatment regimen based on eNO and symptoms, compared to symptoms alone, resulted in a significant reduction in disease-related parameters, including the severity of airway hyperresponsiveness, with a concomitant (but non-significant) reduction in asthma exacerbations requiring oral prednisone (27). On the other hand, a recent study reported that addition of eNO as an indicator of asthma control on top of standard disease monitoring resulted in the prescription of higher doses of inhaled corticosteroids, without additional clinically relevant improvements in asthma control (28). Currently, employing the single flow technique it is difficult to fully assess the airway origin of increased eNO. If eNO is measured at multiple expired

flow rates, it can be portioned into NO from the bronchial or alveolar compartment. It has already been demonstrated that alveolar NO is increased in severe asthma in comparison with mild to moderate asthma while there is no difference in eNO between these groups (29). In the same study it was also shown that alveolar NO is refractory to inhaled corticosteroids but is decreased following a course of oral steroids. This suggests alveolar NO is a potential marker for distal airway inflammation, possibly also useful in COPD and in clinical trials examining the effect of systemic anti-inflammatory therapy (30). The multiple flow technique is laborious and has not been fully standardized but in the future measuring NO at different flow rates may further refine this biomarker.

Overall, eNO could serve as a biomarker of allergic airway inflammation in clinical trials. In clinical practice, it can help in the diagnosis of asthma and dose titration of subsequent anti-inflammatory treatment. Whether it can improve asthma control in patients treated according to current best practice is open for debate (31,32).

IS EBC READY FOR IMPLEMENTATION IN DRUG EVALUATION AND CLINICAL PRACTICE?

EBC is an appealing sampling method, enabling repeated collections of fluid from the airways in a completely non-invasive and patient-friendly fashion (33). For the purpose of cooling and collection of the exhaled breath, many custom-made devices were initially developed – unsuprisingly, this yielded an array of sampling methods. Presently, commercially available devices (most widely used are the EcoScreen from CardinalHealth and the RTube from Respiratory Research) may help to overcome problems arising from the use of the early collectors using different collecting protocols. A recent ATS/ERS taskforce issued guidelines for better standardization of collecting procedures allowing comparison across research centers (34). However, studies comparing commercially available devices have shown mixed data. Following identical collection, EBC levels of total protein, eotaxin and cysteinyl leukotrienes were found to be significantly higher in samples collected with the EcoScreen collector compared to the RTube device (35,36). The pH values also differed between samples from both asthmatics and allergic rhinitics (35-37). In addition, the volume of EBC collected with the EcoScreen was consistently higher as compared to the RTube (1.8 ± 0.1 and 1.4 ± 0.1 mL, respectively). This may be due to the differences in cooling the exhaled air: the Ecoscreen has a refrigeration device at a constant temperature of -20 oC, while the RTube uses a cooling sleeve (at -20 oC), that heats up to 15 oC after a 10 minute collection period. This 'warming process' may cause the degradation of heat labile substances, which may also account for the differences in

protein and lipid levels found between the two devices. A clear advantage of the RTube is its small size, which makes it suitable for home use.

Apart from these sampling issues, problems with detection/quantification of inflammatory biomarkers in EBC is of even greater concern (33,38). This may be due to a limited sensitivity of the ELISA technique to measure inflammatory compounds in the EBC (39). However, using the very sensitive multiplex technology we were still unable to detect most inflammatory biomarkers in EBC samples collected with the EcoScreen from steroid-naïve patients with mild persistent allergic asthma. It is conceivable, that higher EBC concentrations of these biomarkers are present in more severe asthma-phenotypes or under different conditions, e.g. following an exacerbation. Metabolomic analysis of EBC seems a promising approach in both adults and children (40,41). This analytical technique, using high-resolution proton nuclear magnetic resonance (NMR) spectroscopy, enables characterization of the metabolic compounds, providing a 'fingerprint' of the individual samples. This approach seems especially promising since it reflects the heterogeneous nature of asthma and can be obtained from generally small EBC volumes generally containing low levels of inflammatory biomarkers.

Alternatively, coating the collecting tube or employing glass tubing may improve the sample yield. A study by Tufvesson *et al* found that coating the plastic surfaces with Tween 20 detergent or BSA improved the detection of eicosanoids and cytokines, respectively (42). However, since these coating substances may interfere with several detection assays, a more sophisticated approach may be to employ a glass condenser. In a recent study, the in-vitro recovery of albumin and 8-isoprostane sprayed into the collection system was found to be significantly higher in glass and silicone condensers as compared to teflon or polypropylene condensors (43). In a subsequent study in healthy volunteers, significantly more EBC volume yielding detectable biomarker was recovered using an optimized glass condenser compared to a silicone condenser and the EcoScreen (44).

In conclusion, despite optimization of the EBC collection and detection of biomarkers in recent years, this sampling technique awaits full validation and standardization before it can be implemented into research or clinical practice. For this purpose, it is worthwhile to incorporate EBC along with more standardized biomarkers sampling techniques in clinical trials and asthma management to help further development of this promising, non-invasive sampling technique.

IS NASAL NO USEFUL BIOMARKER TO MONITOR AR AS EXHALED NO IN ASTHMA?

Nasal NO can be sampled from upper airways and may be useful for non-invasive monitoring of airway inflammation in AR similarly to eNO in asthma.

The first studies with nasal NO were encouraging. Increased nNO levels were found in both symptomatic and asymptomatic patients with AR - as opposed to non-allergic controls (45,46). Recently, nNO measurements by the portable NO-analyzer, MINO, were validated against the gold standard chemiluminescence NO-analyzer in both healthy volunteers and patients with AR (47). Hence, this simple, non-invasive sampling methodology could easily be added to the existing diagnostic and research tools.

To further explore the applicability of nNO as a potential biomarker for AR, the longitudinal reproducibility of nNO levels in patients with clinically stable AR was investigated. A good reproducibility over short time periods, diminishing over time was found. In chapter 8, we conclude that despite the large "natural" variability in nNO, the large effect of anti-inflammatory treatment (nasal corticosteroids) seen in a previous study would still allow to detect a significant intervention effect, being a prerequisite of a suitable biomarker (46). In contrast, other authors found a poor reproducibility of nNO over longer time periods in subjects with AR and in this study nasal steroids did not significantly decrease nNO levels (48). Therefore, it is still controversial if nNO is a suitable marker to monitor upper airways inflammation.

What is the effect of intranasal allergen-induced inflammation on nNO? Surprisingly, NO levels significantly decreased following nasal challenge with a relevant allergen, as compared with sham challenge. This phenomenon cannot be explained by a biological mechanism, but is most probably caused by mechanical obstruction of the nasal cavity during the acute phase post-allergen with subsequent decrease in nNO diffusion. This assumption has been previously confirmed in a study in patients with AR and nasal polyps (49). Nasal NO levels appeared to be inversely correlated with polyp size and raised following treatment of the polyps, suggestive of a mechanical blockade of NO diffusion from the nasal sinuses. Similarly to eNO measurements in asthma, appropriate decongesting was thought to counteract the nasal blockade and would allow correct nNO measurements during the allergen-induced acute phase. However, an acute fall in nNO is seen following administration of intranasal xylometazoline, possibly decreasing NO-diffusion as a result of xylometazoline-induced vasoconstriction (50,51). During the late phase response when most of the obstruction had subsided, nNO was increased up to 24 h. Similarly, a challenge with intranasal eotaxin in patients with AR previously caused a significant increase in nNO, at 24h post challenge (52). Thus, nNO may be a useful biomarker in nasal allergen challenge studies, especially during the late phase in patients with AR, preferably not pre-treated with nasal decongestants.

In conclusion, nNO measurement is a simple, fast and non-invasive biomarker in patients with AR. Substantial long-term intra-subject variation and the

marginal effect of anti-inflammatory therapy found in some studies, make it less suitable for disease monitoring (48,53). Nonetheless, nNO may potentially serve as a biomarker of (allergen-induced) nasal inflammatory response in clinical trials with novel anti-inflammatory therapy although confounding factors such as massive rhinorrhea, (partial) occlusion of the paranasal sinuses or pretreatment with nasal decongestants need to be taken into account.

CAN NASAL LAVAGE AND BRUSH BE USED TO SAMPLE BIOMARKERS OF NASAL INFLAMMATION?

Nasal lavage and NAB are more or less standardized sampling methods, commonly applied in clinical research and early drug development (54). Both techniques share the following advantages: they are simple, relatively non-invasive, suitable for repeated samplings, take little time and cause minor discomfort which makes them suitable to study the kinetics of the inflammatory airway response/process and their responsiveness to anti-inflammatory therapy (55). In addition, processing of the acquired samples is not as laborious as biopsy processing. Nevertheless, this flexibility seems to come at an expense. With the exception of IgE, data suggest a modest reproducibility of soluble biomarker levels in NAL in clinically stable allergic rhinitics. Also, many biomarkers remain below detection level. Similar observations were made by other authors employing a comparable nasal lavage technique (56,57). An alternative is to increase the dwelling time by occluding the nostril with an inflated balloon and subsequently instilling the lavage fluid (58). This technique yields higher levels of biomarkers and a better reproducibility. However, this is a less flexible procedure which does not allow repeated sampling. Another technique applies filter paper into the nose to absorb nasal secretions. This technique circumvents the problem of excessive dilution, encountered with nasal lavage and allows direct sampling of a region of interest. Indeed, in a recent study in patients with seasonal AR, Erin *et al.* showed generally higher levels of several inflammatory cytokines and chemokines in nasal filter paper than in lavage fluid (59). Whether this technique yields a superior reproducibility compared to lavage needs to be investigated. Similarly to EBC, more sensitive detection assays could aid in the quantification of biomarkers in nasal lavage fluid. In the study by Erin a multiplex bead array system was used for biomarker detection. Almost all cytokines and chemokines were above the detection limit both in nasal filter paper and lavage fluid. This is in contrast to our study where traditional ELISA/FEIA quantification was used. Apart from traditional and more sophisticated quantification techniques, other evaluation techniques, such as metabolomics and proteomics, may have potential to assess upper airway inflammation, although both techniques await evaluation in AR. Most NAB material was

suitable for analysis (87%) in our study. However, eosinophil counts showed a modest reproducibility and appear to be more variable than the established nasal biopsies (60).

Nasal lavage and brush techniques allow multiple sampling in a relatively short time period. Significantly increased levels of several soluble inflammatory biomarkers in NAL and eosinophils in NAB in combination with increased nasal symptom scores followed a nasal allergen challenge (chapter 7). The cell samplings from the nasal mucosa also allow RNA isolation and quantification in small amounts of material. Using this technique, Dreskin *et al* reported an increase in mRNA for IL-5 following nasal allergen challenge in patients with seasonal AR (61). Other authors previously reported decreases in nasal symptoms accompanied by reduced inflammatory markers sampled with NAL and NAB in AR patients treated with topical corticosteroids versus placebo-treated patients (62).

In conclusion, these techniques allow simple and safe sampling of (clinically) relevant biomarkers of upper airway inflammation that are responsive to treatment effects. Their low reproducibility is a clear disadvantage that may be improved by employing refined sampling and detection procedures or multiple baseline measurements.

COULD CLEAVED SLPI SERVE AS A POTENTIAL BIOMARKER OF BOTH ASTHMA AND AR?

Development of biomarkers for both diseases seems rational because of the shared pathophysiology. Chymase is a potent pro-inflammatory protease that is released from mast cells, important effector-cells in the pathophysiology of allergic airway inflammation (63-65). The protease inhibitor SLPI is both a chymase inhibitor and a substrate of chymase and its cleaved product, CSLPI, is a biomarker of chymase activity in vitro (66). In contrast with chymase that has a short half-life upon release, CSLPI is stable and readily measurable in relevant body fluids including saliva, sputum and nasal lavage. We further explored the potential of CSLPI as a biomarker of chymase activity in subjects with mild to moderate persistent allergic asthma and found increased baseline sputum levels compared to healthy controls. Inhaled corticosteroid-treated asthmatics had overall lower sputum chymase levels compared to untreated asthmatics, suggesting that CSLPI is sensitive to anti-inflammatory treatment effects. In untreated patients with AR, CSLPI was increased in NAL, 20 min post intra-nasal allergen challenge, accompanied by an increase in symptoms and other (local) inflammatory markers. These events did not occur following placebo challenge. This is in agreement with previous findings in atopic patients showing increased SLPI levels in NAL following a nasal allergen challenge (67). SLPI could also serve as a (target for)

novel drugs. In vitro experiments showed that SLPI can inhibit IgE-mediated histamine release probably from mast cells and basophils in human nasal mucosa (68). This effect was confirmed in sensitized guinea-pigs in vivo: treatment with topical SLPI suppressed the conjunctival recruitment and degranulation of eosinophils after antigen challenge for 6 h, thus inhibiting the development of allergic conjunctivitis. In the same study it was demonstrated that SLPI inhibited chymase activity in a dose-dependent manner, without affecting tryptase (69). Alternatively, direct chymase inhibitors are being developed as anti-allergic therapy and presently the first compounds are entering the early clinical phases.

Detection of CSLPI is feasible in patients with asthma and AR and suggests that CSLPI could act as a novel biomarker of chymase activity. The relationship with disease activity and the effect of anti-inflammatory therapy on CSLPI levels awaits prospective evaluation by well-powered, placebo-controlled studies. It may be speculated that CSLPI may not only serve as a future biomarker of allergic disease activity but may also become a novel therapeutic agent.

Overall conclusion

An applicable biomarker possesses the characteristics of clinical relevance, sensitivity and specificity for the disease and responsiveness to treatment effects, in combination with simplicity, reliability and repeatability of the sampling methodology. All biomarkers for asthma and AR obtained with the sampling techniques described in this thesis meet at least some of these criteria. On a case by case basis, a decision will need to be made what biomarker suits best the purpose of the research pursued and fulfills most of the relevant selection criteria.

With the future development of more sophisticated sampling and detection techniques, the described biomarkers will be further refined and possibly replaced by new ones.

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NON-INVASIVE SAMPLING METHODS OF INFLAMMATORY BIOMARKERS IN ASTHMA
AND ALLERGIC RHINITIS

Samenvatting

Allergisch astma en allergische rhinitis (AR) zijn verwante chronische ontstekingsziekten van respectievelijk de onderste en bovenste luchtwegen met wereldwijd een hoge prevalentie (astma 1-18% en AR 10-25%). Hoewel zelden levensbedreigend, hebben beide aandoeningen een grote invloed op de kwaliteit van leven en kunnen veel patiënten niet adequaat behandeld worden met de beschikbare medicatie. De klachten die patiënten met astma en/of allergische rhinitis uiten, zijn verschillend per patiënt, en kunnen gepaard gaan met de volgende symptomen: kortademigheid, piepende ademhaling, drukkend gevoel op de borst, hoesten, en 'overgevoelige luchtwegen voor allerlei stoffen', dan wel nasale congestie, jeuk, niezen en oogsymptomen. Beide aandoeningen komen vaak gezamenlijk voor en hebben hun oorsprong in de interactie van genetische constitutie met bepaalde omgevingsfactoren.

Het allergische ontstekingsproces kenmerkt zich door een immunoglobuline E (IgE) gemediëerde reactie op specifieke, externe prikkels ('allergenen'), zoals huisstofmijten, pollen of huidschilfers van dieren. De hierop volgende ontstekingscascade wordt getypeerd door een complex netwerk van effectorcellen (voornamelijk mestcellen en eosinofiele granulocyten), ontstekingsmediatoren, cytokinen, chemokinen, neuropeptiden en signaalmoleculen met onderlinge interacties. Dit leidt enerzijds tot migratie van ontstekingscellen naar de mucosa in de longen en de neus, en anderzijds tot een reactie met verschillende targetcellen (zoals gladde spiercellen, vaatwandcellen en mucusproducerende cellen) met als gevolg nasale cq. bronchiale obstructie en hyperreactiviteit. Onderzoek bij patiënten heeft aangetoond dat symptomen en longfunctieparameters, die veelal als leidraad voor de behandeling van astma worden gebruikt, niet de beste indicatoren zijn voor de mate van de onderliggende luchtwegontsteking. Om die reden staan huidige richtlijnen astmamanagement op geleide van astmacontrole voor - hetgeen een 'multi-dimensionale' parameter is.

Een biomarker is een te meten variabele die gebruikt kan worden als een indicator voor normale biologische processen, pathologische processen en/of het effect van een behandeling. Een goede biomarker is klinisch relevant, gevoelig voor veranderingen in het ziekteproces, betrouwbaar, reproduceerbaar, en eenvoudig uitvoerbaar. Het ontstekingsproces bij astma en AR biedt verschillende potentiële biomarkers. Eosinofiele granulocyten, verkregen via niet-invasieve samplings van de onderste of bovenste luchtwegen, zijn een goed voorbeeld van een ontstekingsbiomarker. Het percentage eosinofielen kan enerzijds een indicator zijn voor het type en de activiteit van het onderliggende ziekteproces en als graadmeter dienen voor het monitoren van anti-inflammatoire therapie. Eosinofielen-tellingen kunnen worden gedaan op gekleurde cytospins uit het sputum (slijm uit de onderste luchtwegen). Sputum kan op een niet-invasieve manier worden geïnduceerd

middels inhalaties van o.a. hypertone zoutoplossingen, waarna de patiënten worden aangemoedigd om op te hoesten. Naast eosinofielen, kunnen uit het sputum diverse andere ontstekingsbiomarkers worden bepaald, waarvan er verscheidene gevalideerd zijn. Sputum is dus een geschikt medium om het ziekteproces bij astma te onderzoeken. Een andere sampling-techniek voor ontstekingsbiomarkers uit de longen bestaat uit het verzamelen en analyseren van gekoeld uitgeademd luchtcondensaat (EBC). Hierbij ademt de patiënt gedurende een tiental minuten in een collector die de uitgeademde lucht koelt. Met behulp van gevoelige detectietechnieken kunnen vervolgens ontstekingsproducten in de EBC gemeten worden.

Een derde manier om de luchtwegontsteking bij astma te vervolgen is het meten van stikstofoxide (NO) in uitgeademde lucht. Dit kan op een betrekkelijk eenvoudige wijze met behulp van moderne apparatuur. Uit diverse studies is gebleken dat de concentratie uitgeademd NO de ernst van de luchtwegontsteking bij astma reflecteert en dat de NO waarde daalt na adequate behandeling met anti-inflammatoire therapie.

Ook in de neus kunnen verschillende niet- of semi-invasieve samplingtechnieken toegepast worden om ontstekingsbiomarkers te bemonsteren en analyseren. Zo kan op vergelijkbare wijze als uitgeademd NO bij astma, nasale NO (nNO) worden gemeten bij patiënten met allergische rhinitis. Twee andere technieken, waarbij men op 'semi'-invasieve wijze meerdere biomarkers in één keer verzamelt, bestaan respectievelijk uit neuslavage (NAL), waarbij de neus wordt gespoeld met een isotone zoutoplossing, en neusbrush (NAB), waarbij het neusslijmvlies lokaal wordt geborsteld met een klein borsteltje. Middels beide methoden kunnen verschillende biomarkers (respectievelijk ontstekingsmediatoren en ontstekingscellen) worden bepaald die deel uitmaken van het ontstekingsproces bij AR.

Dit proefschrift omvat een aantal klinische studies waarin de beschreven technieken werden toegepast om componenten van de luchtwegontsteking bij patiënten met allergisch astma en AR, al dan niet in een interventie setting, te meten. Het doel van dit proefschrift was om deze samplingtechnieken te optimaliseren als ook om de reproduceerbaarheid van biomarkers en toepasbaarheid van nieuwe detectiemethoden te onderzoeken.

Het eerste deel van dit proefschrift betreft biomarkeronderzoek bij allergisch astma. Hoofdstuk 3 beschrijft het onderzoek naar de farmacologische en klinische effectiviteit van een gecombineerde Neurokinine1/Neurokinine2 (NK1/NK2) receptor antagonist (AVE5883) in twee aparte studies. In de eerste studie bood geinhaleerd AVE5883 bescherming tegen Neurokinine A-geïnduceerde luchtwegvernauwing bij patiënten met allergisch astma. Echter, bij toepassing in een exacerbatie model van astma, bleek dezelfde dosis AVE5883 niet effectief tegen allergeen-geïnduceerde

luchtwegvernauwing en hyperreactiviteit. Bovendien was de toename in luchtwegontstekingscomponenten (cq biomarkers), namelijk sputum eosinofielen en uitgeademd NO, niet verminderd door behandeling met AVE5883. In een volgende studie (hoofdstuk 4) werd uitgeademd NO tijdens een allergeen-geinduceerde late reactie voor en na recuperatie met salbutamol bepaald. Er was een sterke stijging van uitgeademd NO na opheffing van de allergeen-geïnduceerde luchtwegvernauwing na salbutamol-inhalatie. Hierbij correleerde de toename in uitgeademd NO met de toename in longfunctie (FEV_1). Uit onderzoek bij patiënten met klinisch stabiel astma, bleek eerder dat luchtwegvernauwing gepaard gaat met lagere NO waarden. Gezien de relatie tussen uitgeademd NO en luchtwegdiameter, concluderen wij dat voor een juiste vergelijking en interpretatie van deze biomarker in de klinische setting, NO gemeten dient te worden na een adequate luchtwegverwijding. In het volgende hoofdstuk werd de betrouwbaarheid van een draagbare NO meter (NIOX MINO®) in vergelijking met de 'gouden standaard' onderzocht (hoofdstuk 5). De NO waarden gemeten met de NIOX MINO bij gezonde vrijwilligers, rokers en patiënten met onbehandeld allergisch astma waren reproduceerbaar en kwamen overeen met metingen met behulp van een gevalideerde uitgeademd NO analyser (Ecomedics). De vergelijking tussen NIOX MINO met 'zijn grote broer', NIOX stationary, toonde destijds vergelijkbare resultaten. De NIOX MINO is draagbaar, goedkoper, eenvoudiger in gebruik en onderhoud en daardoor potentieel beter inzetbaar in de kliniek dan de traditionele (stationary) analysers, de NIOX en de Ecomedics. Het meten van biomarkers in sputum en EBC wordt vaak bemoeilijkt door te kleine hoeveelheden materiaal, gevoeligheid van biomarkers voor voorbehandeling en beperkt meetbereik van de meeste traditionele detectie-technieken. In hoofdstuk 6, werden nieuwe voorbehandelings- en detectietechnieken in sputum en EBC van patiënten met allergisch astma getest. In deze exploratieve studie, bleek ultracentrifugatie van sputum hogere spiegels aan diverse biomarkers op te leveren dan de traditionele voorbehandeling met dithiotreitol (DTT). Dit laatste ondanks toepassing van een (eveneens nieuwe) dialyse techniek, waarmee het voor biomarkers schadelijke DTT na de homogenisatie uit het monster werd gezuiverd. In het ultragecentrifugeerde sputum konden met twee gevoelige analyse technieken, i.e. Mesoscale en Luminex, biomarkers worden gemeten, die normaliter niet detecteerbaar zijn na voorbehandeling met DTT. Daarnaast was het mogelijk om RNA te isoleren uit de sputumcellen en het expressie niveau te bepalen. In EBC bleven de meeste biomarkers onder de detectielimiet, ondanks toepassing van Luminex.

Het tweede deel van dit proefschrift beschrijft biomarkerstudies bij patiënten met AR. Hierin bleken de veelvuldig gebruikte allergiemarkers, serum IgE waarden en huidpriktesten, een goede reproduceerbaarheid te

vertonen (hoofdstuk 7). Daarentegen waren biomarkers in NAL en NAB vaak niet meetbaar of vertoonden over een periode van 3 weken een aanzienlijke variabiliteit ondanks de stabiele klinische status van deelnemers. Mogelijk dat een andere sampling-procedure, met behulp van filtreerpapier dat vloeistof in de neus absorbeert, een oplossing kan bieden voor dit probleem dat deels samenhangt met een te sterke verdunning van de biomarkers vooral in de NAL. Ondanks dit minpunt, bleek in tegenstelling tot de placebo challenge, een nasale provocatietest met een relevant allergeen een duidelijk effect te hebben op verschillende ontstekingsmarkers in de neus. Hierbij waren met name $\alpha2$ macroglobuline, eosinofiel cationisch proteïne (ECP) en tryptase verhoogd in de NAL, en de eosinofielen in de NAB. In dezelfde patiëntengroep werd eveneens de reproduceerbaarheid van de 'baseline' nasale NO waarden getest als ook het effect van een nasale allergeen provocatietest hierop (hoofdstuk 8). Uit onze bevindingen bleek dat bij patiënten met klinisch stabiele AR, nNO metingen gedurende korte observatie-perioden (<1 week) reproduceerbaar zijn, maar dat reproduceerbaarheid afneemt over langere perioden (al vanaf 2-3 weken). Mogelijk kunnen a-symptomatische seizoens- of weersinvloeden de nNO schommelingen verklaren. Interessant genoeg induceerde nasaal allergeen enerzijds wel een toename van symptoomscores en ontstekingsmarkers in de NAL, maar anderzijds een daling van nNO waarden tijdens de vroege allergische reactie. Deze daling werd waarschijnlijk veroorzaakt door een mechanische blokkade van de sinus maxillares door massieve slijmsecretie en slijmvlieszwelling en was vermoedelijk niet het gevolg van een biologische reactie. Vergelijkbare bevindingen werden eerder gedaan bij patiënten met neuspoliepen. Hierbij waren de nNO concentraties initieel omgekeerd evenredig aan de poliepomvang en stegen na behandeling van de poliepen. Vierentwintig uur na de allergeenprovocatie, toen de nasale obstructie grotendeels was geweken, bleken de nNO waarden significant verhoogd.

Het onderliggende ontstekingsproces bij astma en AR heeft vergelijkbare ontstekingskenmerken. In de laatste jaren wordt wederom veel aandacht besteed aan de mestcel als belangrijke effectorcel in beide aandoeningen. In hoofdstuk 9 werd exploratief onderzoek verricht naar een mogelijke rol van chymase (een protease afkomstig uit mestcellen) en diens afbraakproduct (cleaved secretory leukocyte protease (CSLPI)) bij beide aandoeningen. CSLPI geeft een indicatie van chymase activiteit (hetgeen een relevanter parameter is dan de concentratie) en is stabieler dan chymase, en ergo, een betrouwbaarder biomarker. In deze studie vonden wij hogere chymase- en CSLPI concentraties in het sputum van patiënten met onbehandeld allergisch astma in vergelijking met monsters van astmapatiënten die met anti-inflammatoire therapie werden behandeld en gezonde vrijwilligers. Bij patiënten

met AR induceerde intranasaal allergeen (in tegenstelling tot placebo) een significante stijging van chymase en CSLPI in de NAL. Tevens was er een positieve correlatie tussen de chymase concentraties en de ratio SLPI/CSLPI (maat voor chymase activiteit).

Conclusies en aanbevelingen:

IS SPUTUM EEN GESCHIKTE TECHNIEK OM BIOMARKERS TE BEPALEN?

Een belangrijk voordeel van sputumbepalingen is dat een groot aantal biomarkers, afkomstig uit het ontstekingsproces in de luchtwegen, tegelijk kan worden bepaald. Moderne detectiemethoden maken bepalingen van een steeds toenemend aantal ontstekingsmarkers mogelijk uit zowel het supernatant als uit sputumcellen. Echter, naast de belasting van de patiënt, houden factoren als het ingewikkelde opwerkproces, de geavanceerde detectietechnieken als ook de celdifferentiatiebepalingen, het gebruik van deze techniek beperkt tot specialistische centra en klinisch onderzoek

IS UITGEADEMD NO EEN GOEDE BIOMARKER VOOR ASTMA?

Uitgeademd NO is niet-invasief, eenvoudig meetbaar en reproduceerbaar. Daarnaast kan een verhoogde NO waarde ondersteuning bieden bij het stellen van de diagnose 'astma'. Andere voordelen zijn diens gevoeligheid voor veranderingen in de ontstekingsgraad van de onderste luchtwegen (zowel voor als tijdens behandeling met anti-inflammatoire geneesmiddelen) en de voorspellende waarde voor een verminderde astmacontrole/exacerbatie. Minpunten zijn diverse storende factoren die de uitkomsten kunnen beïnvloeden, zoals de luchtwegdiameter, sigarettenrook en bepaalde voedingsmiddelen. Het is vooralsnog onduidelijk welke rol uitgeademd NO zal spelen bij het helpen optimaliseren van de lange-termijn behandeling van astma.

KAN EBC GEBRUIKT WORDEN BIJ KLINISCH ONDERZOEK EN IN DE KLINIEK?

Vooralsnog biedt EBC als enige samplingmethode de mogelijkheid om op volledig niet-invasieve en eenvoudige wijze biomarkers uit uitgeademde lucht te verzamelen. Echter, de thans beschikbare apparatuur en techniek zijn nog niet volledig gestandaardiseerd hetgeen aanzienlijke verschillen in studieresultaten heeft opgeleverd tussen de verschillende onderzoekcentra. Bovendien grenzen de concentraties van verschillende biomarkers aan de detectielimieten van de meest gangbare assays. Ter verdere ontwikkeling en optimalisatie, dient deze veelbelovende techniek te worden gecombineerd met gestandaardiseerde methoden en gespiegeld aan meer gevestigde biomarkers.

ZIJN NAL EN NAB BRUIKBARE SAMPLINGTECHNIEKEN VOOR BIOMARKERS?

NAL en NAB zijn gestandaardiseerde, relatief patiëntvriendelijke en eenvoudige, semi-invasieve technieken die herhaaldelijk kunnen worden aangewend om meerdere biomarkers te monsteren. Belangrijkste nadelen vormen de lage concentraties en grote variabiliteit van de meeste biomarkers in de verkregen monsters. Grote(re) patiëntpopulaties, langere 'dwellingtime' in de neus (NAL) en het gebruik van gevoelige detectietechnieken kunnen mogelijk uitkomst bieden.

IS NNO NET ZO BRUIKBAAR VOOR AR ALS UITGEADEMD NO VOOR ASTMA?

Nasale NO kan op een eenvoudige en niet-invasieve manier gemeten worden en de concentraties zijn over het algemeen hoger bij patiënten met onbehandelde AR vergeleken met patiënten die anti-inflammatoire therapie gebruiken en gezonde controles. Echter, andere studies rapporteerden een behoorlijke overlap in nNO waarden tussen gezonde vrijwilligers en patiënten met AR. Wellicht worden de nNO waarden in belangrijke mate beïnvloed door de variabele slijmvlieszwelling. Mogelijk is dit - naast weers- en seizoensinvloeden - een belangrijke reden, waarom nNO metingen over langere perioden variabel zijn en dus minder geschikt voor klinische monitoring bij patiënten met AR. Nasale NO metingen kunnen misschien wel gebruikt worden in 'korte termijn studies', bijvoorbeeld als biomarker van (allergeen geïnduceerde) acute nasale inflammatie als gecontroleerd wordt voor interfererende factoren, zoals nasale congestie.

IS CSLPI DE TOEKOMSTIGE BIOMARKER VOOR ZIEKTEACTIVITEIT BIJ ALLERGISCH ASTMA EN ALLERGISCHE RHINITIS?

Cleaved SLPI bleek in vitro een marker te zijn voor chymase activiteit. Deze observaties worden bevestigd door onze *in vivo* data, waarbij hoge concentraties CSLPI werden gemeten bij patiënten met onbehandeld astma en bij AR patiënten met allergeen geïnduceerde nasale ontsteking. Gelet op de centrale rol die de chymase-bevattende mest- cellen mogelijk spelen bij allergische luchtweginflammatie en hyperreactiviteit, dient de rol van CSLPI verder te worden onderzocht in grote prospectieve studies.

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AND ALLERGIC RHINITIS

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Johan Diderik Boot was born on October 30, 1977 in Chertsey, England. After graduating from secondary school (Christelijk Gymnasium Sorghvliet, The Hague, 1995) he started studying Biopharmaceutical Sciences at the Leiden University in 1996. A research project was performed on antisense therapy for atherosclerosis at the Leiden Amsterdam Centre for Drug Research (Department of Biopharmacy; Prof T. van Berkel) and an internship at the Fortis capital markets & corporate finance Biotech team was completed. A Master of Science degree was obtained in 2002. After briefly working for Sanofi-Synthelabo in the clinical research department he took on the position of clinical scientist at the Centre for Human Drug Research (CHDR; CEO Prof. Dr. A.F. Cohen) in 2003. The research described in this thesis was performed at CHDR. In 2009 the registration as a clinical pharmacologist was obtained. Currently he works for Nycomed BV, Hoofddorp, The Netherlands as Medical Manager.