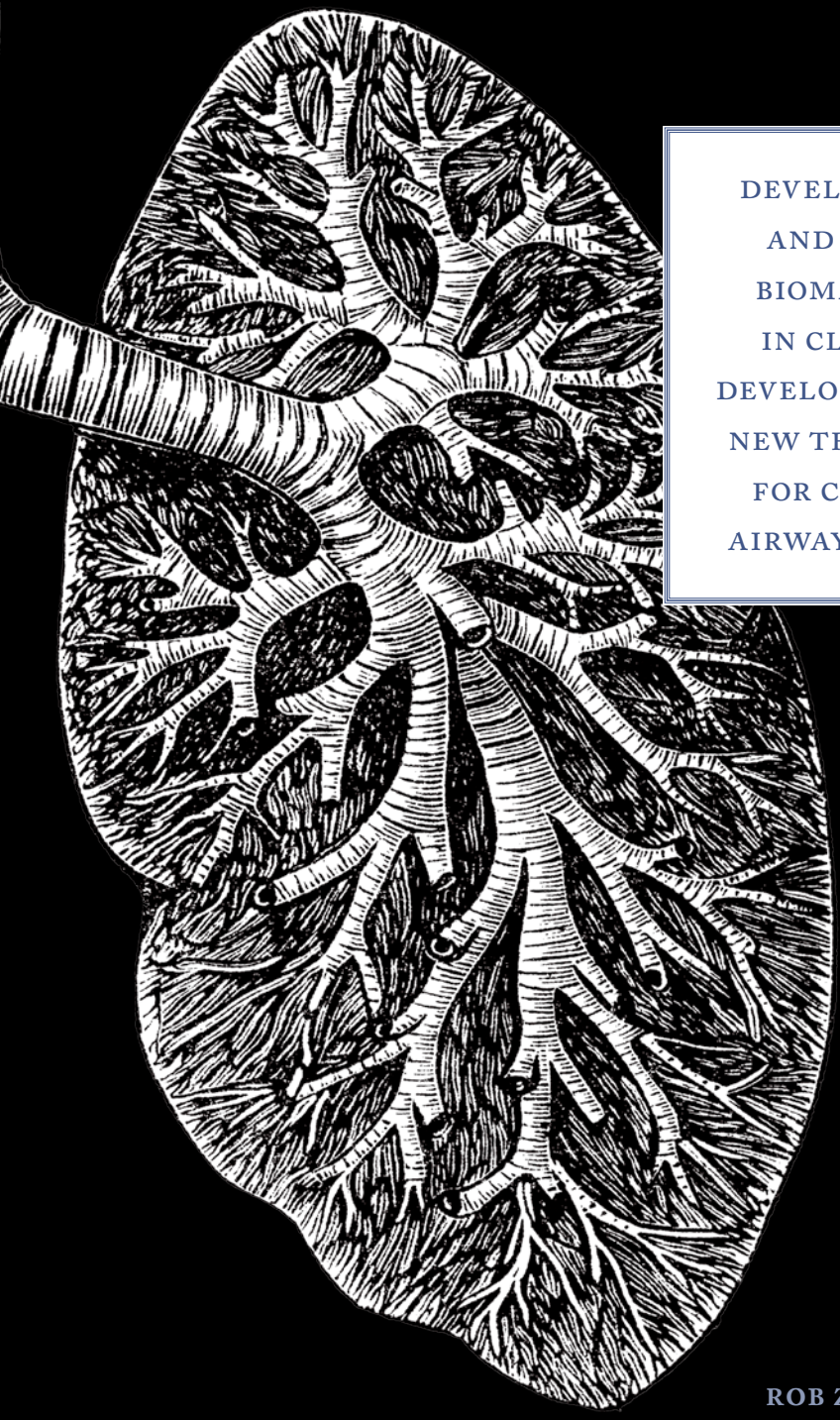
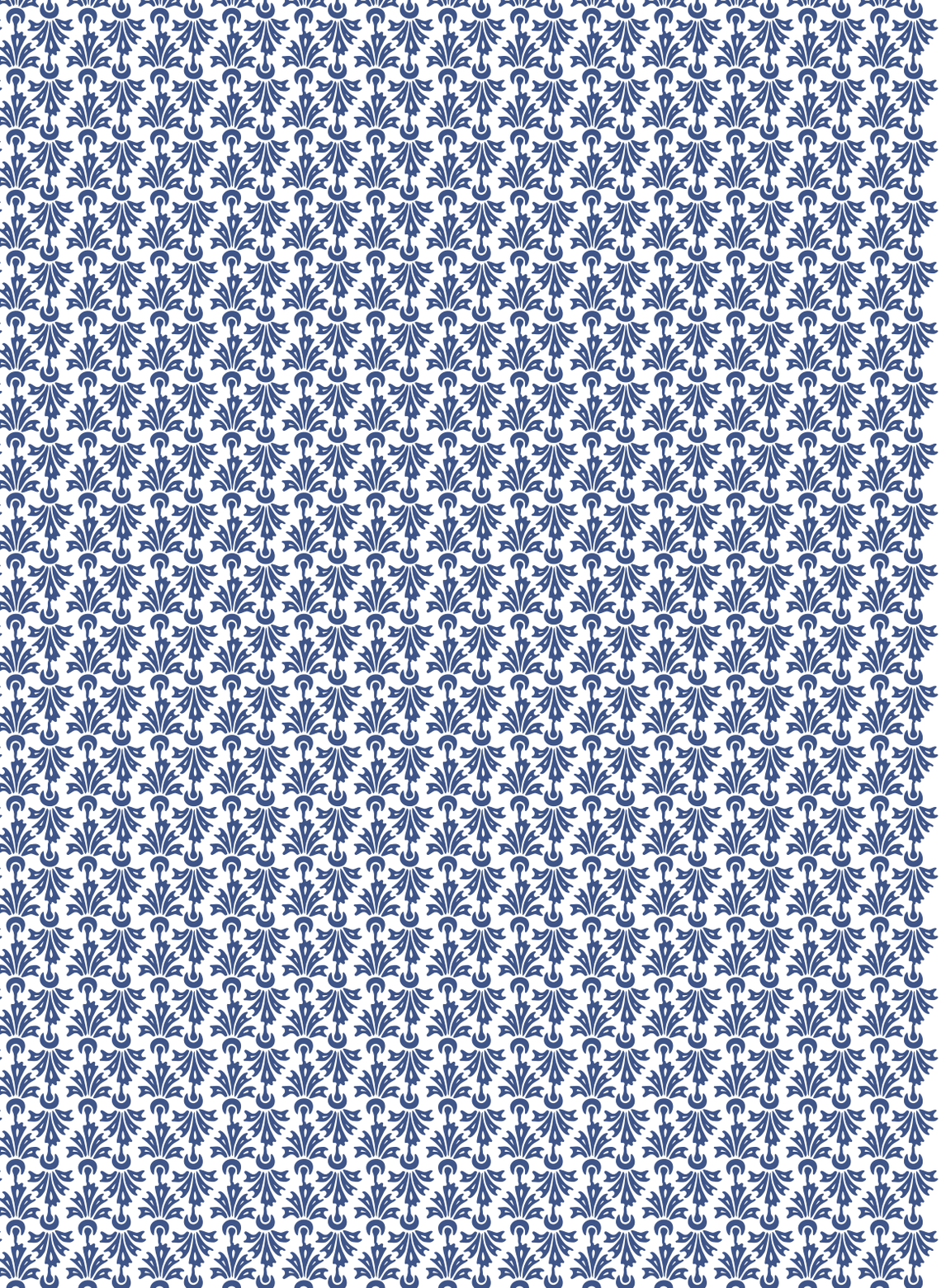


DEVELOPMENT AND USE OF BIOMARKERS IN CLINICAL DEVELOPMENT OF NEW THERAPIES FOR CHRONIC AIRWAY DISEASE

ROB ZUIKER



DEVELOPMENT AND USE OF BIOMARKERS IN CLINICAL DEVELOPMENT OF NEW THERAPIES FOR CHRONIC AIRWAY DISEASE

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DEVELOPMENT AND USE OF BIOMARKERS IN CLINICAL DEVELOPMENT OF NEW THERAPIES FOR CHRONIC AIRWAY DISEASE

Proefschrift
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door Robertus Gerardus Johannes Antonius Zuiker geboren te Wognum in 1968

CHAPTER I

INTRODUCTION

INTRODUCTION

Imagine working as a researcher at an independent research institute, a contract research organization with close connections to academia (www.chdr.nl). Recently, a new pharmaceutical company contacted your organization after they acquired a new compound with a mode of action possibly suitable for the treatment of asthma. Preclinical studies show promising results and expectations are high. Since the pharmaceutical company is small, it seeks external advice on how to perform a first in human study specifically, and on a drug development plan in general. As a clinical pharmacology researcher you are asked to advise the company how to proceed in demonstrating safety and unlock the blockbuster potential of the compound, under the constraint of limited time and budget.

This introduction describes several aspects that should be considered in the development of potential anti-asthmatic drugs as an example for drugs that may be developed for respiratory diseases. The same strategy could also be considered for other respiratory diseases such as cystic fibrosis, chronic obstructive pulmonary disease, but these disease entities and possible therapeutics are not discussed in this vignette case. The clinical features and pathophysiology of asthma, the added value of animal models, a general overview of asthma drug research and finally an overview of commonly uses biomarkers for asthma are described.

ASTHMA

EPIDEMIOLOGY ❦ Asthma is estimated to affect 300 million individuals worldwide [1;2]. The prevalence of asthma and associated allergic syndromes is increasing worldwide and the currently available drugs are not equally effective in all patients [3]. Hence there is still a demand for novel, targeted anti-asthma therapy.

CLINICAL CHARACTERISTICS ❦ Asthma is a chronic inflammatory disorder of the airways, often associated with atopy (allergy). Clinically, variability of symptoms and airway obstruction is the most striking phenomenon. The pathogenesis is still not fully elucidated, but chronic airway inflammation and airway hyper-responsiveness (AHR), an excessive response to triggers that have little effect in normal individuals, represent the key characteristics in the pathophysiology of asthma [4]. Along with these more or less dynamic features, structural changes of the airways (the so-called airway remodeling) occur early on in the disease, *Figure 1*.

All these asthma characteristics appear to be interrelated [5] and if the disease is persistently treated inadequately, this may result in the loss of asthma control and accelerated decline of lung function. Since none of these asthma features or underlying inflammatory mechanisms are present in healthy, non-asthmatic individuals, it is necessary to conduct a trial with a novel therapy on patients as soon as possible in early clinical proof of concept (POC) studies.

When a relevant allergen is inhaled by sensitized patients with allergic asthma, it can induce various airway responses [6]. An immediate or early asthmatic response (EAR) is characterized by a fall in the forced expiratory volume in one second (FEV1) of ≥ 15 % from baseline within 10 to 30 minutes following inhalation of the allergen. The EAR is an IgE-triggered phenomenon, mainly caused by mast cell release inducing acute bronchoconstriction, and usually resolves within one to three hours. In about 50 % of such patients an EAR is followed by a late asthmatic response (LAR), characterized by prolonged airway narrowing, often defined as a fall in FEV1 of ≥ 15 % occurring between three and eight hours post-allergen exposure in which TH2 lymphocytes, activated eosinophils, their pro-inflammatory and toxic products play a key role, *Figure 2*. These sequelae may last for several days or weeks and result in the development of the allergen-induced AHR.

PATHOPHYSIOLOGY OF ASTHMA ❦ Two main types of T helper lymphocytes have been characterized: TH1 and TH2. TH1 cells produce (amongst others) interleukin (IL)-2 and IFN-γ, which in general are critical in cellular defense mechanisms when responding to infection. TH2-cells generate a family of interleukins that can mediate allergic inflammation, *Figure 3* [7]. The TH2-cells produces IL-4 and IL-13 (causing B cell immunoglobulin (Ig) E production), IL-5 (causing eosinophilic inflammation) and IL-9 (promoting mast cells growth). Traditionally, it is thought that in atopic asthmatic patients, allergens tend to induce an unbalanced production of the TH2 type cytokines, but little of the TH1 cytokines.

NEW INSIGHTS ❦ For many years asthma was considered a straight forward disease driven by aberrant TH2 immunity. This hypothesis made it possible to understand classic allergic asthma, its association between atopy, eosinophilic lung inflammation, and the effect of corticosteroid treatments to reduce the TH2 type inflammation. However, it cannot explain why residual disease remains after optimized anti-inflammatory treatment. Also, numerous new targets for the treatment of asthma like key anti-interleukins 4 and 13 [8] and anti-interleukin 5 [9], have been based on this TH2 inflammation model but this approach has shown no or marginal

effect in the clinic. In addition, the TH2 inflammation model cannot explain some of the endophenotypes for asthma [10]. Asthma is nowadays recognized as a complex heterogeneous syndrome consisting of many different subtypes (E.G. intrinsic, extrinsic, seasonal, exercise induced, virally induced, aspirin sensitive, allergic, non-allergic, nocturnal and steroid resistant) each with different pathophysiology, expression of symptoms, response to treatment and prognosis. Lately, the role of the innate immune system with its Toll like receptors (TLR) and macrophages, originally thought of as a first defense against infections, has been more emphasized in relation to asthma [11;12].

TOLL LIKE RECEPTORS AND ASTHMA ❋ Before the immune system reacts, it must recognize the virus, bacterium or other infection. For the innate immune system, these recognitions are carried out by soluble elements (E.G. complement, binding proteins) or by pattern recognition receptors (PRRS) on macrophages, dendritic cells or polymorph nuclear leucocytes. The Toll like receptor is an example of a PRR recognizing lipopolysaccharides (LPS), a component of the outer membrane of Gram-negative bacteria. Provocation of the lung in asthma patients with LPS induces a predominantly neutrophilic type of inflammation, acute and chronic forms of airway obstruction and even airway remodeling [13]. More specific, bone marrow derived dendritic cells produce IL-12 in response to high doses of LPS, stimulating the TH1 response, *Figure 3*. This suggests that the role of other pathways, like the TH1 pathway, may play a more important role in the pathophysiology than originally acknowledged.

ADDED VALUE OF ANIMAL MODELS

The first β2 agonist for the treatment of asthma was introduced to the market in 1969 and corticosteroids were introduced since 1974. Since then, few new drugs have made it to the clinic. Multiple drugs performed well in preclinical animal models of asthma but did not live up to their promise in humans. Leukotriene antagonists (E.G. montelukast) and antibodies directed to immunoglobulin E (E.G. omalizumab) are an exception, even though both have restricted clinical indications.

There are several reasons for the limited utility of translating animal models to humans. It is suggested that asthma is a human disease only [14]. Several species and study designs have been used to try and mimic asthma. However, no animals, including those frequently used to study asthma, exhibit an asthma like syndrome that is similar to the disease in humans [15;16]. Moreover, many antigen challenge tests used in animals result in an acute inflammation phenotype and bypasses the etiology of asthma which develops over time through multiple step processes [17]. Even if longer duration models are used in animals with repeated exposure to allergens for many weeks, important clinical endpoints of human asthma do not develop such as chronic inflammation of the airway wall and airway remodeling [18;19].

THE DRUG DEVELOPMENT PROCESS FOR POTENTIAL NEW PULMONARY THERAPEUTICS

There are several possible classifications for the process of clinical drug development. Classically, drug development is considered to be a linear consecutive process in which drugs passes through four clinical phases of development [20], *Figure 4*. After each phase a Go/No Go decision is made where the drug progresses into later development, or the drug development program is discontinued. Generally, phase I trials, usually in healthy volunteers, are used to determine safety and sometimes also aim to investigate the pharmacokinetic profile of the drug (absorption, distribution, metabolization and excretion); phase II trials are used to get an initial understanding of efficacy and further explore safety in small numbers of patients; phase III trials are large, pivotal trials to determine safety and efficacy in sufficiently large numbers of patients and are often used to request market approval; and phase IV trials are post-approval trials and sometimes a requirement from the agencies for the evaluation of medicinal products.

The classification of drug development into proof of mechanism, proof of concept and proof of principle studies are related to the concept of these four phases. The underlying principle for these studies is use of biomarkers as surrogate endpoints [21]. In early development establishing the drug's effectiveness in the targeted disease population is not required, and instead surrogate endpoints are often used to guide decisions on further testing.

Proof of Mechanism (PoM). These studies often refers to early drug development in the preclinical phase (animal models), but sometimes this term is also used for testing pharmacodynamic effect of the drug in healthy volunteers. The intention is to show that the drug is available at the targeted site of action and that its interaction with the intended receptor results in a desired biological effect. It serves as an indication for the intended pharmacodynamic effect and is an important tool for

selection of appropriate dose for Proof of Concept studies. Proof of Concept (PoC) studies refers to clinical studies with a small group of patients. The aim of these studies is to show a useful clinical effect. Proof of Principle (PoP) studies are related to Phase III studies and are generally used for registration purposes. Some 15 years ago it was realized by the FDA and EMA that classification of the drug development process into four distinct phases provided an inadequate basis for classification of clinical trials as a single trial could occur in several phases. Furthermore, they concluded that the typical sequence is not applicable for each drug. Therefore they came up with the classification of studies using study objectives, summarized in *Table 1*.

Although the FDA and the EMA have abolished the use of the four-phase terminology for drug development, it is still widely applied amongst professional drug developers. The main reason for this is that this four-phase approach provides guidance for the planning of clinical development of any new drug. However, as this is likely to be a valid approach for drugs with low uncertainty regarding the development, it seems invalid for drugs with a high level of uncertainly. The uncertainty is based on the link between the molecular mechanism and its clinical effect, as well as its methodology to study it. It may be argued that a more useful model for drug development is the so called question based approach to drug development [22;23]. Question based drug developers make use of a logical progression of questions as shown in *Figure 5*. Does the drug get into the lungs? Does it reach the targeted receptors at its site of action? Will it affect the bronchodilation or its underlying inflammatory process in asthmatic patients? Does the drug have its pharmacological effect? For example, the mode of action of the new anti-asthmatic has to address the underlying endophenotype of asthma in the targeted patient population. What is the optimal dose with regards to meaningful clinical effects, and what is the therapeutic window? Other issues are whether the new anti-asthmatic drug has unwanted side effects, and how variations in the targeted asthmatic population, for instance genetic variations or lifestyle differences, affect the effectiveness of the drug. In addition, an investigation whether the applicable biomarkers and linkage markers are validated for this purpose can be deemed of value.

A schematic determination of objectives using this question based approach can be used to design a set of research questions to build the drug development plan. An integrated understanding of the fundamental principles of exposure at the site of action, target binding and expression of functional pharmacological activity determines the likelihood of the compound's potential survival in early phase trials, and improves the chance of progression to subsequent development phases. Questions that remain unanswered make up the development risk.

APPLICABLE BIOMARKERS IN ASTHMA DRUG DEVELOPMENT

A biological marker (biomarker) is a physical sign or laboratory measurement that can serve as an indicator of normal biological processes, pathophysiological processes or a pharmacological response to a therapeutic intervention. In asthma there are several validated pathophysiological and immunological biomarkers, and many others are being validated. In principle, all biological compounds of the asthma inflammatory cascade could serve as biomarkers. Ideally, a biomarker should have the following characteristics [24]:

- Clinical relevance: a clear relationship between the biomarker and the pathophysiological events leading to a well-defined clinical endpoint;
- Reliability and repeatability: the measurements of the biomarker should be precise and reproducible;
- Simplicity of sampling methodology, preferably via non- or semi-invasive sampling techniques, and measurement to promote widespread use; and,
- Sensitivity and specificity for treatment effects.
- Dose response relationship

Alongside safety data, it has become increasingly important to get as much information on the drug's potential efficacy as soon as possible in the early phases of drug development. Also, regulatory authorities have advocated the incorporation of validated biomarkers into early clinical studies to speed up drug development [25]. Therefore, in proof of pharmacology or proof of concept (POC) studies of asthma, biomarkers as read outs of pharmacological efficacy should be added. To obtain biomarkers containing tissue in asthma, bronchial biopsies are the 'gold standard'. However, this invasive technology requires trained staff and expensive equipment, and therefore it is increasingly being replaced by more patient-friendly, non- or semi-invasive sampling techniques, which enables evaluation of biomarkers for instance in blood, sputum or exhaled air.

In the last decade, several non-invasive airway sampling techniques have been validated, yielding several biomarkers in induced sputum and exhaled air to better characterize respiratory disease entities.

INDUCED SPUTUM ❋ During the standard procedure [26] sputum is induced by serial inhalations of hypertonic saline solutions. This semi-invasive method has been validated over the past 15 years and requires the combination of a patient's collaboration, investigator's expertise, a well-equipped lab and a certified cytopathologist. Obtained sputum samples are processed and can be divided into a solid phase

and a fluid phase containing soluble (bio)markers. The cell pellet is cyto-spinned, stained, and cell differentials are then counted [27]. Ample evidence exists that sputum eosinophil and neutrophil counts are reproducible biomarkers for allergic and non-allergic airway inflammation, allowing phenotyping and assessment of asthma severity [28;29]. Moreover, sputum eosinophil counts in patients with moderate to severe persistent asthma have been shown to be a superior guide of asthma control than traditional disease markers like symptom scores and lung function [30]. The fluid phase or the supernatant of the sputum can be analyzed by bioassays and many soluble markers can be quantified. This reflects on the disease severity and activity E.G. pro-inflammatory mediators, cytokines, chemokines, neuropeptides and growth factors [31]. The technique of sputum processing is currently being optimized to allow detection of a larger array of potential biomarkers in the supernatant. Novel methods including ultracentrifugation, sputum-dialysis and protease inhibition are being introduced and tested in combination with sensitive detection techniques, such as Luminex assays, proteomics and metabolomics.

EXHALED NITRIC OXIDE ❦ Exhaled nitric oxide (ENO) is a sensitive marker of acute airway inflammation and its measurement is increasingly applied to diagnose and monitor asthma [32]. Over the past decades various methods have been reported to measure ENO. The currently recommended ENO sampling method is performed by validated chemoluminescence analyzers during a single-breath exhalation against a fixed resistance, allowing reproducible (online) measurements [33]. This is a simple and patient-friendly procedure allowing serial measurements even in children. More recently, another device has been introduced for online ENO measurements: the handheld electromechanic analyzer Niox Mino® whose measurements are reproducible and in agreement with the chemoluminescence analyzers [34]. In patients with allergic asthma uncontrolled by inhaled corticosteroids (ICS), ENO has been found to correlate with sputum eosinophils, and hence may qualify as a biomarker of asthma control [35]. Indeed, ICS and other anti-eosinophil therapies, including leukotriene modifiers and anti-IgE, dose-dependently reduced ENO [36;37]. In the same way, several tapering studies have demonstrated that loss of asthma control is associated with an increase in ENO [38]. Hence, ENO is increasingly applied as a biomarker in the diagnosis, treatment monitoring and clinical trials of asthma.

EXACERBATION MODELS ❦ Exacerbation models are useful tools in clinical POC studies for asthma [39]. The crux of these models consists of a validated stimulus that is capable of inducing a reproducible, more or less specific, inflammatory response within the asthmatic airways (*Figure 2*). The endpoint measurements in such studies are the maximum early and late decrease in FEV1, and the areas under the curve for the early response (EAR; 0-2 H post challenge) and the late response (LAR, 3-8 hour post challenge).

The most commonly used exacerbation model for the investigation of potential anti-inflammatory therapy is the allergen inhalation challenge. The reproducibility in this model is high [40]. In a cross over designed study, less than 10 subjects are needed to have 95% power for a 50% reduction of percent of eosinophils [41]. Applying the allergen challenge is revealed as an overall good predictor regarding a drug's efficacy in asthma.

Methacholine has a direct effect on the airway smooth muscle via the muscarine-3 receptor. Its challenge test is the gold standard to quantify airway hyper responsiveness (AHR) by PC20. However, the test is less related to inflammation [42].

Another reproducible model inducing bronchoconstriction and airway hyper responsiveness (AHR) in healthy volunteers or asthmatic patients is the inhalation of lipopolysaccharide (LPS) [43], which causes neutrophilic inflammation [44;45]. Like allergen challenges, LPS challenges require close safety monitoring for airway and systemic effects. There are many more exacerbations models utilizing exposure to virus, ozone, adenosine 5 monophosphate, ETC. These models are not described in detail as in this thesis only the allergen and methacholine challenges were applied.

IN CONCLUSION ❦ For rapid and optimal evaluation of an investigative drug's clinical efficacy, the use of both clinical measures and biomarkers is advocated. For asthma research with potential anti-inflammatory compounds, this could indicate the combination of an exacerbation model (E.G. allergen or LPS challenge) with validated noninvasive airway sampling techniques (ENO, sputum induction). In addition, drug development programs should proceed in a much more adaptive manner, using a question based approach.

The aim of this thesis is to discuss and highlight several aspects that should be considered in the development of potential new respiratory therapeutics. In early clinical research the goal is to bridge preclinical development of potential new drugs into successful next phase studies. Therefore, early phase clinical studies are designed generate understanding of clinical characteristics of the drug, to determine its safety and to predict effectiveness in the targeted population. Information about this is often obtained by effective integration of modern technologies and tools like biomarkers into clinical development plans.

This thesis consists of two main parts; it covers biomarker development and evaluation in section 1 and the early clinical development of a new anti-asthmatic drug in section 2.

SECTION 1

BIOMARKER DEVELOPMENT AND EVALUATION

CHAPTER 2 In this chapter the development of a novel method to evaluate well-known and unknown biomarkers is described. By applying an allergen challenge in patients with clinical stable asthma, a large set of inflammatory TH2 derived markers obtained from induced sputum were evaluated. The ability to quantify changes in these cytokines in sputum after an allergen challenge could be useful to assess effects of anti-asthma therapeutics.

CHAPTER 3 Microarray assessment of gene expression in induced sputum obtained after allergen challenge was applied in the same study as described in chapter 2. Using this method, it was evaluated whether an RNA signature could be identified from induced sputum following an inhaled allergen challenge, whether a gene signature could be modulated by limited doses of inhaled fluticasone, and whether these genes would correlate with the clinical endpoints measured in this study.

CHAPTER 4 Fractional exhaled nitric oxide FeNO is a sensitive marker of acute airway inflammation. However, it has been shown that FeNO levels may be reduced after sputum induction by hypertonic saline in asthmatics and healthy controls. It is unknown if this phenomenon also occurs in asymptomatic chronic smokers, a population at risk to develop COPD. This was investigated and described in chapter 4.

CHAPTER 5 The reproducibility of the measurements of many soluble mediators in the supernatant of hypertonic saline-induced sputum and serum is still unknown. This hampers their use in clinical trials as possible read-out for treatment effect. Therefore we evaluated the reproducibility of a specific panel of soluble biomarkers in sputum and serum on healthy non-smokers and asymptomatic chronic smokers and this is described in chapter 5.

SECTION 2

CLINICAL STUDIES WITH A NEW ANTI-ASTHMATIC DRUG

CHAPTER 6 Chapter 6 describes the development process of a potential new anti-asthmatic drug, the combined PDE 3/4 inhibitor RPL554. The purpose of this first in man study was to identify a potentially effective dose and to assess in an early stage whether RPL554 possesses bronchodilative, bronchoprotective and anti-inflammatory properties in patients with allergic asthma and rhinitis. To this purpose a step by step adaptive design was used in which a safe dose of RPL554 was selected in healthy volunteers, a potentially effective dose was selected in patients with allergic asthma and finally, RPL554 effects were evaluated in more depth in both patients with allergic asthma and patients with allergic rhinitis. The use and evaluation of several technologies and biomarkers while using an adaptive study design are described in chapter 6.

CHAPTER 7 The next step in the development of RPL554 was to assess its effectiveness after repeated administrations. In this chapter the results on lung function are presented after repeated daily dosing of RPL554 for several consecutive days in order to evaluate the sustainability of effect on FEV1.

CHAPTER 8 Chapter 8 covers the discussion and conclusion section. It includes a critical evaluation of the biomarker selection and drug development program for RPL554.

Table 1 Classification of study objectives according to the EMA clinical guide guidance

Type of Study	Objective of Study	Study Examples
Human Pharmacology	Assess tolerance Define/describe PK and PD Explore drug metabolism and Drug interactions Estimate activity	Dose-tolerance studies Single and multiple dose PK and/or PD studies Drug interaction studies
Therapeutic Exploratory	Explore use for the targeted indication Estimate dosage for subsequent studies Provide basis for confirmatory study design, endpoints, methodologies	Earliest trials of relatively short duration in well- defined narrow patient populations, using surrogate or pharmacological endpoints or clinical measures. Dose-response exploration studies
Therapeutic Confirmatory	Demonstrate/confirm efficacy Establish safety profile Provide an adequate basis for assessing the benefit/risk relationship to support licensing Establish dose-response relationship	Adequate, and well controlled studies to establish efficacy Randomized parallel dose response studies Clinical safety studies Studies of mortality/ morbidity outcomes Large simple trials Comparative studies
Therapeutic Use	Refine understanding of benefit/ risk relationship in general or special populations and/or environments Identify less common adverse reactions Refine dosing recommendation	Comparative effectiveness studies Studies of mortality/morbidity outcomes Studies of additional endpoints Large simple trials Pharmaco-economic studies

Figure 1 Schematic relationship among asthma characteristics

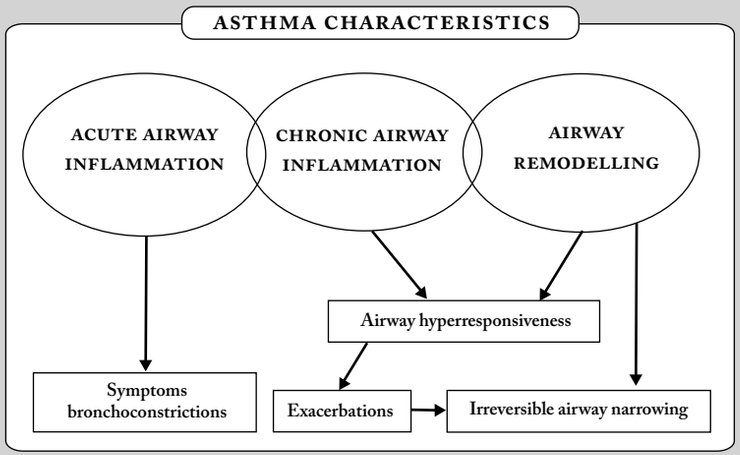


Figure 2 Early and late airway response after inhalation of grass pollen (this thesis)

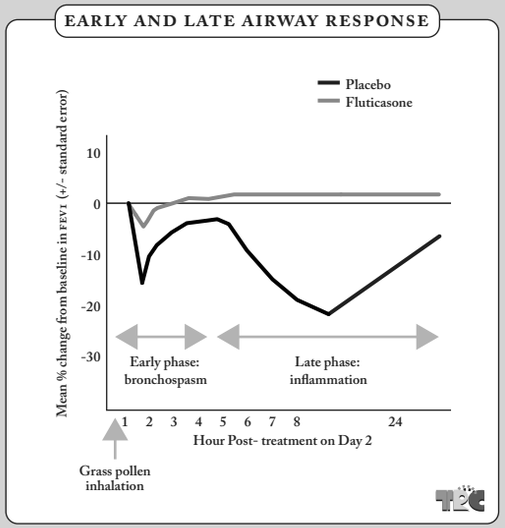


Figure 3 Pathogenesis of asthma

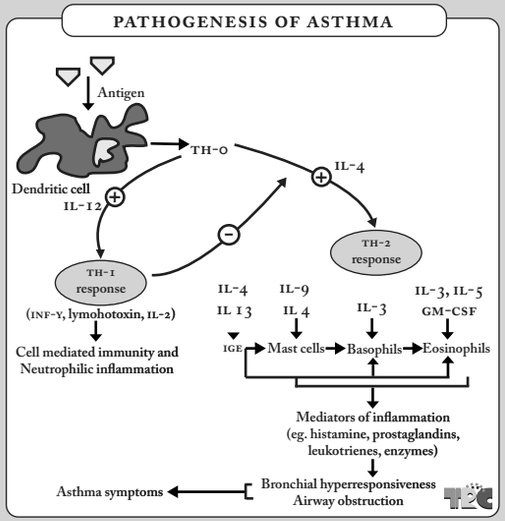


Figure 4 The classical drug development plan

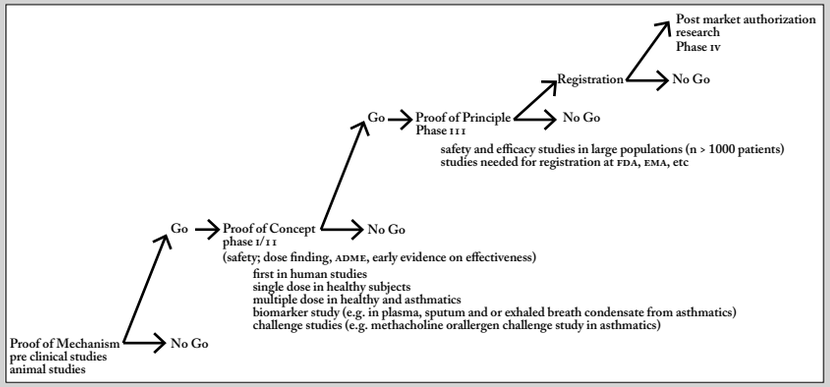
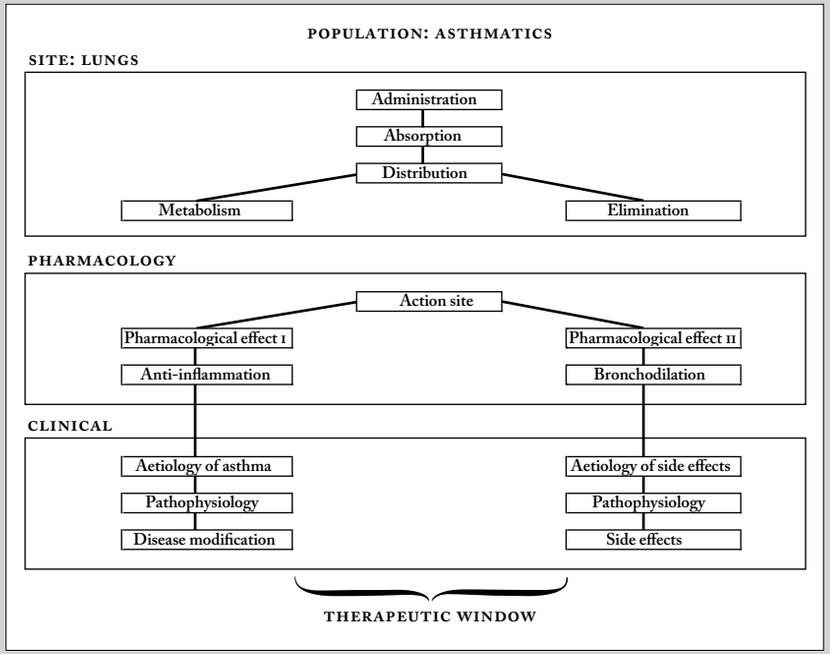


Figure 5 Schematic determination of objectives using the question based approach to drug development (Figure is reproduced and adapted with permission from reference [22])



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SECTION I

BIOMARKER DEVELOPMENT AND EVALUATION

CHAPTER 2

KINETICS OF TH2 BIOMARKERS IN SPUTUM OF ASTHMATICS FOLLOWING INHALED ALLERGEN

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ABSTRACT

BACKGROUND Allergen-induced late airway response offers important pharmacodynamic targets, including T helper 2 (TH2) biomarkers. However, detection of inflammatory markers has been limited in dithiothreitol-processed sputum.

OBJECTIVES To test whether allergen-induced TH2 inflammatory markers can be reproducibly quantified by sensitive detection techniques in ultracentrifuged sputum and the effect of fluticasone on these endpoints.

METHODS Thirteen allergic asthmatics with dual allergen-induced airway responses documented during a single-blind placebo run-in period, participated in a double-blind, 2-period cross-over study. Each period consisted of 3 consecutive days, separated by ≥3 weeks. Following randomization, subjects inhaled fluticasone (500 µG BID, 5 doses total) or placebo. On day 2 in each study period, allergen challenge was performed and airway response measured by FEV1 until 7 H post-challenge. Sputum was induced 24 H pre- and 7 & 24 H post-allergen. Sputum samples were split into 2 portions: TH2 biomarkers were quantified by Mesoscale multiplex platform following ultracentrifugation and cell differentials were counted on Giemsa-May-Grünwald-stained cytospins. Allergen-induced changes in inflammatory endpoints were compared between fluticasone and placebo using a mixed model ANCOVA.

RESULTS Inhaled allergen-induced dual airway responses in all subjects during both placebo periods with reproducible late asthmatic response (LAR) and increases in sputum inflammatory biomarkers (IL-2, IL-4, IL-13 and eotaxin-1) and eosinophil counts. Fluticasone effectively blunted both the LAR and the inflammatory biomarkers.

CONCLUSIONS Combining novel, sensitive quantification methods with ultracentrifugation allows reproducible quantification of sputum biomarkers following allergen challenge, reversed by fluticasone. This approach allows non-invasive identification of pharmacodynamic targets for anti-asthma therapies. This study is registered under EUDRACT number 2007-003671-40.

INTRODUCTION

Inhaled allergen challenge is a highly reproducible, integral disease model enabling the investigation of several features of asthma [1]. Allergen challenge can be applied to study the pathophysiology and, if complemented with (non-) invasive airway samplings, the immune-biology to allergic stimuli within the airways. In drug development, allergen challenge is an established tool predicting clinical efficacy of novel anti-allergic and anti-asthma treatments [2].

Non-invasive airway sampling by hypertonic saline-induced sputum [3] has been shown to yield reproducible increases in inflammatory cells and biomarkers following allergen-induced late asthmatic response (LAR) [4] with subsequent response to novel and existing anti-inflammatory therapies [2;4-6]. While animal studies provided evidence of TH2 cytokine response following allergen challenge, supported by some human studies applying bronchoscopy [7;8], no consistent data exist on reproducible quantification of TH2 cytokines and chemokines in sputum. Accountable factors include degradation by standard sputum processing with dithiothreitol (DTT), which destroys the disulphide bounds of these inflammatory markers [9], overall low baseline concentrations and relatively insensitive detection techniques. Some of these hurdles could be overcome by physical homogenization of sputum samples by ultracentrifugation causing cellular disruption with subsequent release of intracellular products in combination with sensitive detection techniques [10;11].

Combining sputum ultracentrifugation with novel, sensitive quantification techniques using Mesoscale multi-array microplates [12] in the allergen challenge model, we aimed to study: 1) the feasibility of the quantification of TH2 cytokines and chemokines in sputum at 7 and 24 H post-challenge, 2) their reproducibility and 3) their reversibility after a short course of inhaled fluticasone (FP). Furthermore, to allow comparison with other established markers of allergen-induced airway inflammation, we also measured the allergen-induced airway responses (*I.E.*, the early (EAR) and late (LAR) asthmatic response), exhaled nitric oxide (ENO), sputum cell differentials and the provocative concentration of methacholine causing a fall in forced expiratory volume in 1 second (FEV1) of 20% (PC20FEV1Methacholine) at baseline and 24 H post allergen, during all study periods.

METHODS

STUDY POPULATION AND DESIGN ❋ Thirteen non-smoking subjects with clinically stable, mild to moderate allergic asthma [13] using prn short-acting

beta2-agonists only and with dual airway responses to inhaled house dust mite (HDM), documented during the single-blind placebo run-in screening period, participated in a double-blind, 2-way cross-over study. Each period consisted of 3 consecutive days, with ≥3 weeks washout between periods [*Figure 1*]. The screening, allowing to test the reproducibility of the variables, was identical to the subsequent treatment periods during which subjects randomly received inhaled FP (MDI, 500 µg BID, total of 5 doses) or matching placebo. On day 1, baseline measurements including ENO, spirometry, followed by methacholine challenge (PC20FEV1Methacholine) and subsequent sputum induction (3 x 5 MIN NaCL 4.5%) were performed prior to study medication. On day 2, 1 H post-study medication, subjects underwent a titrated allergen challenge [1]. The subsequent airway response was repeatedly measured by FEV1 until 7 H post-allergen. ENO was measured pre- and 3 H and 7 H post-allergen; the latter followed by sputum induction. At 24 H post-allergen (day 3), test-procedures were repeated as on day 1 [*Figure 2*]. All test-procedures were conducted according to standardized, validated methods and at the same time of the day (within 2 hours) [1;14-16].

A dual airway response to inhaled HDM extract consisted of an early (EAR) and a late asthmatic response (LAR) defined as a fall in FEV1 > 15% from baseline occurring between 0-3 H and 3-7 H post-allergen, respectively.

The study was approved by the Ethics Committee of Leiden University Medical Center, Leiden, The Netherlands, and all participants gave a signed informed consent (EUDRACT number 2007-003671-40). All procedures were performed in accordance with the Helsinki Declaration of 1975, revised in 2008.

STUDY MEDICATION AND DOSING RATIONALE Fluticasone 250 µG/puff (Allen & Hanburys, Glaxo Wellcome Ltd, Middlesex, UK) and matching placebo (Armstrong Pharmaceuticals Inc., Canton, MA, USA, packaged at Merck Frosst, Kirkland, Canada) were supplied in identical metered dose inhalers (MDIS) and inhaled per single puff through an Aerochamber (Volumatic, GlaxoSmithKline, Zeist, The Netherlands). The rationale for the dose regimen was based on a previous study showing substantial reductions in allergen-induced LAR, non-specific airway hyperresponsiveness (AHR) and sputum eosinophils already following one single dose of inhaled FP 250 µG [6]. Thus, to ensure optimal reversal of the allergen-induced inflammatory markers versus placebo, a total of 5 FP doses (500 µG per dose) were administered throughout the active treatment period.

ALLERGEN CHALLENGE The allergen challenge was performed using the 2 minutes tidal breathing method that has been previously validated [1]. The run-in period served as a dose (range) finding procedure, while during study periods 1 & 2 each subject inhaled the same 2 or 3 cumulative doses of the allergen extract that had caused a fall in FEV1 of at least 15% from baseline during the run-in period. Following diluent, incremental doubling concentrations (7.81 to 2,000 BU/mL) of HDM extract (Dermatophagoides pteronyssinus; SQ 503, ALK-BPT, ALK-Abelló, Almere, The Netherlands) in phosphate-buffered saline (PBS) were aerosolized by a calibrated jet-nebulizer (DeVilbiss 646, output 0.13 mL/MIN, Somerset, Pennsylvania, USA) and inhaled at approximately 12 MIN intervals, until the EAR was reached (defined as a decrease in FEV1 of > 15% from post-diluent baseline within 1 H post-allergen). Airway response to inhaled allergen was measured by FEV1 in duplicate on a calibrated spirometer (Vmax Spectra; Sensor Medics, Bilthoven, The Netherlands) according to standard procedures [17], at 10, 20, 30, 45, 60, 90 and 120 minutes and then hourly until 7 H after the last allergen inhalation. The highest, technically valid measurement was expressed as percentage decrease from post-diluent baseline FEV1 and included into the analysis.

METHACHOLINE CHALLENGE The methacholine challenge was performed using standard methodologies [15]. Serial doubling concentrations of methacholine bromide (MBR, Janssen Pharmaceutical, Beerse, Belgium) diluted in normal saline (NaCL 0.9%) to serial doubling dilutions of 0.15-80 µMOL/mL, were aerosolized by a calibrated jet-nebulizer (DeVilbiss 646) at 5 minutes intervals and inhaled by the subjects by tidal breathing for 2 minutes through the mouthpiece with the nose clipped. Airway response was measured by FEV1 at 30 and 90 seconds (and potentially at 180 seconds as well) following each concentration, and the lowest, technically satisfactory FEV1 was implicated into analysis. Nebulization was continued until a > 20% fall in FEV1 from post-diluent baseline.

After both bronchoprovocation tests, subjects received salbutamol through an aerochamber, until the FEV1 returned within 10% of the baseline value.

EXHALED NITRIC OXIDE (ENO) All ENO measurements were performed according to current guidelines [14] using a chemiluminescence analyzer (Ecomedics CLD88sp; Ecomedics, Duernten, Switzerland), which had to be replaced by a NIOX MINO®(Aerocrine AB, Solna, Sweden) during the study. NIOX MINO was used for subjects 8, 9, 10, 11, 12 and 13 during both study periods. In a previous study at our institute, both analyzers yielded similar values [18].

SPUTUM INDUCTION, PROCESSING AND ANALYSIS Sputum induction was performed as previously described [16;19] using a DeVilbiss Ultraneb 2000

ultrasonic nebulizer (Tefa Portanje, Woerden, The Netherlands) connected to a 100-cm long plastic tube, with an internal diameter of approximately 22 mm, connected to a two-way valve (No.2700; Hans-Rudolf, Kansas City, MO, USA) with a mouthpiece. Hypertonic saline (NaCl 4.5%) was nebulised and inhaled through the mouth, with the nose clipped, during three periods of 5 minutes. At approximately 7 minutes following each induction, spirometry was performed as a safety measure.

Collected sputum samples were divided into two portions of equal weight. The cell pellet of the first portion was processed as a full sample according to guidelines [16;20], using 0.1% DTT (Sputolysin, Calbiochem, La Jolla, CA, USA). Cell viability and total cell count were assessed using Trypan Blue; sputum samples containing > 80 % squamous cells were excluded from analysis. Differential cell counts were performed by a qualified cytologist on May-Grünwald-Giemsa stained, coded cytospins and expressed as percentage of 500 nucleated, non-squamous cells.

The second sputum portion was used to quantify soluble inflammatory markers. At Merck Research Laboratories, defrosted samples were pretreated with a protease-inhibitor cocktail (50 µL per 200 mg sputum), prepared by dissolving one protease cocktail tablet (Complete Protease Inhibitor Cocktail tablets, Roche Applied Science #11 697 498 001) into 50 mL of PBS (Invitrogen cat. no. 14040). Prepared sputum samples were subsequently ultracentrifuged in an ultracentrifuge (Beckman Coulter Inc. Optima Max Ultracentrifuge 130,000 RPM; Fullerton, CA, USA) at 35,000 RPM (53,500 x g) for 90 minutes at 4˚C. Subsequently, sputum supernatant was collected and analyzed.

CYTOKINE AND CHEMOKINE MEASUREMENTS ❦ Quantification of soluble biomarkers in sputum samples was performed using an MSD (Mesoscale Discovery, Gaithersburg MD, USA) Singleplex kit (IL-13), an MSD duplex kit (eotaxin-3 and TARC) and two MSD multiplex assays (IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IFN-γ, TNF-α, eotaxin, IP-10, MCP-1, MCP-4, and MIP-1β). All concentrations were expressed as PG/ML.

STATISTICAL ANALYSIS ❦ Data of all randomized subjects were included into the analysis. The effect of FP versus placebo on the TH2 cytokines, chemokines and other inflammatory markers at 7 H and 24 H post-allergen was assessed using a mixed effects analysis of variance (ANOVA) model. The model included fixed factors for sequence, treatment, and period, and a random effect for subjects within sequence. Between treatment differences were estimated by the difference in least-square means from the model with 90% CI (one-sided alpha = 5%). Sputum cell differentials were analyzed using the actual change from baseline, while absolute cell counts were analyzed using the change from baseline for the square root transformed values. Geometric mean baseline sputum biomarker concentrations were calculated; half of the lower limit of quantification (LLOQ) was used in case of negative outcomes. Changes in sputum biomarker concentrations were analyzed after log-transformation and expressed as fold change from baseline.

The airway response to inhaled allergen was expressed as percentage decrease in FEV1 from post-diluent baseline and plotted as time–response curves during all treatment periods. The difference in FEV1 during both the EAR and the LAR was analyzed using the time weighted average of percentage change and the maximum percentage charge from baseline. Subject 1 had an initial FEV1 decrease of slightly under 15% at 7 H post-allergen, but met the inclusion criterion at 8 H post-allergen and was included in the study. Therefore, for this subject FEV1, cytokines, chemokines and ENO were consequently measured at 8 H during all periods. FEV1 results at 8 H were not included into the analysis.

PC20FEV1 Methacholine was calculated by linear interpolation on a plot of log-concentrations versus response using methacholine concentrations below and above a 20% fall in FEV1. The (allergen-induced and FP-reverted) changes in PC20FEV1Methacholine were expressed in doubling doses. ENO was expressed as a fold change from baseline at 3, 7 and 24 H post-allergen.

Reproducibility of the allergen-induced airway responses and sputum inflammatory markers was assessed using data from the run-in and study placebo periods. The intra-class correlation coefficient (ICC) was calculated, and a 2-sided paired t-test was performed.

SAMPLE SIZE ❦ In the absence of information about variability in TNF- α and IL-13 concentrations in sputum, eosinophil count was used as an approximate variable for sample size estimation [21]. Power calculation showed that the study would have > 90% power (α = 0.05, one tailed) to detect a five-fold increase from baseline at 7 H post-allergen challenge with 12 completing subjects.

RESULTS

SUBJECTS ❦ Fifteen subjects were considered eligible after completion of the run-in period. Before randomization, two subjects were withdrawn: one had a positive cotinine test, while the other repeatedly presented with a clinically relevant bronchoconstriction (baseline FEV1 <70% predicted). Thus, 13 subjects were randomized and all completed the study [*Table 1*].

SAFETY No serious adverse events occurred. Headache and fatigue were the most frequently reported adverse events. All events were mild in intensity and classified as unrelated to the study medication or procedures.

ALLERGEN-INDUCED AIRWAY RESPONSES Inhaled HDM induced both an EAR and an LAR in all subjects during both placebo periods. Compared to placebo, FP significantly reduced the EAR and completely blunted the LAR [*Figure 3*]. The reproducibility of the allergen-induced LAR during both placebo periods was good, both in terms of the maximum %fall in FEV1 from baseline and as time weighted average (3-7 H post-allergen), with an ICC of 79.7% and 69%, respectively [*Table 2*].

ALLERGEN-INDUCED NON-SPECIFIC AIRWAY HYPERRESPONSIVENESS (AHR) During both placebo periods, allergen challenge increased non-specific AHR, by decreasing PC20FEV1Methacholine at 24 H post allergen by on average 1.18 (90%CI: 1.73; 0.64) doubling doses. In contrast, FP increased 24 H post-allergen PC20FEV1Methacholine by on mean 1.60 doubling doses (90%CI: 1.06; 2.15), resulting in a mean difference of 2.79 doubling doses (90%CI: 2.07; 3.51; $p < 0.001$) between placebo and FP [*Figure 4*].

SPUTUM INFLAMMATORY CELLS A sputum sample was obtained from all subjects at all occasions. The average squamous cell contamination was 36% (range: 2-71%). Sixteen of 117 samples were not analyzable. Inhaled allergen significantly increased sputum eosinophils both at 7 and 24 H post-challenge during both placebo periods. This effect was significantly reduced by FP [*Table 3*]. The reproducibility for both sputum eosinophil count (ICC: 76%) and percentage (ICC: 88%) was high at 7 H post-allergen, but poor (ICC 0 %) at 24 H.

SPUTUM (TH2) CYTOKINES AND CHEMOKINES During placebo treatment, inhaled allergen increased sputum inflammatory cytokines and chemokines both at 7 and 24 H post-allergen, yielding the most robust increase at 7 H, *Table 4*. Fluticasone significantly blunted the allergen-induced increases in sputum concentrations of IL-5, IL-13, TARC, eotaxin-3, MCP-1, eotaxin-1 and IL-4 at 7 H post-allergen challenge and of IL-5, IL-13, eotaxin-3, IL-12p70 and MCP-1 at 24 H post-allergen challenge. None of the other sputum soluble markers were significantly affected by FP compared to placebo treatment. At 24 H post-allergen, there was no difference in any sputum inflammatory markers, with the exception of TARC between both placebo treatments.

At 7 H post-allergen many soluble markers were reproducible, especially IL-2, IL-4, IL-13, and eotaxin-1 showed an interclass correlation coefficient (ICC) values greater than 50%, with more variation between subjects than within subjects. At 24 H, none of the inflammatory markers had ICC values greater than 50%. Cytokine baseline values on day 1 for each treatment period are provided in *Table 5*.

CHANGE IN ENO Compared to baseline, ENO levels did not significantly increase at 3 and 7 H post-allergen and were not different between placebo and FP. At 24 H post allergen, however, a significant increase in ENO was measured (1.63 fold and 90%CI: 1.2; 2.3) which was blunted by FP (0.83 fold, 90%CI: 0.6; 1.2), resulting in a significant difference between placebo and FP of 49% ($p=0.012$, 90%CI: 19;68).

DISCUSSION

In this study, we have been able to reproducibly quantify several TH2 inflammatory cytokines and chemokines in sputum from allergic asthmatic subjects following inhaled allergen. The increase in these soluble sputum biomarkers was consistent with other established allergen-induced inflammatory responses and most robust at 7 H post-allergen, coinciding with the maximal fall in FEV1 during the LAR. Fluticasone significantly blocked both the allergen-induced airway response and the majority of the inflammatory markers in sputum. Although other researchers previously showed a similar inflammatory response in bronchoalveolar lavage [7] and in sputum [22-24], none of them has investigated such wide range of allergen-induced TH2-cytokines and chemokines or their reversibility to corticosteroid treatment.

The use of sulfhydryl-reducing reagents, such as DTT, has complicated the detection of inflammatory cytokines and chemokines and alternative processing techniques enabling the measurement of E.G. eotaxin have previously been published [9]. In our study sputum samples were ultracentrifuged [10] instead of being processed with DTT to avoid potentially degrading effects on several TH2 cytokines and chemokines [9]. Following this 'boosting' step, substantial allergen-induced increases in several cytokines and chemokines could be reproducibly quantified using sensitive detection techniques (Mesoscale multi-array microplates). However, reproducibility was lost for most soluble markers and sputum eosinophils at 24 H post-allergen.

In parallel with reproducible increases in the TH2-derived inflammatory markers, we were able to demonstrate reproducible changes in the established allergen-induced

inflammatory outcome [4;25;26], including the late asthmatic airway response, non-specific airway hyperresponsiveness and sputum eosinophils, underscoring the validity of our data. In agreement with previous evidence, we also found increased eNO levels at 24 h post-allergen [27] , while no significant eNO increases could be observed at our cut off point during the LAR, *i.e.*, at 7 h post-allergen. Although previous studies showed increased eNO levels at 9 and 10 h post-allergen, respectively [27] [28], the present findings can be explained by the use of two different measuring devices (for logistic reasons) and the time-lag required for the synthesis of inducible NO synthase (i-NOS), responsible for the synthesis of NO [29].

Although in the present study no direct comparison was made with soluble markers from the DTT-processed sputum portion, the current approach yielded reproducible data. In addition, the observation that FP can reverse the allergen-induced increase in these inflammatory markers in parallel with its inhibitory effects on the other inflammatory events including the airway responses and cellular markers, suggests that this approach is sensitive enough to offer evaluation of therapeutic interventions in asthmatic subjects.

In conclusion, combining novel, sensitive quantification methods with ultracentrifugation allows reproducible quantification of sputum biomarkers following an allergen-induced LAR, which can be reversible by fluticasone. This approach allows non-invasive identification of pharmacodynamic targets for anti-asthma therapies.

Table 1 Baseline characteristics of randomized subjects

Number of subjects	13
Age (years)	25.9 (21-43)
Gender	4M/9F
BMI, (KG/M2)	24.4 (16.6-39.8)
FEV1 (L)	3.57 (2.92-4.50)
FEV1 (% pred)	94.0 (74.5-112.3)
PC20FEV1 Methacholine (µMOL/ML)	12.8 (0.8-81.5)
SPT HDM Wheal (MM)	5.5 (2.5-10.5)
eNO (PPB)	53.4 (11.2-160.8)

Numbers are expressed in mean (range), BMI = Body Mass Index,
SPT HDM = Skin Prick Test for House Dust Mite, ppb = parts per billion

Table 2 Analysis of the airway response to inhaled allergen

FEV1 Endpoint	Treatment	% Estimate* (90% CI)	Diff. in % Change Fluticasone-Placebo (90% CI)	1-sided p-value**	Reproducibility		
					CHANGE† (90% CI)	2-sided P-VALUE ††	ICC (%)
EAR Time – weighted mean	Placebo	-7.09 (-8.90, -5.28)	5.28 (3.28, 7.28)	<0.001	4.0 (0.8, 7.3)	0.020	0.0
	Fluticasone (500 MCG BID)	-1.81 (-3.62, 0.00)					
EAR Max. Decrease	Placebo	-17.7 (-21.3, -14.1)	11.12 (8.26, 13.99)	<0.001	6.3 (1.0, 11.7)	0.025	21.3
	Fluticasone (500 MCG BID)	-6.56 (-10.1, -2.99)					
LAR Time – weighted mean	Placebo	-13.8 (-17.0, -10.6)	15.08 (10.97, 19.18)	<0.001	2.6 (-0.8, 6.0)	0.121	69.0
	Fluticasone (500 MCG BID)	1.28 (-1.89, 4.44)					
LAR Max. Decrease	Placebo	-25.9 (-31.1, -20.7)	24.00 (16.70, 31.31)	<0.001	0.0 (-4.7, 4.6)	0.982	79.7
	Fluticasone (500 MCG BID)	-1.89 (-7.06, 3.28)					
24 Hours	Placebo	-5.30 (-8.16, -2.44)	7.05 (4.45, 9.64)	<0.001	3.7 (0.4, 6.9)	0.030	59.4
	Fluticasone (500 MCG BID)	1.75 (-1.11, 4.61)					

EAR = Early Asthmatic Response, LAR = Late Asthmatic Response, CI = confidence interval, ICC = Intraclass correlation coefficient, * % change form period baseline, **p-value: fluticasone vs placebo, one-sided alpha = 5%, † Placebo period vs. (Placebo) run-in period, †† Paired t-test

Table 3 Analysis of the eosinophils and neutrophils to inhaled allergen.

Sputum differential	Time (hours)	Treatment	% Estimate (90%CI)*	% Change from Baseline Fluticason vs Placebo (90%CI)	p-value*	Reproducibility		
						CHANGE† (90% CI)	2-sided P-VALUE††	ICC (%)
Eosinophils (Count)	7	Placebo	250.8 (165.5; 336.1)	-277 (-394; -160)	0.002	-37.4 (-168, 93.5)	0.528	88.1
		Fluticasone	-25.9 (-106; 54.5)					
	24	Placebo	217.9 (142.7; 293.0)	-271 (-377; -165)	0.001	-118 (-244, 8.17)	0.063	0.0
		Fluticasone	-52.9 (-128; 22.3)					
Eosinophils (Percent)	7	Placebo	11.5 (5.7; 17.4)	-11.8 (-19.9; -3.8)	0.014			
		Fluticasone	-0.3 (-5.8; 5.2)					
	24	Placebo	6.8 (1.7; 11.8)	-7.8 (-12.3, -3.3)	0.007			
		Fluticasone	-1.1 (-6.1; 3.9)					
Neutrophils (Count)	7	Placebo	173.6 (62.5, 284.6)	-101 (-253, 52.0)	0.124	58.8 (-206, 324)	0.623	15.1
		Fluticasone	73.0 (-31.7, 177.7)					
	24	Placebo	224.6 (33.1, 416.2)	-190 (-450, 70.6)	0.103	219 (-48.2, 487)	0.095	28.0
		Fluticasone	35.0 (-157, 226.5)					
Neutrophils (Percent)	7	Placebo	-5.5 (-18.8, 7.9)	6.3 (-9.3, 21.8)	0.768			
		Fluticasone	0.8 (-11.9, 13.5)					
	24	Placebo	-5.3 (-20.9, 10.3)	-4.6 (-24.7, 15.5)	0.335			
		Fluticasone	-9.9 (-25.5, 5.6)					

CI = confidence interval, ICC = Intra class correlation coefficient, *Sputum eosinophils and neutrophils expressed as %change from baseline at 7 and 24 h post allergen challenge., **p-value: fluticasone vs placebo, one-sided alpha = 5%, † Placebo period vs. run-in period, †† Paired t-test

Table 4 Analysis of sputum cytokines and chemokines to inhaled allergen

c	Hours post allergen challenge	Treatment	Estimate* (90%CI)	% ChangeFluticasone vs Placebo(90% CI) P-VALUE **	Reproducibility. CHANGE† (90% CI)	2-sided P-VALUE††	ICC (%)
IL-5	7	PLACEBO	5.67 (3.63; 8.86)	82.6 (71.4; 89.4)	1.27 (0.53; 3.06)	0.555	48.1
		FLUTICASONE	0.99 (0.63; 1.55)	P <0.001			
	24	PLACEBO	2.57 (1.54; 4.30)	66.1 (29.9; 83.6)	0.69 (0.24, 2)	0.465	34.7
		FLUTICASONE	0.87 (0.52; 1.46)	P = 0.011			
IL-13	7	PLACEBO	9.76 (6.25; 15.25)	88.6 (81.2; 93.1)	1.13 (0.63; 2.02)	0.655	73.6
		FLUTICASONE	1.11 (0.71; 1.73)	P <0.001			
	24	PLACEBO	2.11 (1.52; 2.94)	59.7 (37.0; 74.2)	0.66 (0.36, 1.19)	0.148	41.2
		FLUTICASONE	0.85 (0.61; 1.18)	P = 0.002			
TARC	7	PLACEBO	2.13 (1.68; 2.72)	41.9 (22.8; 56.3)	0.9 (0.49; 1.66)	0.709	43.3
		FLUTICASONE	1.24 (0.97; 1.58)	P = 0.003			
	24	PLACEBO	1.75 (1.32; 2.32)	21.4 (-9.4; 43.6)	0.49 (0.29, 0.82)	0.012	17.9
		FLUTICASONE	1.37 (1.04; 1.82)	P = 0.108			
Eotaxin-3	7	PLACEBO	2.24 (1.71; 2.94)	44.6 (18.4; 62.4)	1.25 (0.55; 2.81)	0.556	0.0
		FLUTICASONE	1.24 (0.94; 1.64)	P = 0.010			
	24	PLACEBO	2.56 (1.80; 3.63)	39.2 (12.0; 58.0)	0.73 (0.29, 1.81)	0.458	22.2
		FLUTICASONE	1.55 (1.09; 2.21)	P = 0.018			
MCP-1	7	PLACEBO	1.27 (1.03; 1.56)	28.2 (9.1; 43.3)	1.14 (0.91; 1.44)	0.230	32.2
		FLUTICASONE	0.91 (0.74; 1.12)	P = 0.015			
	24	PLACEBO	0.96 (0.81; 1.15)	22.8 (4.1; 37.8)	1.07 (0.82, 1.4)	0.571	8.0
		FLUTICASONE	0.74 (0.62; 0.89)	P = 0.028			
Eotaxin-1	7	PLACEBO	2.01 (1.43; 2.82)	47.5 (15.2; 67.6)	0.68 (0.36; 1.3)	0.219	52.0
		FLUTICASONE	1.05 (0.75; 1.48)	P = 0.018			
	24	PLACEBO	1.25 (0.89, 1.75)	-0.7 (-61.9, 37.3)	0.65 (0.26, 1.63)	0.329	0.0
		FLUTICASONE	1.26 (0.90, 1.76)	P = 0.511			
IL-4	7	PLACEBO	1.52 1.21; 1.92)	34.0 (8.9; 52.2)	1.02 (0.74; 1.41)	0.899	72.3
		FLUTICASONE	1.01 (0.80; 1.27)	P = 0.021			
	24	PLACEBO	0.98 (0.92, 1.03)	-4.7 (-13.6, 3.5)	0.9 (0.72, 1.12)	0.312	0.0
		FLUTICASONE	1.02 (0.96, 1.08)	P = 0.836			
MCP-4	7	PLACEBO	1.34 (0.96, 1.87)	32.4 (-3.3, 55.7)	1.05 (0.6; 1.85)	0.839	0.0
		FLUTICASONE	0.91 (0.65, 1.27)	P = 0.063			
	24	PLACEBO	1.36 (0.98, 1.89)	14.6 (-36.2, 46.5)	0.87 (0.44, 1.7)	0.646	1.6
		FLUTICASONE	1.16 (0.83, 1.61)	P = 0.277			

IL-12	7	PLACEBO	1.47 (0.97, 2.24)	29.0 (-24.9, 59.6)	1.56 (0.91; 2.68)	0.095	38.5
		FLUTICASONE	1.05 (0.69, 1.59)	P = 0.149			
	24	PLACEBO	1.61 (1.07; 2.42)	50.6 (12.2; 72.2)	0.85 (0.46, 1.54)	0.555	26.5
		FLUTICASONE	0.79 (0.53; 1.19)	P = 0.025			
IP-10	7	PLACEBO	1.11 (0.85, 1.46)	19.2 (-16.6, 44.0)	1.12 (0.64; 1.95)	0.671	0.0
		FLUTICASONE	0.90 (0.69, 1.18)	P = 0.159			
	24	PLACEBO	1.04 (0.71, 1.53)	15.4 (-42.1, 49.7)	0.94 (0.52, 1.69)	0.809	0.0
		FLUTICASONE	0.88 (0.60, 1.30)	P = 0.286			
MIP-1β	7	PLACEBO	1.70 (1.16, 2.48)	17.8 (-40.5, 51.9)	1.52 (0.66; 3.52)	0.296	14.6
		FLUTICASONE	1.40 (0.96, 2.04)	P = 0.261			
	24	PLACEBO	1.70 (1.08, 2.67)	-11.9 (-97.1, 36.5)	0.8 (0.25, 2.55)	0.687	2.7
		FLUTICASONE	1.90 (1.21, 2.99)	P = 0.637			
IL-8	7	PLACEBO	1.01 (0.80, 1.28)	4.0 (-31.6, 30.0)	0.93 (0.64; 1.36)	0.697	39.3
		FLUTICASONE	0.97 (0.77, 1.23)	P = 0.409			
	24	PLACEBO	1.26 (0.95, 1.66)	8.4 (-35.4, 38.0)	0.88 (0.53, 1.46)	0.583	29.9
		FLUTICASONE	1.15 (0.87, 1.52)	P = 0.347			
IL-10	7	PLACEBO	1.18 (0.94, 1.48)	-1.8 (-40.0, 26.0)	1.17 (0.85; 1.63)	0.300	30.5
		FLUTICASONE	1.20 (0.96, 1.50)	P = 0.539			
	24	PLACEBO	1.24 (0.95, 1.61)	9.7 (-17.2, 30.3)	0.97 (0.61, 1.52)	0.867	11.0
		FLUTICASONE	1.12 (0.86, 1.45)	P = 0.247			
IL-1β	7	PLACEBO	1.02 (0.77, 1.34)	-10.6 (-60.0, 23.6)	1.02 (0.64; 1.62)	0.933	42.6
		FLUTICASONE	1.12 (0.85, 1.48)	P = 0.684			
	24	PLACEBO	1.17 (0.80, 1.72)	3.2 (-45.1, 35.5)	1.16 (0.6, 2.28)	0.628	8.9
		FLUTICASONE	1.13 (0.77, 1.67)	P = 0.443			
IL-2	7	PLACEBO	0.90 (0.70, 1.16)	-13.9 (-59.2, 18.5)	0.81 (0.64; 1.02)	0.069	72.4
		FLUTICASONE	1.03 (0.80, 1.32)	P = 0.752			
	24	PLACEBO	1.01 (0.72, 1.42)	-19.6 (-79.6, 20.3)	0.82 (0.42, 1.6)	0.530	13.4
		FLUTICASONE	1.21 (0.86, 1.70)	P = 0.778			
IFN-γ	7	PLACEBO	1.05 (0.78, 1.43)	-41.1 (-101, 1.0)	1.19 (0.64; 2.23)	0.552	0.0
		FLUTICASONE	1.49 (1.10, 2.02)	P = 0.946			
	24	PLACEBO	1.03 (0.77, 1.38)	-10.8 (-66.7, 26.4)	0.84 (0.4, 1.75)	0.611	0.0
		FLUTICASONE	1.14 (0.86, 1.53)	P = 0.670			
TNF-α	7	PLACEBO	1.14 (0.88, 1.50)	-43.6 (-84.1, -12.0)	1.18 (0.73; 1.92)	0.460	0.0
		FLUTICASONE	1.64 (1.26, 2.15	P = 0.988			
	24	PLACEBO	1.21 (0.93, 1.56)	-4.3 (-43.0, 23.9)	0.86 (0.43, 1.71)	0.634	0.0
		FLUTICASONE	1.26 (0.97, 1.63)	P = 0.593			

CI = Confidence Interval, ICC = Intra class correlation coefficient ; * Sputum cytokines and chemokines in fold change from baseline at 7 and 24 h post-allergen challenge after placebo and fluticasone treatment.; **p-value: fluticasone vs placebo, one-sided alpha = 5%; † Placebo period vs. Run-in period. ; †† Paired t-test

Table 5 Mean* baseline values of cytokines and chemokines during placebo and fluticasone treatment

Cytokine/ chemokine	IL-5	IL-13	TARC	Eotaxin-3	MCP-1	Eotaxin-1	IL-4	MCP-4	IL-12	IP-10	MIP-1β	IL-8	IL-10	IL-1β	IL-2	IFN-γ	TNF-α
Placebo Day 1 (-3 H)**	2.43	1.40	3.69	9.15	21.33	8.55	0.86	4.49	1.99	33.19	9.80	35.17	3.51	10.29	2.98	1.63	3.85
Fluticasone Day 1 (-3 h)**	2.41	1.41	3.61	8.78	22.85	9.07	0.85	5.50	2.49	39.63	8.91	32.45	3.17	8.73	2.69	1.63	3.48

*Geometric means (pg/ml); ** Measured at 3 hours before the start of treatment on study day 1.

Figure 1 Overview of the single-blind placebo run-in period and double blind cross-over study periods 1 and 2

Figure 2 Overview of study assessments. IS = induced sputum, ENO = exhaled nitric oxide. Time zero is time of first study medication dosing. The single-blind placebo run-in screening period and the subsequent study periods 1 & 2 were identical

Figure 3 Time-response curves (mean ± SEM) to inhaled allergen during run-in period, placebo treatment and fluticasone treatment, respectively

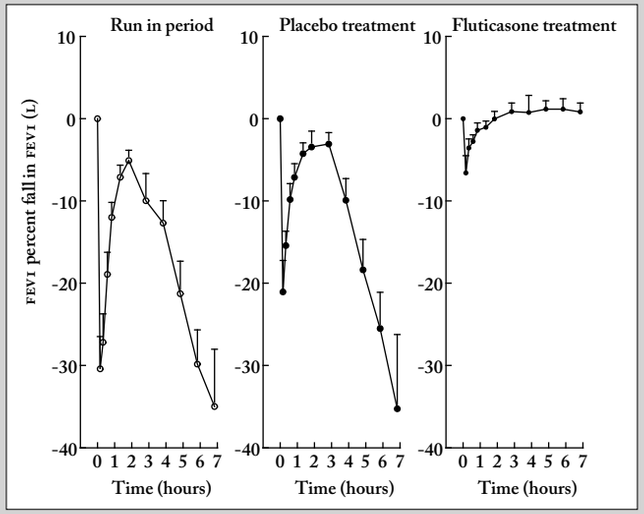
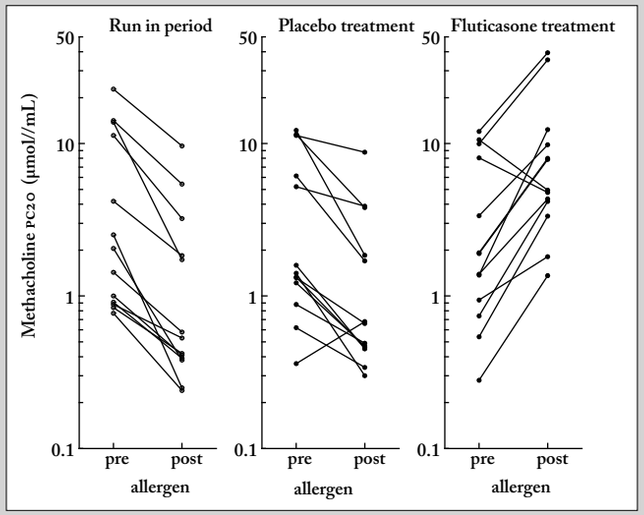


Figure 4 Changes in airway hyperresponsiveness 24 h pre- versus 24 h post-allergen during run-in period, placebo treatment and fluticasone treatment, respectively



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CHAPTER 3

SPUTUM RNA SIGNATURE IN ALLERGIC ASTHMATICS

SUBMITTED FOR PUBLICATION

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ABSTRACT

RATIONALE Inhaled allergen challenge is a validated exacerbation model of allergic asthma offering useful pharmacodynamic assessment of pharmacotherapeutic effects in a limited number of subjects.

OBJECTIVES To evaluate whether an RNA signature can be identified from induced sputum following an inhaled allergen challenge, whether a RNA signature could be modulated by limited doses of inhaled fluticasone, and whether these gene expression profiles would correlate with the clinical endpoints measured in this study.

METHODS Thirteen non-smoking, allergic subjects with mild to moderate asthma participated in a randomized, placebo-controlled, 2-period cross-over study following a single-blind placebo run-in period. Each period consisted of 3 consecutive days, separated by a wash-out period of at least three weeks. Subjects randomly received inhaled fluticasone ((FP) MDI; 500 MCG BID X 5 doses in total) or placebo pre- until 24 H post-allergen. On day 2, house dust mite (HDM) extract was inhaled and airway response was measured by FEV1 at predefined time points until 7 H post-allergen. Sputum was induced by NaCl 4.5%, processed and analysed at 24 H pre- and 7 & 24 H post-allergen. RNA was isolated from eligible sputum cell pellets (<80% squamous of 500 cells), amplified according to NUGEN technology and profiled on Affymetrix arrays. Gene expression changes from baseline and fluticasone treatment effect were evaluated using a mixed effects ANCOVA model at 7 and at 24 hours post allergen challenge.

RESULTS Inhaled allergen induced statistically significant gene expression changes in sputum, which were effectively blunted by fluticasone (adjusted p-value <0.025). 47 RNA signatures were selected from these responses for correlation analyses and further validation. This included TH2 cytokines, chemokines, high affinity IgE receptor FCERIA, histamine receptor HRH4, and enzymes and receptors in the arachidonic pathway. Individual messengers from the 47 RNA signature correlated significantly with lung function and sputum eosinophil counts.

CONCLUSION Our RNA extraction and profiling protocols allowed reproducible assessments of inflammatory signatures in sputum including quantification of drug effects on this response in allergic asthmatics. This approach offers novel possibilities for development of PD biomarkers in asthma.

INTRODUCTION

Inhaled allergen challenge can be applied to study the pathophysiology and the immune-biology to allergic stimuli within the airways. Allergen challenge is highly reproducible and serves as an integral disease model enabling the investigation of several features of asthma [1]. In drug development, allergen challenge is an established tool predicting clinical efficacy of novel anti-allergic and anti-asthma treatments [2]. Hypertonic saline-induced sputum [3] has been shown to yield reproducible increases in inflammatory cells and biomarkers following allergen-induced late asthmatic response (LAR) [4] with subsequent response to novel and existing anti-inflammatory therapies [2;4-7].

Microarray technology allows to profile gene expression of the entire genome and has been widely applied in several asthma studies [8;9]. A large majority of these gene profiling studies involved tissue obtained from asthmatics like airway epithelium [10;11], bronchial biopsies [12] or nasal mucosal cells [13]. Although gene expression has also been studied in fluids from asthmatics like blood [14], broncho-alveolar lavage [15], and induced sputum [16], little is published on extensive gene expression profiling on induced sputum cells following allergen challenge.

In this study Affymetrix 2.0 microarray technology was used to measure the gene expression levels of > 50.000 transcripts in induced sputum obtained from 13 allergic asthmatics before and after allergen challenge. In a refined set of 47 genes signatures we aimed to study: 1) the feasibility and reproducibility of quantification of gene expression in induced sputum at 7 and 24 hour post-challenge 2) their reversibility after a short course of inhaled fluticasone (FP) treatment, and 3) the correlation with lung function and eosinophil measurements.

METHODS

STUDY POPULATION AND DESIGN Thirteen non-smoking subjects with clinically stable, mild to moderate allergic asthma [17] using prn short-acting beta2-agonists only and with dual airway responses to inhaled house dust mite (HDM), documented during the single-blind placebo run-in screening period, participated in a double-blind, 2-way cross-over study. Each period consisted of 3 consecutive days, with ≥ 3 weeks washout between periods, *Figure 1*. The screening was identical to the subsequent treatment periods during which subjects randomly received inhaled FP (MDI, 500 µg BID, total of 5 doses) or matching placebo. On day 1, baseline measurements including, spirometry and subsequent sputum induction (3 x 5 MIN

NaCl 4.5%) were performed prior to study medication. On day 2, 1 h post-study medication, subjects underwent a titrated allergen challenge [1]. The subsequent airway response was repeatedly measured by FEV1 until 7 h post-allergen. At 24 h post-allergen (day 3), test-procedures were repeated as on day 1. All test-procedures were conducted according to standardized, validated methods and at the same time of the day (within 2 hours) during the different treatment periods [1;18-20].

A dual airway response to inhaled HDM extract consisted of an early (EAR) and a late asthmatic response (LAR) defined as a fall in FEV1 > 15% from baseline occurring between 0-3 h and 3-7 h post-allergen, respectively.

This study was part of an allergen study measuring allergen induced TH2-profile in sputum [7].

The study was approved by the Ethics Committee of Leiden University Medical Center, Leiden, The Netherlands, and all participants gave a signed informed consent (EUDRACT number 2007-003671-40).

STUDY MEDICATION ❦ Fluticasone 250 µg/puff (Allen & Hanburys, Glaxo Wellcome Ltd, Middlesex, UK) and matching placebo (Armstrong Pharmaceuticals Inc., Canton, MA, USA, packaged at Merck Frosst, Kirkland, Canada) were supplied in identical metered dose inhalers (MDIs) and inhaled per single puff through an Aerochamber (Volumatic, GlaxoSmithKline, Zeist, The Netherlands).

ALLERGEN CHALLENGE ❦ The allergen challenge was performed using the 2 minutes tidal breathing method that has been previously validated [1]. The run-in period served as a dose (range) finding procedure, while during study periods 1 & 2 each subject inhaled the same 2 or 3 cumulative doses of the allergen extract that had caused a fall in FEV1 of at least 15% from baseline during the run-in period. Following diluent, incremental doubling concentrations (7.81 to 2,000 BU/mL) of HDM extract (Dermatophagoides pteronyssinus; SQ 503, ALK-BPT, ALK-Abelló, Almere, The Netherlands) in phosphate-buffered saline (PBS) were aerosolized by a calibrated jet-nebulizer (DeVilbiss 646, output 0.13 mL/MIN, Somerset, Pennsylvania, USA) and inhaled at approximately 12 MIN intervals, until the EAR was reached (defined as a decrease in FEV1 of > 15% from post-diluent baseline within 1 h post-allergen). Airway response to inhaled allergen was measured by FEV1 in duplicate on a calibrated spirometer (Vmax Spectra; Sensor Medics, Bilthoven, The Netherlands) according to standard procedures [21], at 10, 20, 30, 45, 60, 90 and 120 minutes and then hourly until 7 h after the last allergen inhalation. The highest, technically valid measurement was expressed as percentage decrease from post-diluent baseline FEV1 and included into the analysis.

SPUTUM INDUCTION, PROCESSING AND ANALYSIS ❦ Sputum induction was performed as previously described [20;22] using a DeVilbiss Ultraneb 2000 ultrasonic nebulizer (Tefa Portanje, Woerden, The Netherlands) connected to a 100-CM long plastic tube, with an internal diameter of approximately 22 MM, connected to a two-way valve (No.2700; Hans-Rudolf, Kansas City, MO, USA) with a mouthpiece. Hypertonic saline (NaCl 4.5%) was nebulised and inhaled through the mouth, with the nose clipped, during three periods of 5 minutes. At approximately 7 minutes following each induction, spirometry was performed as a safety measure.

The cell pellet was processed as a full sample according to guidelines [20;23]. The processing took place within 2 h of collection. A DTT 0.1% solution (Dithiothreitol, Calbiochem, La Jolla, CA, USA) was mixed with a protease inhibitor pill (Complete Protease Inhibitor Cocktail tablets, Roche Applied Science #11 697 498 001; 1 pill per 50 mL of solution). The volume of the entire sputum sample was determined and an equal amount of 0.1% DTT/protease inhibitor solution was added. Subsequently, the sample was mixed with a pipette and placed in a warm shaking bath for 15 minutes at 37 °C. The homogenized mixture was centrifuged at 390G (1500 RPM) during 10 minutes. The supernatant was removed.

To determine cell viability and the total cell count, the cell pellet was re suspended in 2 mL PBS and filtered; 50 µL of the suspension was mixed with 50 µL of Trypan Blue. Total cell counts were determined in a counting chamber (Bürker; Omnilab 402521) using a cell counter (Omnilab 7005333). Cytospin slides (50 µL/cytospin; Shandon Cytospin 4, Thermon Electron Corporation, Runcorn, UK) were prepared by diluting the cell suspension with PBS in order to obtain approximately 0.5x 106 cells per mL, and subsequently centrifuged for 3 minutes at 254 G. 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 each sputum sample, at least 500 nucleated cells, excluding squamous cells, were counted twice and the average percentage of each cell type was determined and expressed as percentage of nonsquamous cells. If > 80% of the cell count consisted of squamous cells, the quality of the sputum sample was judged unsatisfactory and was excluded from analysis.

The remaining suspension was centrifuged a second time. The resulting cell pellet was resuspended in 1 ml of TRIzol® (Invitrogen, Cat. # 15596-018, Life Technologies, Carlsbad, California, USA). RNA was amplified using WT-Ovation® amplification technology (NuGEN, San Carlos, California, USA). The amplified material was labeled and hybridized using a standard Affymetrix protocol. Gene expression studies were performed using the Rosetta/Merck Human RSTA Custom Affymetrix 2.0 microarray (Affymetrix, Santa Clara, California, USA) containing 51,562 probe sets

interrogating 50,159 human transcripts predominantly from REFSEQ, GenBank, dbEST and ENSEMBL databases as described on the Gene Expression Omnibus website (https:// http://www.ncbi.nlm.nih.gov/geo/). The accuracy of sample processing was monitored through quality metrics assessing RNA yield, RNA quality: 18S/28S ribosomal RNA ratio, RNA Integrity Number (RIN) score, and hybridization parameters: 3'/5' ratios for GAPDH MRNA and scale factor. In addition, the amount of bacterial RNA contamination was evaluated by calculating the area under the curve for the 16S and 23S (bacterial) versus the 18S and 28S (eukaryotic) ribosomal RNA peaks using a bio analyzer electropherograms (Agilent, Santa Clara, California, USA). Specimens with more than 80% bacterial contamination were removed from the analysis. Data were normalized using the Robust Multichip Average (RMA) algorithm prior to statistical analysis.

STATISTICAL MODEL FOR DATA ANALYSIS ❦ A mixed effect ANCOVA model was selected including terms for baseline gene expression, treatment, sequence and period as fixed effects and subject nested in sequence as a random effect. Gene expression change from the appropriate baseline was used as the dependent variable. The baselines for each of the periods were used as covariates.

ANALYSIS OF TREATMENT EFFECTS ❦ For each time point, 7 hours and 24 hours, the allergen challenge effect and the FP treatment effect were calculated. The allergen challenge effect (ACE) was calculated as the change from baseline when the subject received placebo treatment. The FP treatment effect (FTE) was calculated as the difference in change from baseline between the FP treatment group and the placebo group. P-values for each gene in each treatment effect were adjusted using the Benjamini-Hochberg's procedure with a false discovery rate (FDR) level pre-specified at 0.025 to select significant genes.

CORRELATION ANALYSES ❦ Pearson correlation coefficient and the associated p-value were computed for correlation between the estimated individual subject-level effect, separately for allergen challenge effect and FP treatment effect, for a given clinical endpoint and gene of interest. Assuming no period or sequence effect, subject-level allergen challenge effect was calculated as the log-transformed change from baseline, for a clinical endpoint or gene of interest, when the subject received placebo treatment. Similarly, subject-level FP treatment effect was calculated as the difference in change from baseline for a clinical endpoint or gene of interest when the subject received fluticasone vs placebo. Type I error of 10% (two-sided) was used to select significant results, and no multiplicity adjustment was applied for declaring statistical significance.

RESULTS

Sputum specimens were collected from asthmatic subjects who provided a baseline specimen in period 1 and period 2 and which passed the quality control. The reproducibility of the sputum induction and collection procedures for RNA profiling after hybridization on microarrays, were evaluated by comparing individual gene expression intensities in combination with hierarchical clustering using Pearson correlation coefficients [24], *Figure 2*. The results of this cluster analysis revealed that 14 out of 18 sputum specimens clustered appropriately in subject specific pairs, validating our sputum collection and isolation protocol.

The whole microarray contained 51,562 probe sets. At 7 hours post allergen challenge, and applying a false discovery rate of < 0.025, a total of 4.175 and 1.001 statistical significant probe sets were identified for the allergen effect (ACE) and the FP treatment effect (FTE), respectively. Likewise, 1.143 and 1.018 statistical probe sets were identified at 24 hours post allergen for the allergen effect and the FP treatment effect, respectively. 714 probes sets were regulated by both the allergen challenge effect and FP treatment at 7 hours and 311 probe set at 24 hours post challenge, *Table 1*. All the genes regulated by both the allergen challenge and FP at each time point were reversed from their allergen induced levels in presence of fluticasone, *Figure 3*. In other words, fluticasone effectively blunted the response to the allergen challenge at the gene expression level.

Quantification of the individual genes that contribute to the key cytokines of the TH1, TH2 and TH17 pathways was performed by displaying the change from baseline in gene expression at 7 hours and 24 hours following allergen challenge in presence or absence of FP treatment (*Figure 4*). This analysis revealed the up-regulation by the allergen challenge and the down-regulation by FP treatment of the gene expression for several key TH2 cytokines (Interleukin (IL)-4, IL-5 and IL-13) and an absence of an effect on key TH1 cytokines (Interferon (INF)-γ and Tumor Necrosis Factor (TNF)). Chemokine ligand 13 (CCL13)/Monocyte Chemoattractant Protein (MCP)-4 [25], CCL17/ Thymus and Activation Regulated Chemokine (TARC) [26] and CCL26/ eotoxin-3 [27] are Inflammatory chemokines mediating TH2 cell recruitment and known to be induced by IL-4. Their gene expressions were up-regulated by the allergen challenge and down-regulated by FP treatment following a similar pattern as the TH2 cytokines (*Figure 5*). Likewise, the same pattern was observed for genes belonging to pathways controlling the release of inflammatory parameters like: HDC (histidine decarboxylase) known to catalyze the production of histamine [28]; histamine receptor 4 HRH4 which is specific for eosinophils and basophils [29]; FCERIA, the alpha subunit of the high affinity IgE receptor which directly binds IgE and through crosslinking induces the release of preformed histamine and proteases as

well as the generation of leukotrienes and prostaglandins; the messengers for the enzyme GGT5 (gamma glutamyl transferase 5, which converts leukotrienes C_4 to D_4) [30]; ALOX15 (15-lipoxygenase) and the receptor PTGER3 (prostaglandin receptor 3) were also up regulated by the allergen challenge and down regulated by FP treatment. In most of the cases, the fold change from baseline was higher at 7 hours versus 24 hours and the p-values smaller. This suggests that the 7 hours' time point provides the most useful readouts of the strict inflammatory response following an allergen challenge.

In order to facilitate the correlation analyses, the union of the genes affected by the allergen challenge and fluticasone 7 hours or 24 hours post-challenge was reduced to a set of 47 RNA signatures based on statistical significance, intensity of the change from baseline, biological relevance and classified based on druggable structural and functional categories (*Figure 6*). All the genes represented in the 47 RNA signatures harbor robust expression changes, and the large majority of them is up-regulated after 7 hours with the exception of FLT3 and CRLF2, which are regulated only after 24 hours.

The 47 RNA signature set was then used to identify genes correlating with lung function measurements (*Table 2*) and eosinophil cell counts and percentages (*Table 3*). Allergen challenge and FP treatment-mediated correlations were independently assessed for each probe set in the signature by estimating correlations at the subject level at 7 and 24 hours post allergen challenge. Correlation plots for the most significant probe sets from each correlation analysis type are represented in *Figure 7*. High correlation for some of the probe sets, E.G. ILIRLI and HRH4 and the eosinophil counts from the allergen challenge and the fluticasone treatment effect were observed, with correlation coefficients greater than 0.9 and p-value between < 0.001-0.002. In the allergen challenge effect analysis, probe sets for NRG1, CCR2, CD1C, MAP2K6, IL26 were negatively correlated with FEV1 measurements at 7 hours. In the fluticasone treatment effect, probe sets for, NRG1, RUNX3, FLT3, negatively correlated to the FEV1 measurements at 7 hours and 24 hours. NRG1 was the most significant gene consistently negatively correlated to lung function measurements at 7 hours in both the allergen effect and the fluticasone effect analysis with p-values of and coefficients of correlations in the range of -0.75 (p-value 0.054) to -0.90 (p-value 0.002).

DISCUSSION

In this study a RNA signature in sputum induced by the allergen challenge and reversed with fluticasone was identified. A subset of these genes, known to regulate the key inflammatory responses associated with allergic asthma, correlated with clinical endpoints and may constitute potential PD biomarkers of response to fluticasone.

TH2 responses have been traditionally described as playing a central role in the pathophysiology of asthma, although not all patients share a TH2 inflammatory pattern [31]. It is striking that in our study the shift toward the TH2 differentiation pathway is a major element of the transcriptional response to the HDM challenge in sputum and is down regulated following response to fluticasone treatment in the mild asthmatic atopic subjects enrolled in this study. The implications of these results are several-fold.

First, the screening of subjects for dual EAR and LAR responses and the strong homogeneity of our results are consistent with the concept of clustering of clinical asthma phenotypes in which presence of eosinophilic infiltration was identified as one of the key variables [32]. Furthermore, clinical phenotypes of asthma have been linked to molecular signatures and pathways in a study where TH2 "high" and "low" phenotypes, characterized by differences in airway responsiveness, eosinophilia and airway remodeling, could be differentiated at the molecular level [33]. The observed low variability and high effect size obtained for the gene expression measurements in this study is likely due to the careful selection of a homogeneous allergic, corticosteroid responsive subject population characterized by eosinophilic inflammation in response to an allergen challenge.

Second, our results also suggest that gene expression measurements collected in such an allergen challenge platform could guide the development of novel quantitative assays. For instance, one direct application of this technology could be the quantification of the RNAs that correlate the best with eosinophil numbers as a surrogate to the standard sputum eosinophil cell count assays. Another application of our technology would be the selection of PD biomarkers of response to anti-inflammatory treatment in asthma identified from a set of markers that correlate with clinical endpoints.

The results presented here also raised important questions. We identified from our data set two cytokines, IL-22 and IL-26, induced by the allergen challenge and reverted to baseline by fluticasone, which have been associated with the TH17 pathway. IL-22 is preferentially produced by TH17 cells in psoriatic skin and mediates the epithelium hyperplasia induced by IL-23 [34]. IL-26 is often co-expressed together with IL-17 and IL-22 by activation of TH17 cells, however, its function remains to be further investigated [35]. Despite the significance of IL-22 and IL-26, we were however unable to detect any up or down-regulation of the cytokines IL-17A and IL-17 F, as well as other genes associated with the TH17 pathway [36], therefore providing more support to the concept of a dominant TH2 response in this study.

Another question is whether the observed signature in sputum is due to i) changes in cell counts, in particular eosinophil cell counts since this cell type is predominantly increased in sputum following a segmental allergen challenge, ii) up or down-regulation of messengers within a given cell type or, iii) a combination of the above. The only way to address this question is to profile individual cell types isolated from sputum, however, the results from our analysis indicated some changes in gene expression that were correlated with cell type specific eosinophil cell counts and some that are not, therefore supporting option iii). On the one hand, we have identified two genes IL1RL1 and HRH4 that correlate extremely precisely with eosinophil cell counts (correlation coefficients > 0.9, p-values <0.002) and are known to be expressed predominantly in eosinophils, basophils and mast cells. RNAs for both genes therefore appear to be excellent surrogates of eosinophil measurements in sputum. Interestingly, polymorphisms in the HRH4 gene were found to be associated with atopic dermatitis [37], while variants of the IL1RL1 gene have been associated with atopic dermatitis and atopic asthma [38]. Given the important role that IL1RL1 has in eosinophil function as a receptor for IL-33, this gene might therefore also represent a promising drug target in inflammatory diseases characterized by a strong eosinophilic component correlating with disease symptoms. Then again, we have identified from this study multiple examples of genes that display similar expression pattern upon allergen challenge and fluticasone treatment and which are known to have very different cell type specificity. In particular chemokines CCL13 and CCL17 have a dendritic specific expression while CCL26 is epithelial specific; similarly CD1A and CD1B are T-cell specific markers. However, as the expression of those genes is up-regulated by the allergen challenge and down-regulated by fluticasone, this suggests that the identified signature cannot be explained uniquely by variations in eosinophil cell counts or percentages and also reflects major transcriptional changes in a large variety of cell types. An analysis of the transcriptional signatures of isolated sputum cell types in combination with the identification of transcriptional modules of genes co-expressed in asthma as previously described in blood [39] could map the relative contribution of each gene and cell type to the inflammatory response.

Finally, we also identified from our analysis a set of RNAs that uniquely correlates with classical lung function measurements. At 7 hours, chemokines or chemokine receptors (CCL13, CCL17, CCL26, and CCR2) and membrane bound glycoproteins such as CD1B, CD1C and CD209 correlate to lung function measurements. NRG1, the gene that most significantly correlated to FEV1 measurements at 7 hours, is a member of the neuregulin family, which signals through tyrosine kinases of the ErbB3 family. NRG1 induces the expression of the globlet cell mucin proteins MUC5AC and MUC5B in human airway epithelium [40]. Its inhibition may therefore represent a novel therapeutic approach for decreasing mucus hypersecretion in respiratory diseases. In conclusion, our RNA extraction and profiling protocols allowed sensitive assessments of allergen-induced inflammatory signatures in sputum and precise quantification of drug effects on this response in allergic asthmatics. This approach offers novel possibilities for development of pharmacodynamic biomarkers in asthma.

Table 1 Number of statistically significant probe sets identified from each contrast analysis, allergen challenge effect and fluticasone treatment effect, at 7 hours and 24 hours. The analysis was conducted on the whole microarray containing 50,159 human transcripts. The number of probe sets in common between the allergen challenge effect and the fluticasone effect analyses at a given time point are displayed on the right hand side. FDR: false discovery rate

	FDR < 0.025	
	# of probe sets	in common
Allergen Challenge Effect (ACE) at Hour 7	4,175	714
Fluticasone Treatment Effect (FTE) at Hour 7	1,001	
Allergen Challenge Effect (ACE) at Hour 24	1,143	311
Fluticasone Treatment Effect (FTE) at Hour 24	1,018	

Table 2 Correlations between gene expression measurements from the 47 RNA signatures and various FEV1 measurements. FEV1 measure i: % change in maximal drop of FEV1 during LAR, FEV1 measure ii: % change in time weighed average of FEV1 during lar, fevi measure iii: % change in FEV1 at hour 24. Significant probe sets (p-values < 0.1 and correlation coefficient > 0.73) are displayed.

Effect of interest	Probe set	Gene symbol	Clinical endpoint	Correlation coefficient (90% confidence interval)	P-value
mRNA at hour 7 and clinical endpoint at hour 7					
Allergen challenge effect	100150696_TGI_at	NRG1	FEV1 Measure II[2]	-0.90 (-0.98, -0.62)	0.002
	100300593_TGI_at	NRG1	FEV1 Measure II[2]	-0.83 (-0.96, -0.44)	0.01
	100124067_TGI_at	CCR2	FEV1 Measure I[1]	-0.79 (-0.95, -0.32)	0.02
	100312593_TGI_at	NRG1	FEV1 Measure I[1]	-0.77 (-0.94, -0.28)	0.025
	100161022_TGI_at	CDIC	FEV1 Measure II[2]	-0.77 (-0.94, -0.27)	0.027
	100303601_TGI_at	CCR2	FEV1 Measure I[1]	-0.76 (-0.94, -0.26)	0.027
	100145467_TGI_at	CDIC	FEV1 Measure II[2]	-0.76 (-0.94, -0.26)	0.027
	100161022_TGI_at	CDIC	FEV1 Measure I[1]	-0.76 (-0.94, -0.25)	0.03
	100147484_TGI_at	CCR2	FEV1 Measure II[2]	-0.75 (-0.94, -0.24)	0.031
	100300556_TGI_at	MAP2K6	FEV1 Measure II[2]	-0.75 (-0.94, -0.24)	0.03
	100311406_TGI_at	CDIC	FEV1 Measure II[2]	-0.74 (-0.93, -0.21)	0.036
	100159528_TGI_at	IL26	FEV1 Measure II[2]	-0.74 (-0.93, -0.21)	0.036
	100303601_TGI_at	CCR2	FEV1 Measure II[2]	-0.74 (-0.93, -0.20)	0.038
Fluticasone treatment effect	100135727_TGI_at	CDIA	FEV1 Measure II[2]	0.87 (0.46, 0.97)	0.012
	100127751_TGI_at	NRG1	FEV1 Measure I[1]	-0.84 (-0.97, -0.39)	0.017
	100302360_TGI_at	NRG1	FEV1 Measure I[1]	-0.84 (-0.97, -0.37)	0.019
	100155853_TGI_at	RUNX3	FEV1 Measure I[1]	-0.77 (-0.95, -0.19)	0.044
	100302360_TGI_at	NRG1	FEV1 Measure II[2]	-0.76 (-0.95, -0.16)	0.049
	100309572_TGI_at	FLT3	FEV1 Measure I[1]	-0.75 (-0.95, -0.16)	0.051
	100133255_TGI_at	MMP1	FEV1 Measure III[3]	-0.74 (-0.91, -0.34)	0.01
mRNA at hour 7 and clinical endpoint at hour 24					
Allergen challenge effect	100148726_TGI_at	CCL17	FEV1 Measure III[3]	-0.80 (-0.95, -0.36)	0.016
	100133255_TGI_at	MMP1	FEV1 Measure III[3]	-0.74 (-0.93, -0.22)	0.035
Fluticasone treatment effect	100145401_TGI_at	MAOA	FEV1 Measure III[3]	0.80 (0.27, 0.96)	0.031
	100301747_TGI_at	ADAM19	FEV1 Measure III[3]	0.80 (0.26, 0.96)	0.031
	100127751_TGI_at	NRG1	FEV1 Measure III[3]	-0.75 (-0.95, -0.14)	0.054
	100155853_TGI_at	RUNX3	FEV1 Measure III[3]	-0.74 (-0.94, -0.12)	0.058
	100309572_TGI_at	FLT3	FEV1 Measure III[3]	-0.74 (-0.94, -0.12)	0.058
mRNA at hour 24 and clinical endpoint at hour 24					
Allergen challenge effect	100157709_TGI_at	ALOX15	FEV1 Measure III[3]	0.77 (0.28, 0.94)	0.025
	100149346_TGI_at	SOCS2	FEV1 Measure III[3]	-0.75 (-0.94, -0.24)	0.031

[1]% change in maximal drop of FEV1 during LAR; [2]% change in time weighed average of FEV1 during LAR; [3]% change in FEV1 at hour 24

Table 3 Correlations between the gene expression measurements from the 47 RNA signatures and eosinophils (cell counts and percentages).Significant probe sets (p-values < 0.1 and correlation coefficients > 0.86) are displayed

Effect of interest	Probe set	Gene symbol	Clinical endpoint	Correlation coefficient (90% confidence interval)	P-value
MRNA at hour 7 and clinical endpoint at hour 7					
Allergen challenge effect	100302783_TGI_at	ILIRL1	Eosinophil counts	0.92 (0.70, 0.98)	0.001
	100161010_TGI_at	HRH4	Eosinophil counts	0.91 (0.67, 0.98)	0.002
	100149346_TGI_at	SOCS2	Eosinophil counts	0.91 (0.66, 0.98)	0.002
	100312840_TGI_at	ILIRL1	Eosinophil counts	0.88 (0.56, 0.97)	0.004
	100301491_TGI_at	GATA2	Eosinophil counts	0.87 (0.53, 0.97)	0.005
Fluticasone treatment effect	100148162_TGI_at	ILIRL1	Eosinophil counts	0.98 (0.92, 1.00)	< 0.001
	100312840_TGI_at	ILIRL1	Eosinophil counts	0.98 (0.88, 1.00)	< 0.001
	100302783_TGI_at	ILIRL1	Eosinophil counts	0.97 (0.85, 0.99)	< 0.001
	100142511_TGI_at	CCL26	Eosinophil percentage	0.97 (0.84, 0.99)	< 0.001
	100148726_TGI_at	CCL17	Eosinophil percentage	0.96 (0.80, 0.99)	< 0.001
	100161010_TGI_at	HRH4	Eosinophil counts	0.96 (0.79, 0.99)	< 0.001
	100134267_TGI_at	CRLF2	Eosinophil percentage	0.93 (0.69, 0.99)	0.002
	100135727_TGI_at	CD1A	Eosinophil percentage	0.92 (0.64, 0.98)	0.004
	100148210_TGI_at	ILIRL1	Eosinophil counts	0.90 (0.59, 0.98)	0.005
	100157709_TGI_at	ALOX15	Eosinophil counts	0.89 (0.52, 0.98)	0.008
	100302151_TGI_at	ILIRL1	Eosinophil counts	0.88 (0.52, 0.98)	0.008
	100162200_TGI_at	GATA2	Eosinophil percentage	0.88 (0.52, 0.98)	0.008
	100125222_TGI_at	IL13	Eosinophil percentage	0.88 (0.51, 0.98)	0.008
	100163032_TGI_at	VDR	Eosinophil counts	0.88 (0.51, 0.98)	0.008
	100302151_TGI_at	ILIRL1	Eosinophil percentage	0.88 (0.50, 0.98)	0.009
	100142202_TGI_at	CD1B	Eosinophil percentage	0.87 (0.48, 0.97)	0.01
	100124660_TGI_at	GGT5	Eosinophil counts	0.87 (0.46, 0.97)	0.011
	100143473_TGI_at	HDC	Eosinophil counts	0.87 (0.46, 0.97)	0.012
MRNA at hour 7 and clinical endpoint at hour 24					
Allergen challenge effect	100135727_TGI_at	CD1A	Eosinophil percentage	0.91 (0.65, 0.98)	0.002
	100132327_TGI_at	ADAM19	Eosinophil percentage	0.87 (0.55, 0.97)	0.004
	100136515_TGI_at	CD209	Eosinophil percentage	0.87 (0.53, 0.97)	0.005
Fluticasone treatment effect	100148210_TGI_at	ILIRL1	Eosinophil counts	0.93 (0.67, 0.99)	0.003
	100309438_TGI_at	MAOA	Eosinophil counts	-0.92 (-0.98, -0.64)	0.003
	100309708_TGI_at	PTGS1	Eosinophil counts	0.92 (0.65, 0.98)	0.003
	100124660_TGI_at	GGT5	Eosinophil counts	0.92 (0.63, 0.98)	0.004
	100156386_TGI_at	PTGS1	Eosinophil counts	0.89 (0.53, 0.98)	0.008
	100302151_TGI_at	ILIRL1	Eosinophil counts	0.88 (0.51, 0.98)	0.009
	100136515_TGI_at	CD209	Eosinophil percentage	0.88 (0.49, 0.97)	0.01

Figure 1 Study design. Overview of the single-blind placebo run-in period and double blind cross-over study periods 1 and 2 (upper section). Overview of study assessments (lower section). Time zero is time of first study medication dosing. The single-blind placebo run-in screening period and the subsequent study periods 1 & 2 were identical. IS: induced sputum.

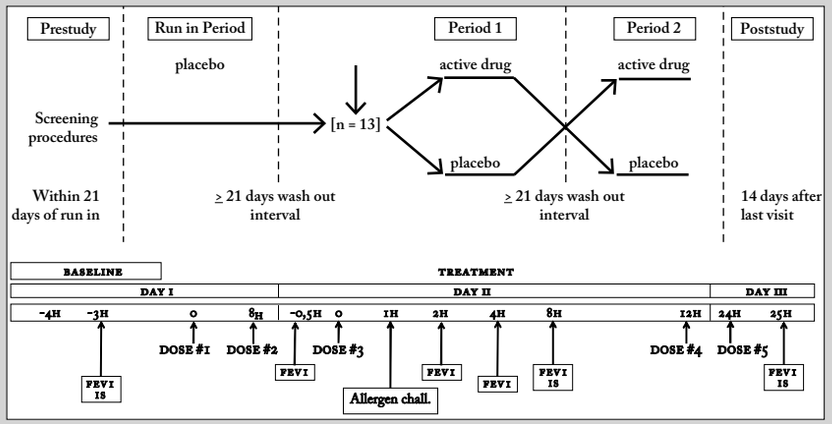


Figure 2 Hierarchal cluster assessment of sputum microarray data. Numbers refer to subject allocation numbers. Log 10 ratios of intensity estimates versus the average of all intensities are displayed. Dark color refers to probe sets that are up-regulated in reference to the pool of all specimens analyzed and light to the probe sets that are down-regulated. Left dark rectangles link specimens from the same subject that co-cluster on the dendrogram

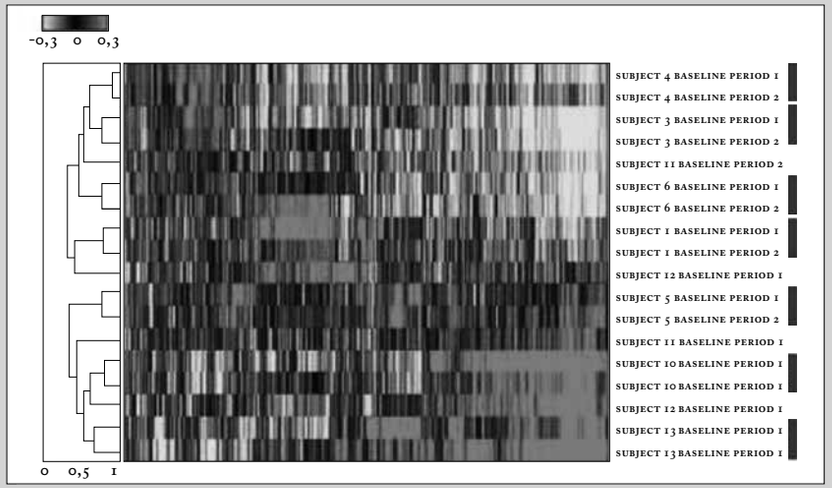


Figure 3 Log 10 Estimates of gene expression changes for the significant genes identified from contrast analysis at 7 hours and 24 hours with an FDR < 0.025. ACE; allergen challenge effect, estimates of changes from baseline in the placebo group. FTE: fluticasone effect, estimates of differences in change from baseline between the placebo and the fluticasone groups

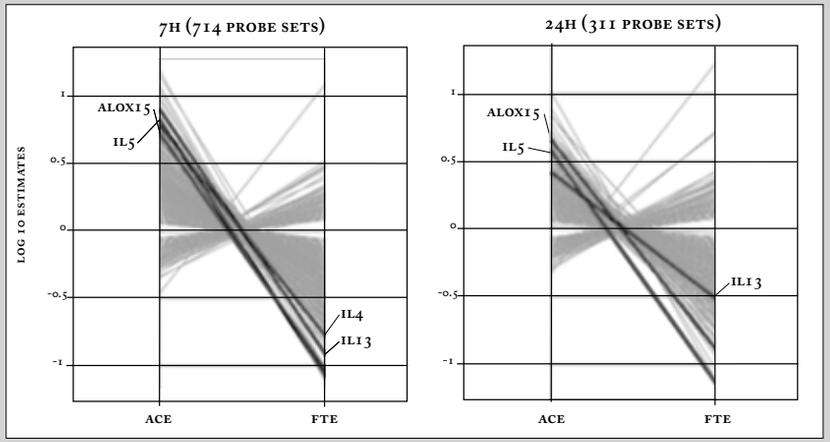


Figure 4 Fold change from baseline in gene expression. Th2 cytokines (IL4, IL5, IL13), Th1 cytokines (IFNG and TNF), Th17 cytokines (IL22, IL26). Fold change from baseline for the placebo group is represented on the left in each bar. Fold change from baseline for the fluticasone group is represented on the right in each bar. P-values are adjusted p-values, error bars represent 90% confidence

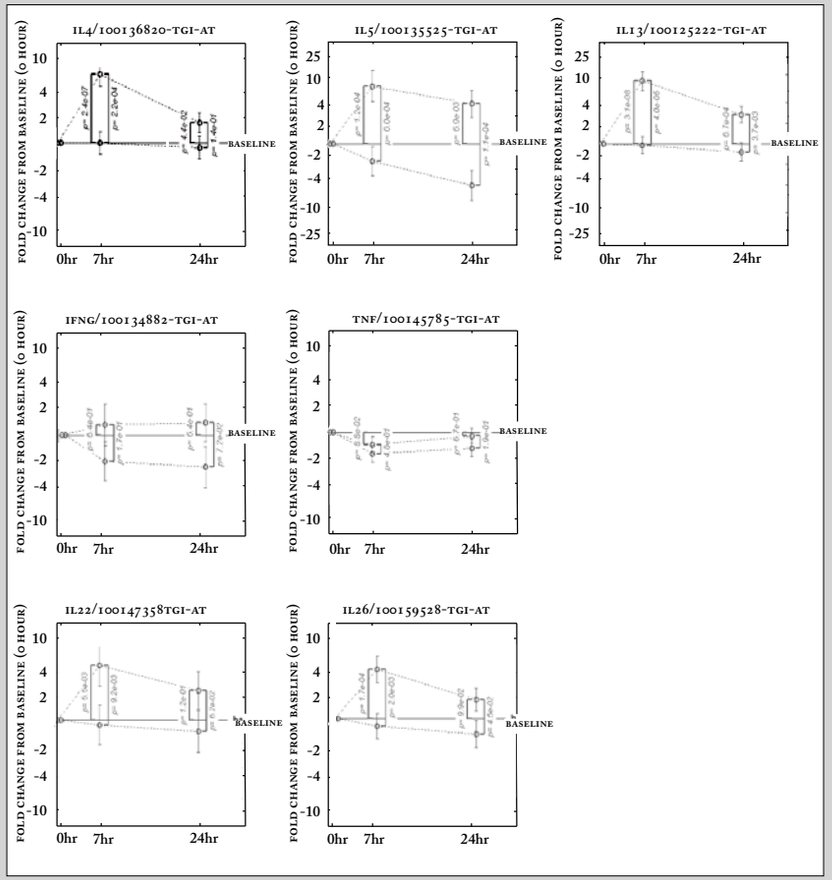


Figure 5 Fold change from baseline in gene expression. Inflammatory chemokines (CCL13, CCL17, CCL26), molecules controlling the release of histamine (HDC, HRH4, FCER1A) prostaglandins and leukotrienes (PTGER3, ALOX15, GGT5). Fold change from baseline for the placebo group is represented on the left in each bar. Fold change from baseline for the fluticasone group is represented on the right in each bar. P-values are adjusted p-values, error bars represent 90% confidence intervals.

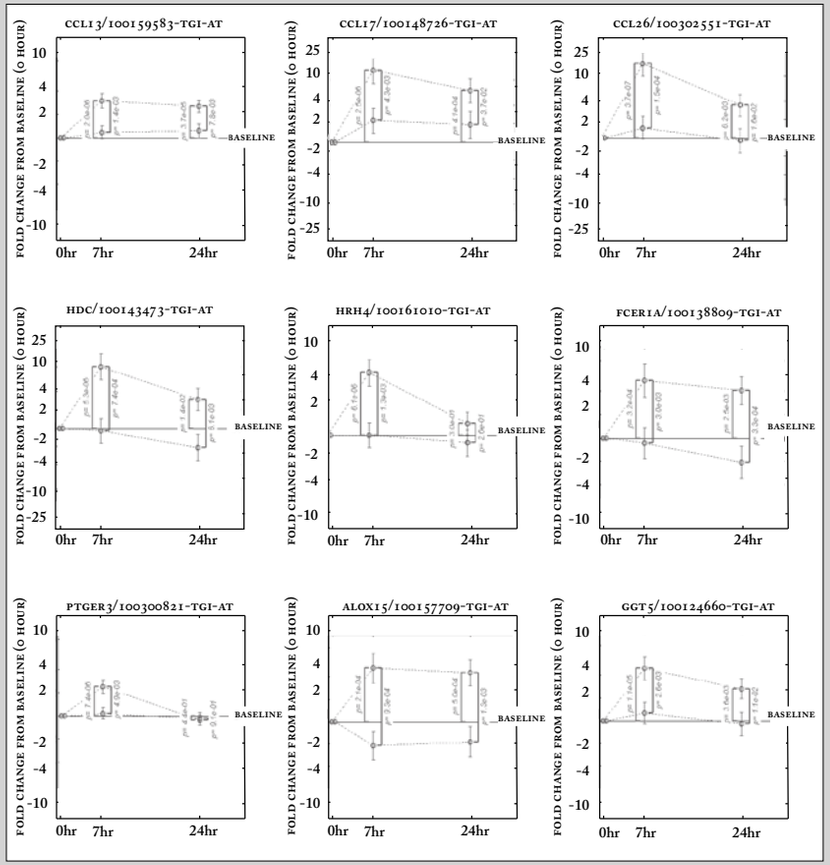


Figure 6 Fold changes over baseline (point estimate and 90% confidence intervals) for the 47 RNA signatures. Light bars represent the change from baseline in the placebo group and dark bars in the fluticasone group. P-values for the allergen challenge effect (ACE) and the fluticasone treatment effect (FTE) are represented. P1: Th2 cytokines, P2: chemokines and chemokine receptors, P3: FCER1 and histamine signaling, P4: enzymes and signaling molecules in prostaglandin, leukotriene pathways, P5: Other cytokines, growth factors and their receptors, P6: Other enzymes, P7: membrane bound glycoproteins, P8: transcription factors, P9: regulators of the inflammatory response"

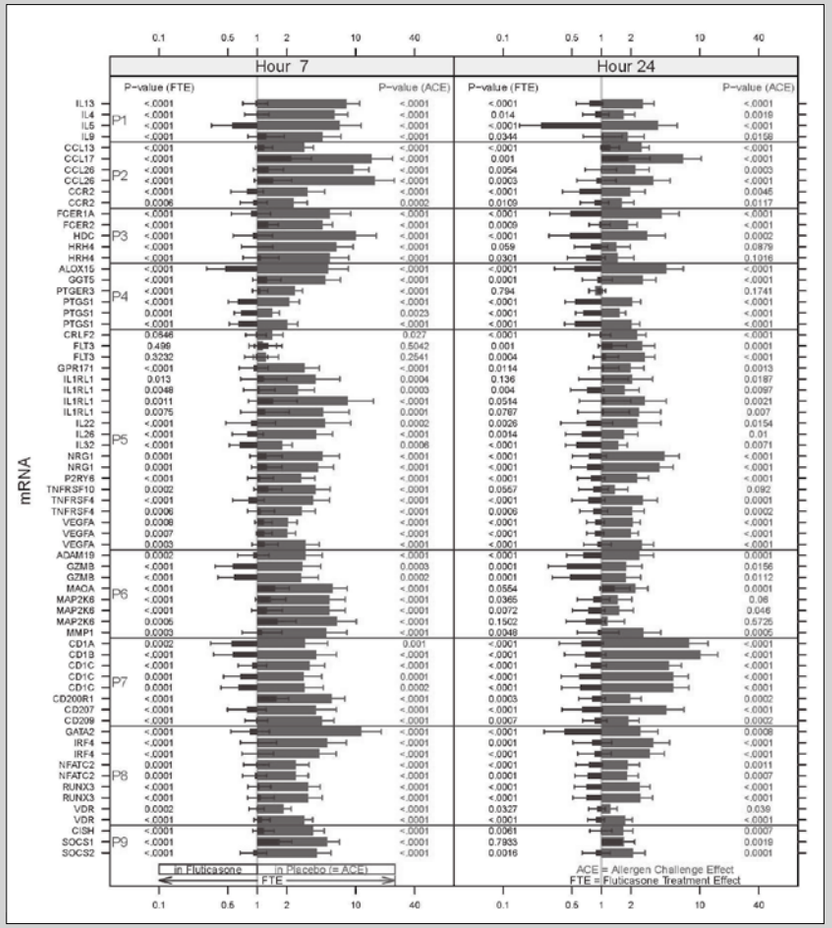
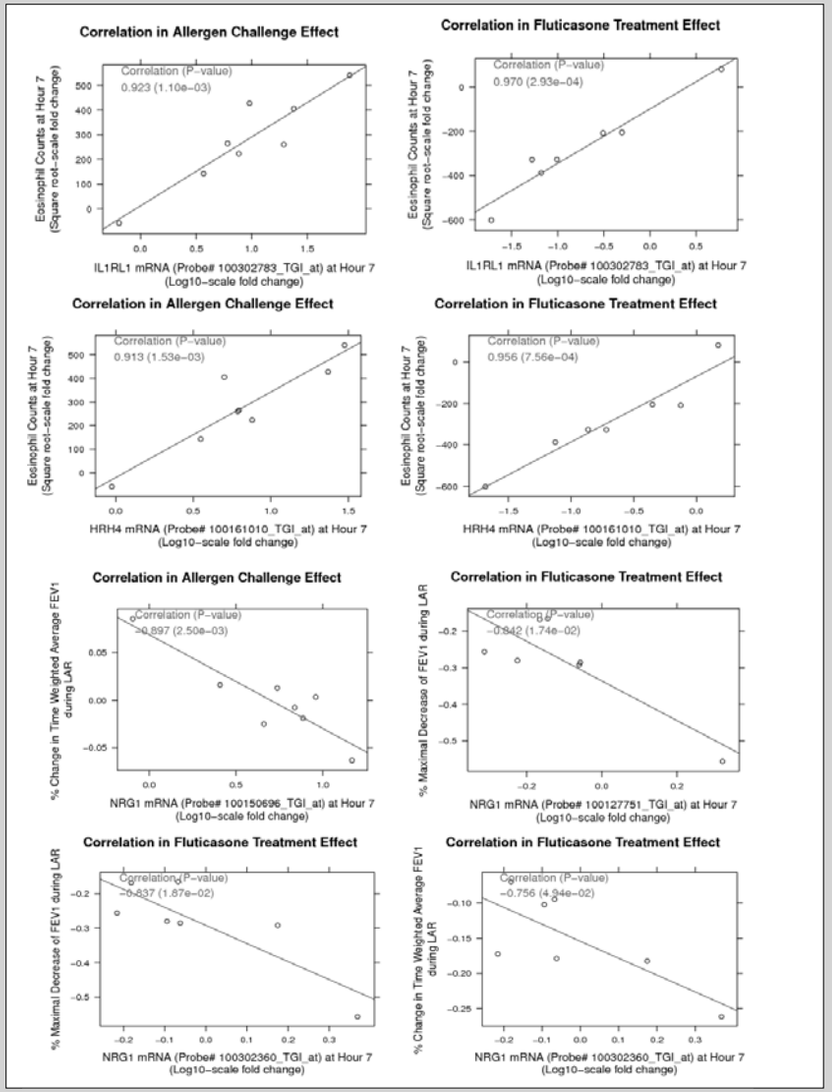


Figure 7 Correlation plots of the most significant probe sets to individual subject clinical measurements for the allergen challenge effect and the FP treatment effect. Correlation coefficients and corresponding p-values in parenthesis are listed in grey. HRH4 (histamine receptor 4); IL1RL1 (IL33 receptor); NRG1 (neuregulin 1)



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CHAPTER 4

SPUTUM INDUCTION WITH HYPERTONIC SALINE REDUCES FRACTIONAL EXHALED NITRIC OXIDE IN CHRONIC SMOKERS AND NON-SMOKERS

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ABSTRACT

BACKGROUND Nitric oxide (NO) measurements in exhaled air and hypertonic saline-induced sputum are commonly used biomarker sampling methods of the lower airways. Both sampling methods have been validated in asthmatic patients and healthy controls, however, data from chronic smokers are scarce.

OBJECTIVES To evaluate the reproducibility and differences in fractional exhaled NO (FENO) values in asymptomatic chronic smokers and healthy, non-smoking controls. Furthermore, to test the effect of hypertonic saline sputum induction (SI) on FENO levels in both study groups.

METHODS 16 asymptomatic chronic smokers and 16 non-smokers participated in this study. Baseline FENO and forced expiratory volume in 1 s (FEV1) were recorded pre- and 30 MIN post-NaCL 4.5% SI (3 x 5 MIN) on 2 study days (± 2 H; 4-10 days apart). Mixed ANOVA was used to estimate the intra-subject Coefficient of Variation (CV) % over days; changes in FENO and FEV1 values before and after SI, were analyzed by a Student's paired t-test. The difference between smokers and non-smokers was estimated by a Student's t-test.

RESULTS On day 1, FENO values in smokers were significantly lower than in non-smokers, 10.6 PPB, and 18.4 PPB, respectively, (42% difference, p = 0.0028, 95% CI: -59%, -19%). In both study groups, FENO measurements were reproducible, with an intra-subject CV of 27.2% and 19.2%, for smokers and non-smokers, respectively. SI significantly decreased FENO levels in both study groups on day 1. In smokers, there was a mean reduction in FENO of almost 37% (p<0.01, 95% CI (-53.2%, -14.2%), and in non-smokers a mean decrease of almost 35% (p=0.047, 95% CI -57%, -0.6%). In both study groups SI did not affect FEV1 (p>0.94).

CONCLUSIONS Our data extend previous findings in asthmatics and healthy controls to asymptomatic chronic smokers: 1. FENO measurements are reproducible in both smokers and non-smokers; 2. baseline FENO levels in chronic smokers are lower than in non-smokers and 3. sputum induction by hypertonic saline reduces FENO levels in both study groups, without affecting lung function.

INTRODUCTION

Sputum induction by hypertonic saline (SI) and fractional exhaled nitric oxide (FENO) are validated, commonly used non-invasive biomarker sampling methods of the lower airways [1;2]. FENO measurements are increasingly applied for diagnosis and monitoring of asthma [3]. Furthermore, both methods are often used as complementary research tools to assess the airway inflammation in response to interventions with (novel) anti-inflammatory therapeutic modalities [4]. However, there is evidence that sampling methods sometimes interfere and thus may affect the levels of biomarkers [5]. So far, two published studies have addressed the effect of SI on FENO values in asymptomatic atopic subjects and asthmatic patients and showed a maximal decrease in FENO directly post-induction with still a substantial decrease up to 4 hours after SI [6;7]. In this study population, FENO levels were reproducible and unrelated to the initial SI-induced decrease in FEV1 [6;7]. So far, few data have been published on chronic smokers [8;9]. Therefore, we tested the reproducibility and differences in FENO levels between asymptomatic chronic smokers and healthy non-smokers. Furthermore, we investigated the effect of SI on FENO levels in both study groups.

METHODS

SUBJECTS The study population consisted of two groups: 16 asymptomatic chronic smokers with a smoking history of at least 10 pack-years (8F/8M; 32-52 years) and 16 healthy non-smokers (8F/8M; 30-49 years) who had not smoked for at least 12 months prior to study enrolment and who had a total smoking history of less than 5 pack-years. For the smoker group, the last cigarette was smoked at least one hour before any study procedure. All subjects had no history of relevant lung disease or any respiratory tract infection for at least 4 weeks before the start of the study. All subjects gave written informed consent. The study was approved by the Ethics Committee of Leiden University Medical Centre, Leiden, Netherlands.

STUDY DESIGN The study comprised two study days, 4 to 10 days apart. On each study day, FENO was measured approximately 55 minutes before and 30 minutes after the SI-procedure. All assessments were performed at the same time of the day (± 2 H). This study was conducted as part of a larger biomarker study; the focus of this manuscript is on methodological issues related to the interaction of SI on FENO levels.

FRACTIONAL EXHALED NITRIC OXIDE (FENO) ❦ FeNO measurements were performed by a chemiluminescence analyser (Ecomedics CLD88sp, Ecomedics, Duernten, Switzerland) according to current guidelines [1]. Briefly, after a deep inhalation of NO-free air, subjects exhaled for approximately 10 seconds against a resistance at a stable flow of approximately 50 mL/s. The mean of the first three technically acceptable measurements (within 10%) were included in the analysis and expressed in parts per billion (PPB).

PULMONARY FUNCTION TESTS ❦ Spirometry was performed according to standardized protocols by a calibrated spirometer (Vmax Spectra Sensor Medics; Cardinal Health, Houten, The Netherlands) [10] connected to a personal computer. The mean of the two out of three (within 5%) highest, technically satisfactory forced expiratory volume in 1 second (FEV1) measurements was included in the analysis.

HYPERTONIC SALINE SPUTUM INDUCTION ❦ Sputum was induced by hypertonic saline (4.5% NaCl) nebulised by an ultrasonic nebulizer (DeVilbiss Ultra NEB 2000, Somerset, PA, USA) according to current guidelines during three periods of 5 MIN each [2]. Spirometry was performed 7 minutes after each SI-period.

ANALYSIS ❦ The reproducibility of the FeNO levels in both study groups was assessed on log-transformed data by a Mixed Analysis of Variance (ANOVA) to estimate the intra-subject Coefficient of Variation (CV). The differences in FeNO levels between both study populations were analyzed with a Student's t-test and the effect of inhaled NaCl 4.5% on FeNO and FEV1 within both study groups was analysed with the paired Student's t-test. Results were back-transformed to ratios and expressed as percentage difference.

RESULTS

STUDY SUBJECTS ❦ The study groups were well-matched with no statistically significant differences in baseline characteristics between the two groups (*Table 1*).

REPRODUCIBILITY AND DIFFERENCE IN FENO BETWEEN STUDY GROUPS ❦ The intra-subject mean CV for baseline FeNO measurements was 19.2% and 27.2% for non-smokers and smokers, respectively (Table 2). Mean FeNO was significantly lower in smokers compared with non-smokers.

EFFECT OF SPUTUM INDUCTION ON FENO AND FEV1 ❦ On day 1, SI decreased FeNO levels in non-smokers by on mean 35% (95% CI: -57%, -0.6%; p=0.047) and in smokers by on mean 37% (95% CI:-53%, -14%; p = 0.0045) (Table 2). SI did not affect FEV1 in either study group.

DISCUSSION

In line with previous observations in allergic asthmatics, we found reproducible FeNO levels in asymptomatic chronic smokers and healthy non-smoking controls. In smokers, FeNO levels were generally lower than in non-smokers and within similar ranges as previously reported [11]. Similarly to previous observations in allergic asthmatics [6], hypertonic saline decreased FeNO levels in both study groups without affecting FEV1. Therefore, our findings confirm and extend previous data (5-7;12).

The sputum inductions in our study were performed according to standardized procedures [1] in age- and gender-matched populations, while in the smokers the time between smoking and any measurements was kept within the same ranges [12]. Hence, the lack of statistical significance between both study groups and pre- and post-SI on study day 2 is most probably due to a larger variability of the FeNO values in a small sample size, possibly caused by external factors.

In line with previous studies we found lower FeNO levels in smokers compared with non-smokers [12]. It appears that smoking inhibits NO formation from inducible nitric oxide synthase in epithelial lung cells [13]. Furthermore, NO synthesis may be reduced by negative feedback as a result of high NO-concentrations in cigarette smoke [12], NO oxidation or interaction with other molecules present in tobacco smoke [14].

In conclusion, FeNO levels in chronic smokers were found to be reproducible and generally lower than in healthy non-smokers. Sputum induction reduced FeNO in both study populations without affecting FEV1. Our data extend previous observations in allergic asthmatics to chronic smokers. In view of the interference of sputum induction with FeNO measurements: FeNO should be measured before sputum induction.

Table 1 Subjects' baseline characteristics

	Non-smokers	Smokers
Number of subjects	16	16
Age (years)	41 (30-49)	41 (32-52)
Gender (M/F)	8M/8F	8M/8F
Height (meters)	1.75 (1.56-1.93)	1.73 (1.58-1.85)
Weight (KG)	77.5 (51.8-110.2)	73.1 (46.5-100.5)
BMI (KG/M2)	25.1 (21.1-29.5)	24.3 (18.6-30.0)
FEV1 (L)	3.66 (2.75-5.16)	3.55 (2.38-4.64)
FEV1 (% predicted)	102.9 (78-119.6)	102.7 (82.1-121.1)
FVC (L)	4.78 (3.21-6.42)	4.46 (3.15–6.38)
FVC (% predicted)	105.1 (82-131)	111.7 (76-127)

Values presented as mean (range)

Table 2 Effect of S1 on FENO and FEV1 values

Study population / parameters	Day 1			
	Pre NACL 4.5%	**30 min post NACL 4.5%**	**Difference (95% CI)**	**p-value**
NON-SMOKERS				
FENO (PPB)	18.4	12.0	-34.9% (-57.3%, -0.6%)	p = 0.047
FEV1 (L)	3.66	3.68	0.02 (-0.38, 0.41)	p = 0.94
SMOKERS				
ENO (PPB)	10.6	6.7	-36.6% (-53.2%, -14.2%)	p =0.0045
FEV1 (L)	3.56	3.55	-0.01 (-0.52, 0.49)	p = 0.95
Study population / parameters	**Day 2**			
	Pre NACL 4.5%	**30 min post NACL 4.5%**	**Difference (95% CI)**	**p-value**
NON-SMOKERS				
FENO (PPB)	15.9	12.3	-22.6 % (-50.5%, 21.1%)	P = 0.25
FEV1 (L)	3.56	3.68	0.12 (-0.31, 0.57)	P = 0.56
SMOKERS				
FENO (PPB)	11.1	8.5	-23.9% (-43.5%, 2.6%)	P = 0.072
FEV1 (L)	3.49	3.53	0.04 (-0.44, 0.52)	P = 0.86
Coefficient of variation (CV%) for FENO (pre NACL 4.5%)	non-smokers		19.2%	
	smokers		27.2%	

CI = confidence interval. FENO values in geometric means; CV = coefficient of variation

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CHAPTER 5

REPRODUCIBILITY OF BIOMARKERS IN INDUCED SPUTUM AND IN SERUM FROM CHRONIC SMOKERS

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ABSTRACT

RATIONALE Soluble inflammatory markers obtained from non-invasive airway sampling such as induced sputum may be useful biomarkers for targeted pharmaceutical interventions. However, before these soluble markers can be used as potential targets, their variability and reproducibility need to be established in distinct study populations.

OBJECTIVE This study aimed to assess the reproducibility of biomarkers obtained from induced sputum and serum in chronic smokers and non-smokers.

METHOD Sputum and serum samples were obtained from 16 healthy non-smokers and 16 asymptomatic chronic smokers (for both groups: 8M/8F, 30-52 years, FEV1 ≥80% pred.; ≥10 pack years for the smokers) on 2 separate visits 4-10 days apart. Soluble markers in serum and sputum were analyzed by ELISA. The differences between smokers vs non-smokers were analyzed with a t-test and variability was assessed on log-transformed data by a mixed model ANOVA.

RESULTS Analyzable sputum samples could be obtained from all 32 subjects. In both study populations neutrophils and macrophages were the predominant cell types. Serum Pulmonary Surfactant Associated Protein D had favorable reproducibility criteria for reliability ratio (0.99), intra-subject coefficient of variation (11.2%) and the Bland Altman limits of agreement. Furthermore, chronic smokers, compared to non-smokers, had significantly higher sputum concentrations of IL-8 (1094.6 pg/mL vs 460.8 pg/mL, p=0.006)), and higher serum concentrations of Pulmonary Surfactant Associated Protein D (110.9 pg/mL vs 64.7 pg/mL, p=0.019), and lower concentrations of Serum Amyloid A (1352.4 pg/mL vs 2297.5 pg/mL, p=0.022).

CONCLUSION Serum Pulmonary Surfactant Associated Protein D proved to be a biomarker that fulfilled the criteria for reproducibility in both study groups.

INTRODUCTION

Smoking is a known risk factor for the development of chronic obstructive pulmonary disorder (COPD) and asymptomatic chronic smokers (without signs of COPD) already show evidence of airway inflammation [1]. Patients should be identified as early as possible in the course of the disease and undoubtedly before disability becomes substantial. Better understanding of the early pathophysiological changes in COPD caused by smoking and the identification of relevant biomarkers may facilitate selection of appropriate molecular targets for pharmacological intervention [2]. Ideally, the identification of such biomarkers should be broadly applicable in the clinical setting and be non-invasive.

Spirometry is a non-invasive method often used as primary endpoint for efficacy in pharmacology studies as it provides information on the early physiological changes in smokers without COPD. However, it does not provide information about the underlying pathology. Sputum induction is another non-invasive method and samples cellular and biochemical constituents of the lower respiratory tract allowing study of asthma and COPD [3]. Analysis of induced sputum may focus on cell counts as an index of inflammation [4], but also sampling of soluble biomarkers in induced sputum from diseased airways has shown promise [5]. Indeed, in COPD several types of biomarkers like interleukin 8 (IL-8), leukotriene B-4 (LTB-4) and tumor necrosis factor-α (TNF-α) amongst others, have been measured in induced sputum that are thought to be related to inflammatory process and disease pathology [6]. Despite these findings, it has been recognized that only few of these biomarkers have been validated and that there is little information about reproducibility and the relationship to disease development, severity or progression [7].

Therefore, we performed a clinical study in asymptomatic chronic smokers and healthy (non-smoking) controls to test reproducibility and differences in a set of soluble inflammatory markers in the supernatant of induced sputum and in serum.

The inflammatory markers were chosen because of their presumed role in the pathogenesis of COPD. Amongst other markers, the selection included IL-8 representing T helper 1 (TH1) driven inflammation; eosinophil derived neurotoxin (EDN) and eotaxin representing TH2 driven inflammation, and serum amyloid A (SAA), and pulmonary surfactant associated protein D (SPD) as markers reflecting inflammation in general. From the same study two manuscripts dealing with methodological aspects have been published [8;9].

MATERIAL AND METHODS

STUDY DESIGN The study consisted of two identical study days separated by 4-10 days. On each study day, eligible subjects underwent in sequential order: spirometry, sputum induction and blood sample drawings. All assessments were performed at the same time of the day (± 2 H) on both study days. The study was performed

at the Centre for Human Drug Research (CHDR) in Leiden, the Netherlands and approved by the Ethics Committee of Leiden University Medical Centre, Leiden, Netherlands. All subjects provided written informed consent.

SUBJECTS Two groups of 16 subjects each were included in the study and matched for gender and age: asymptomatic chronic smokers with a smoking history of at least 10 pack-years (8F/8M) and healthy non-smokers (8F/8M). Non-smoking was defined as: no smoking for a minimum of 12 months prior to study enrolment and less than 5 pack-years of smoking history. For the chronic smoker group, the last cigarette was smoked more than one hour before any study procedure. None of the subjects in both study groups had had any history of relevant lung disease or respiratory tract infection for at least 4 weeks before the start of and during the study.

SPUTUM INDUCTION PROCEDURE Sputum induction was performed as previously described [10;11] using a DeVilbiss Ultraneb 2000 ultrasonic nebulizer (Tefa Portanje, Woerden, The Netherlands) connected to a 100-CM long plastic tube, with an internal diameter of approximately 22 MM, connected to a two-way valve (No.2700; Hans-Rudolf, Kansas City, MO, USA) with a mouthpiece. After 200 µg of salbutamol was administered, hypertonic saline (NaCl 4.5%) was nebulised and inhaled through the mouth, with the nose clipped, during three periods of 5 minutes. Before the start of the procedure and at approximately 7 minutes following each induction, spirometry was performed as a safety measure.

SPUTUM PROCESSING The cell pellet of was processed as a full sample according to guidelines [10;12], using 0.1% dithiothreitol (DTT) (Sputolysin, Calbiochem, La Jolla, CA, USA). Cell viability and total cell count were assessed using Trypan Blue; sputum samples containing > 80 % squamous cells were excluded from analysis. Leucocyte differential cell counts (eosinophils, lymphocytes, macrophage and neutrophils) were performed by a qualified cytologist on May-Grünwald-Giemsa stained coded cytospins. Cell differentials were counted on 500 nucleated nonsqamous cells and expressed as percentage.

BIOCHEMICAL ASSAYS The panel of markers measured in sputum supernatant and serum consisted of eosinophil derived neurotoxin (EDN), fibronectin, interleukin 8 (IL-8), interleukin 17, (IL-17), monocyte chemotactic protein-1 (EDN), serum amyloid A (SAA), pulmonary surfactant associated protein D (SPD), transforming growth factor beta 1 (TGF-b1), eotaxin and thymus and activation regulated chemokine (TARC). All markers were analysed with ELISA (R&D systems, Minneapolis, Minnesota, USA). For each analyte the coefficient of variation was < 10% for both inter and intra assay variability.

STATISTICAL ANALYSIS Because of the exploratory nature of the study, a power calculation to determine the sample size was not performed.
Markers were excluded from analyses if ≥50% of the dataset for a soluble marker was outside the quantification range, or when paired data were available in less than five subjects (*Table 1*).

For the purposes of this manuscript, reproducibility is defined as the consistency of results derived from the ELISA analysis of incurred samples on two independent study days. Each soluble mediator was summarized (mean) by study day and study group. Variability per soluble mediator was evaluated by intra-subject coefficient of variation (calculated by a mixed analysis of variance model) and by the reliability ratio (variance due to subjects /(variance due to subjects + variance due to residual)). Reproducibility for soluble inflammatory markers was evaluated with the values measured on study days 1 and 2 with Bland Altman method [13;14]. The mean ratio of the sample results, along with its 95% confidence interval expressed as Ratio Limit (RL), was taken as a measure for accuracy. The interval for a 25% systematic difference was 0.8 to 1.25. For measuring precision, the Limit of Agreement (LA) was calculated as LA = $e^{d \pm SD}$, where d is the mean difference between two sampling days on log-scale, and SD is the standard deviation of the differences on log-scale. LA was defined as being equivalent to mean ± SD, which indicates that about 68% of all pairs of test data would be within the acceptance range [14].

RESULTS

SUBJECTS All subjects completed both study days and all study procedures were well tolerated. There were no significant differences in demographics and baseline lung function parameters, as measured by FEV1 and FVC, between chronic smokers and non-smokers, *Table 2*.

REPRODUCIBILITY OF SPUTUM BIOMARKERS In both study populations neutrophils and macrophages were the predominant cell types, *Table 3*. No mast cells were found in any of the samples. There was no significant difference between day 1 and day 2 for any of the cell types in each study group. The intra-subject variability was the lowest for eosinophils and neutrophils in both groups. There were no significant differences in cell types between the two groups.

Analysable sputum samples could be obtained from all 32 subjects at both visits. Only 4 soluble markers, eosinophil derived neurotoxin (EDN), Interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1) and prostaglandin E2 (PGE2) met the criteria for analysis. None of those markers met the criteria for reproducibility. IL-8 was significantly higher in chronic smokers compared to non-smokers on day 1 ($p = 0.006$) and on day 2 ($p = 0.026$), *Table 4*.

REPRODUCIBILITY IN SERUM In serum 8 markers (eotaxin, fibronectin, EDN, EDN, SAA, SPD, TARC and TGFBI) met the criteria for analysis. SPD in serum was significantly higher ($p = 0.02$) in chronic smokers compared to non-smokers on both study days (Table 4) and met the criteria for reproducibility when comparing day 1 and 2 in both study groups. Eotaxin was reproducible in chronic smokers and transforming TGFBI was reproducible in non-smokers. In addition, SAA was significantly lower in chronic smokers compared to non-smokers on both study days ($p= 0.022$ and $p = 0042$, RESP.), *Table 5*.

DISCUSSION

Cellular and soluble sputum biomarkers and biomarkers obtained in serum from 16 asymptomatic chronic smokers and 16 non-smokers were measured and investigated for reproducibility. SPD concentration fulfilled the criteria for reproducibility and that chronic smokers compared to non-smokers had significant higher concentrations of IL-8 in induced sputum and lower concentrations of SAA.

SPD in serum showed a two fold increase in chronic smokers compared to non-smokers. SPD is mainly produced by type 2 pneumocytes and non-ciliated bronchiolar cells. Smoking reduces the amount of SPD in the bronchoalveolar lavage fluid, probably as a result of SPD leakage through the pulmonary epithelium into the systemic circulation, but increases SPD serum levels [15]. These increases in serum are apparently independent of age and smoking history but dependent with the degree of airway obstruction [16].

SPD is the only biomarker in this study that showed significant reproducibility in both the chronic smokers and the non-smoking group. Both the intra-subject coefficient of variation (11.2%) and the Bland Altman limits of agreement indicated reproducibility. These properties in addition to its association with exacerbations of COPD [17] and its quality to discriminate significantly between the chronic smokers and non-smokers may make it an attractive lung specific biomarker or drug target in COPD.

The higher concentrations of IL-8 in chronic smokers compared to the non-smoker group, confirmed previously reported data [5;18;19]. Indeed, inhibition of IL-8 or its receptors is a potential target for COPD treatment. Although several compounds are currently in clinical development, so far IL-8 specific antibodies have been largely ineffective in COPD. Similarly, an IL-8 receptor antagonist reduced sputum neutrophils in patients with COPD, but larger studies showed no clinical benefit [20;21].

Our finding on the serum concentrations of SAA was remarkable as non-smokers showed higher levels compared to smokers. SAA is an apolipoprotein and acute phase protein predominantly produced by the liver and it is related to neutrophil chemotaxis. In COPD patients during acute exacerbations elevated SAA levels been reported [22]. The relevance of this finding is not straight forward as also relatively trivial inflammatory stimuli can lead to rapid SAA responses [23]. Nevertheless our findings are at odds with previous reports [24;25] showing higher level of SAA in smokers, for which we have no explanation.

In this study some of the soluble sputum biomarkers were not detectable or did not differentiate between the study groups. One accountable factor includes the degradation by standard sputum processing with dithiothreitol (DTT), which destroys the disulphide bounds of these inflammatory markers [26]. Another observation is the relative neutrophilia in the differential cell count from non-smokers, while in other studies macrophages were the predominant cell type in induced sputum [27-29]. This relative neutrophilia in the non-smokers could not be explained by the influence of outliers nor a recent colds [30] as the latter was an exclusion criterion for the study. Nevertheless, it may be that the seasonal and environmental factors [31], the study was conducted in winter, are reflected in this observation. On the other hand, the predominance of neutrophils in chronic smokers, corresponds well with other studies in similar populations [5;32].

Selection of the inflammatory markers in this study was based on a hypothesis-dependent approach in which markers that may be involved in the pathophysiology of COPD were selected and analysed. An alternative strategy would have been an unbiased approach E.G. not based on known pathways only. This could for example be achieved by large scale analysis of proteins (proteomics) or metabolites (metabolomics). Such studies have shown to result in protein fingerprints, which potentially could be used to investigate the pathophysiology of COPD in more detail, stratify patients for the treatment of COPD and provide a tool to therapy response [33;34]. Despite these promising results, it is important to realize that also this type of biomarkers should properly be validated and preferably underpinned by a mechanistic understanding of the changes in these profiles, before they can be utilized reliably as markers for disease progression or assessment of treatment effects. This may become

a challenge as this paper shows that many, relatively easily obtained markers that have previously been suggested to be associated with chronic airway inflammation fall short on these basic requirements for a useful biomarker.

CONCLUSION

SPD proved to be a biomarker that fulfilled the criteria for reproducibility in both study groups. Furthermore, we found that chronic smokers compared to non-smokers had higher concentrations of IL-8 in induced sputum and SPD in serum and lower concentrations of SAA.

ABBREVIATIONS

CV	Coefficient of Variation
COPD	Chronic Obstructive Pulmonary Disease
EDN	Eosinophil derived neurotoxin
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent essay
F	Female
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
IL-8	Interleukin 8
IS	Induced sputum
LA	Limits of agreement
LTB-4	Leukotriene B-4
M	Male
EDN	Monocyte chemotactic protein-1
PEF	Peak Expiratory Flow
PGE2	Prostaglandin E2
RPM	Round per minute
RL	Ratio Limit
RR	Reliability Ratio
SAA	Serum amyloid A
SEC	Second
SD	Standard deviation
SPD	Pulmonary Surfactant Associated Protein D
TARC	Thymus and activation regulated chemokine
TGFBI	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor-α

Table 1 Overview of collected markers

Matrix	Population	Soluble Mediator	% Data >LLOQ and >ULOQ	# of Subjects with Complete Paired Data**	Included in Analysis?
Serum	Non-Smoker	EDN	96.875	15	Yes
		Fibronectin	100	16	Yes
		IL-8	6.25	0	No
		EDN	100	16	Yes
		SAA	100	16	Yes
		SPD	93.75	15	Yes
		TGFBI	100	16	Yes
		Eotaxin	100	16	Yes
		TARC	93.75	14	Yes
	Smoker	EDN	96.667	14	Yes
		Fibronectin	96.667	15	Yes
		IL-8	12.5	0	No
		EDN	96.875	15	Yes
		SAA	84.375	13	Yes
		SPD	96.667	14	Yes
		TGF_BI	93.75	14	Yes
		Eotaxin	96.667	14	Yes
		TARC	96.667	14	Yes
Sputum	Non-Smoker	EDN	100	16	Yes
		Fibronectin	6.25	0	No
		IL-17	0	0	No
		IL8	100	16	Yes
		EDN	100	16	Yes
		PGE2	100	16	Yes
		SAA	0	0	No
		SPD	34.375	3	No
		TGF_BI	0	0	No
	Smoker	EDN	96.667	14	Yes
		Fibronectin	15.625	2	No
		IL-17	0	0	No
		IL-8	62.5	8	Yes
		EDN	87.5	13	Yes
		PGE2	96.875	15	Yes
		SAA	6.25	1	No
		SPD	70	8	Yes
		TGFBI	0	0	No

Only counted subjects who had concentration values > LLOQ and < ULOQ on both Day 1 and Day 2

Table 2 Baseline demographics and clinical characteristics

	Non-smokers	Chronic smokers
Number of subjects	16	16
Age (years)	41 (30-49)	41 (32-52)
Gender (M/F)	8M/8F	8M/8F
Height (meters)	1.75 (1.56-1.93)	1.73 (1.58-1.85)
Weight (kg)	77.5 (51.8-110.2)	73.1 (46.5-100.5)
BMI (kg/m2)	25.1 (21.1-29.5)	24.3 (18.6-30.0)
FEV1 (L)	3.66 (2.75-5.16)	3.55 (2.38-4.64)
FEV1 (% predicted)	102.9 (78-119.6)	102.7 (82.1-121.1)
FVC (L)	4.78 (3.21-6.42)	4.46 (3.15–6.38)
FVC (% predicted)	105.1 (82-131)	111.7 (76-127)
FEV1/FVC	0.78 (0.59-0.91)	0.79 (0.72-0.89)
PEF (L/sec)	9.98 (7.01-13.07)	9.67 (5.08-13.50)

Values are presented as mean (range)

Table 3 Sputum cell differentials

	Non-smokers			Chronic smokers		
Sputum cell count:	**Day 1**	**Day 2**	**Intra-subject CV (%)****	**Day 1**	**Day 2**	**Intra-subject CV (%)****
leucocytes diff*						
- eosinophils %	0.3 (0.7)	0.4 (1.1)	28.9	0.6 (0.8)	0.6 (0.8)	26.7
- lymphocytes %	1.6 (1.3)	2.1 (1.1)	66.9	2.5 (2.1)	2.6 (2.4)	66.4
- macrophages %	31.6 (23.9)	29.4 (20.4)	54.3	38.3 (19.7)	32.3 (21.0)	42.0
- neutrophils %	64.2 (24.9)	65.5 (21.0)	26.8	55.5 (20.9)	61.6 (23.5)	26.4
Squamous cells %	23.3 (8.1)	29.4 (13.8)	33.5	26.9 (15.8)	24.2 (11.4)	46.8

* Values are given as mean (SD); ** CV(%): coefficient of variation

Table 4 Reproducibility of soluble markers in sputum by ELISA

Soluble Mediator	Study Day	Mean (pg/ml)		p-value	RR	CV%	Non smokers			Smokers		
		Non Smokers	Chronic Smokers			Intra-subject	LA	Mean ratio	RL	LA	Mean Ratio	RL
EDN	1	49.989	108.35	0.1360	0.78	45.7	0.43; 1.56	0.82	0.59; 1.13	0.47; 1.43	0.82	0.61; 1.10
EDN	2	67.394	95.542	0.2860								
IL8	1	460.75	1094.6	0.0060	0.53	67.5	0.27; 1.85	0.71	0.44; 1.14	0.43; 1.84	0.89	0.61; 1.29
IL8	2	654.82	1193.1	0.0260								
EDN	1	257.41	513.75	0.1420	0.81	51.6	0.60; 1.76	1.03	0.79; 1.35	0.56; 1.98	1.06	0.74; 1.50
EDN	2	247.35	326.29	0.3170								
PGE2	1	2088.7	1926.8	0.5190	0.17	27.1	0.73; 1.58	1.07	0.89; 1.30	0.64; 1.40	0.95	0.78; 1.16
PGE2	2	1896.5	1975.9	0.6260								

RR = reliability ratio; CV= coefficient of variation; RL = Ratio Limit; LA = Limit of Agreement

Table 5 Reproducibility of soluble markers in serum by ELISA

Soluble Mediator	Study Day	Mean (pg/ml)		p-value	RR	CV%	Non smokers			Smokers		
		Non Smokers	Chronic Smokers			Intra-subject	LA	Mean ratio	RL	LA	Mean Ratio	RL
EDN	1	33.19	45.291	0.2960	0.60	61	0.78; 1.44	1.06	0.90; 1.24	0.75; 1.46	1.04	0.86; 1.27
EDN	2	29.917	44.693	0.1000								
Eotaxin	1	156.03	161.83	0.8120	0.68	23	0.60; 1.35	0.90	0.74; 1.10;	0.83; 1.22	1.01	0.90; 1.13
Eotaxin	2	163.19	160.5	0.9010								
Fibronectin	1	87.359	112.44	0.0810	0.43	31	0.62; 1.28	0.89	0.74; 1.07	0.68; 1.80	1.11	0.83; 1.46
Fibronectin	2	99.395	98.533	0.9560								
EDN	1	340.54	337.52	0.9420	0.54	26	0.71; 1.25	0.94	0.81; 1.08	0.64; 1.64	1.02	0.79; 1.33
EDN	2	350.76	358.39	0.9070								
SAA	1	2297.5	1352.4	0.0220	0.74	33	0.71; 1.48	1.02	0.85; 1.23	0.63; 1.93	1.10	0.81; 1.50
SAA	2	2315.7	1348.1	0.0420								
SPD	1	64.654	110.87	0.0190	0.99	11	0.92; 1.22	1.06	0.98; 1.14	0.80; 1.11	0.94	0.85; 1.03
SPD	2	62.435	111.47	0.0180								
TARC	1	388.16	444.02	0.5840	0.70	49	0.78; 1.17	0.96	0.86; 1.07	0.87; 1.31	1.07	0.95; 1.20
TARC	2	401.77	410.6	0.9260								
TGFBI	1	27756	28859	0.6770	0.09	124	0.83; 1.16	0.98	0.90; 1.07	0.21; 13.0	1.65	0.52; 5.18
TGFBI	2	28326	28503	0.9600								

RR = reliability ratio; CV= coefficient of variation; RL = ratio limit; LA = limit of agreement

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SECTION II

CLINICAL STUDIES WITH A NEW ANTI-ASTHMATIC DRUG

CHAPTER 6

UTILIZING AN ADAPTIVE TRIAL DESIGN TO ASSESS THE EFFECTS OF THE COMBINED PHOSPHODIESTERASE 3/4 INHIBITOR RPL554

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Efficacy and safety of RPL554, a dual PDE3 and PDE4 inhibitor, in healthy volunteers and in patients with asthma or chronic obstructive pulmonary disease: findings from four clinical trials

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ABSTRACT

RPL554 is a phosphodiesterase-3/4-inhibitor with bronchodilation, bronchoprotective and anti-inflammatory properties in animals. These characteristics were investigated for the first time in humans using an adaptive proof-of-concept-study. After dose escalation with RPL554 in healthy subjects, a potentially effective starting dose was chosen to investigate the protective effects against methacholine induced bronchoconstriction and to explore the effects on FEV1 in asthmatics. The anti-inflammatory properties were assessed using a nasal allergen challenge in rhinitics. In asthmatic patients RPL554 resulted in substantial bronchodilation at doses between 0.009 and 0.072 MG/KG with a mean increase at 1 hour of 520 ML (95%CI: 320; 720 ML) at 0.018 MG/KG. Although increases in eosinophil count after the allergen challenge was lower in the PRL554-treated group compared to placebo, 7.1% ($p > 0.1$, 95%CI -2.2; 16.4%), anti-inflammatory properties of RPL554 could not statistically be established. This study demonstrates the bronchoprotective and bronchodilative properties of RPL554 in asthmatic patients using a swift and efficient clinical development approach.

INTRODUCTION

Maintenance therapy with inhaled corticosteroids (ICS) supplemented with β_2-agonist are the cornerstone for the treatment of asthma [1]. Although treatment with ICS results in a decrease of asthma related symptoms and improve lung function, even the highest doses of ICS do not fully suppress the airway inflammation in all asthma patients [2;3] leaving many patients relatively uncontrolled [4]. Therefore, there is a continuous search for new effective therapeutic options for asthma treatment.

Inhibition of phosphodiesterase (PDE) seems an attractive drug target particularly when it would be possible to utilize the unique tissue distribution and resultant biological effects which characterized this family of enzymes [5]. Specifically, inhibition of bronchial PDE3 produces bronchodilation in patients with asthma [6;7], and PDE4 inhibition would have anti-inflammatory properties and relax airway smooth muscle, which are all involved in the pathophysiology of chronic inflammatory diseases like asthma [5]. It seems attractive to develop dual inhibitors as it has been suggested that dual inhibition results in synergetic effects [8;9]. This concept has been tested clinically before with a few dual PDE3/4 inhibitors of which zardaverine is best known [10;11]. Although zardaverine showed bronchodilation and anti-inflammatory effects in animal models, it only showed modest bronchodilation of short duration after inhaled administration in humans and resulted in CNS and gastrointestinal side effects [12;13].

Classically first in human trials with novel drugs are conducted in healthy volunteers using single and multiple ascending dose (SAD/MAD) designs with a focus on pharmacokinetics, safety and tolerability. This phase is then followed by SAD/MAD studies in patients of the target populations in which also a first impression of the intended pharmacology is obtained. It would appear that such an approach with an inhaled, locally acting, compound is not necessarily informative. It is likely that a study design which combines the objectives mentioned above will be more informative and cost-effective. We explored such an approach with the novel PDE3/4 inhibitor RPL554 which is developed for patients with asthma and COPD. RPL554 is a trequinsin analogue with low human IC50 values for PDE3 (400 PM) and PDE4 (1.5 μM) that has shown bronchodilation, bronchoprotective and anti-inflammatory properties in animal models of allergen-induced asthma and rhinitis [14].

The purpose of this first in human study was to identify a potentially effective dose and to assess in an early stage whether RPL554 possesses bronchodilative, bronchoprotective and anti-inflammatory properties in patients with allergic asthma and rhinitis. To this purpose a step by step adaptive design was used. First, a safe dose of RPL554 was selected in healthy volunteers. Then, the potentially effective dose was selected in patients with allergic asthma. Finally, RPL554 effects were evaluated in more depth in both patients with allergic asthma and patients with allergic rhinitis.

METHODS

DESIGN ❋ In this study healthy volunteers and patients with allergic asthmatic and allergic rhinitis were administered a single dose of nebulized RPL554, *Table 1*. In two groups of 9 healthy volunteers, the safety and pharmacokinetics of 2 single doses of nebulized RPL554 (0.003 and 0.009 MG/KG) were sequentially evaluated. The highest dose was established as safe, and was used as the starting dose in two groups of 3 allergic asthmatics who received a single dose of nebulized RPL554 0.009 MG/KG or 0.018 MG/KG. Efficacy in this group was defined as an increase in PC20MCH of at least 1.5 doubling dose post dose compared to baseline.

The highest established effective single dose of nebulized RPL554 (0.018 MG/KG) was studied in more depth in a subsequent group of 10 patients with mild allergic asthma in 10 patients with allergic rhinitis. In asthmatic patients efficacy was assessed using change in FEV1 and PC20MCH, and in patients with allergic rhinitis,

efficacy was assessed by the inflammatory cell response following a nasal allergen challenge, sampled by a nasal brush. Finally, safety and bronchodilation effects of two higher doses RPL554 (0.036 and 0.072 MG/KG) versus placebo, were sequentially evaluated in two groups of 10 asthmatic patients. Details of the study characteristics are provided in *Table 1*.

All subjects were assessed for eligibility during a screening session. In asthmatics who received 0.009 and 0.018 MG/KG RPL554, a methacholine challenge was performed at 1 hour after study drug administration and in rhinitics, a nasal allergen challenge was performed at 40 minutes after inhalation.

Planned interim analysis after the completion of the first dosing groups (0.003, 0.009 and 0.0018 MG/KG RPL554) were conducted to assess the initial safety and clinical effects of RPL554 and to establish the dose for the next stage. The Medical Ethics Committee of the Leiden University Medical Centre approved the study protocol. The study was performed according to Good Clinical Practice (GCP) and in accordance with the International Conference on Harmonisation (ICH) guidelines.

SUBJECTS ❦ All volunteers were non-smoking males or ex-smokers (> 6 months abstinence and < 10 pack year) and did not suffer from any significant disease. The healthy volunteers used no medication and had a normal lung function; forced expiratory volume in one second (FEV1) and FVC > 90% of predicted and the FEV1/FVC ratio > 80%. Asthmatic patients had mild to moderate persistent clinically stable allergic asthma, require only treatment with short acting β_2-agonists on an as needed basis. Also, they required a FEV1 of > 70% of predicted, a positive provocation test with a fall in FEV1 of > 20% (PC20MCH) after < 4 MG/ML methacholine bromide and a positive skin prick test (SPT > 3mm) for at least one allergen (grass or tree pollen, house dust mite, D. Farinae, cat, dog, or horse-dander, Aspergillus Fumigatus, A. Alternata or Artemisia Vulgaris). Furthermore, FEV1 values had to be within 10% of each other on all study days and no history of respiratory tract infections at least 3 weeks before enrollment or during the study. Rhinitic patients had a history of allergic rhinitis for at least 6 months, a SPT > 3 MM for house dust mite, pollen and or cat, and a Lebel composite symptom score of > 6 following intranasal allergen exposure. The asthma and rhinitis patients had not, except for inhaled short acting β2-agonists, used concomitant anti-asthma or anti-allergy medication for at least 2 weeks.

TREATMENT ❦ RPL554, a 9,10-Dimethoxy-2-(2,4,6-trimethylphenylimino)-3-(Ncarbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2H-pyrimido[6,1-a]isoquinolin-4-one powder was diluted in a citrate-phosphate buffer of pH 3.2. The same buffer was used for placebo. Although the The Food and Drug Administration guidelines [15] for first-in-man studies yielded a maximum starting dose of 0.01 MG/KG, a starting dose of 0.003 MG/KG was selected, as this was assumed to be the initial pharmacological active dose.

Drug administration took place by nasal inhalation of nebulized RPL554 through a facemask (Clement-Clarke adult oro-nasal face mask part no. L3605518), connected to a calibrated electronic nebulizer (Clement-Clarke International AC4000, UK; output: 7L/MIN at 1.00 bar) covering nose and mouth, while the subjects breathed normally through the nose. The nebulization time was 10 minutes for the lower dosing groups (0.003 - 0.018 MG/KG RPL554), and 12 and 24 minutes respectively for the higher dosing groups (0.036 and 0.072 MG/KG RPL554).

SAFETY ❦ Safety evaluation performed during the study to assess RPL554's safety and tolerability consisted of the following safety measures: adverse events, physical examinations, blood biochemistry, haematology and coagulation (analysed at the Leiden University Medical Center), ECG, blood pressure and heart rate measurements. Blood pressure and heart rate measurements were conducted by automated oscillometric equipment (BMK-1101K; Nihon Kohden, Japan, or Dash 4000; GE Healthcare, USA). Twelve-lead ECG recordings were made using Electrocardiograph Marquette 5000/5500 (USA) and in addition continuous ECG monitoring were performed using an H12 recorder, (Mortara Instrument GMBH, Germany) in the lower dose groups.

PHARMACOKINETICS ❦ To investigate the pharmacokinetics of RPL554, plasma samples were drawn at regular intervals (at t = baseline and 15, 20, 30, 45 MIN and 1, 2, 3, 4, 8, 24 hours) in 4 ML EDTA tubes, centrifuged within one hour for 10 m at 4 C, 2000G, and stored for further analysis at -70-80C. RPL554 plasma concentrations were assayed using HPLC methods by ABL bv, Assen, The Netherlands. The pharmacokinetic analyses were performed by GBPK Consulting LTD, UK.

PHARMACODYNAMICS

PULMONARY FUNCTION TESTS ❦ Lung function was measured using the Vmax 20C pulmonary spirometer (SensorMedics, USA) at screening and during MCH challenges. A portable spirometer ZAN 100 (Accuramed, Belgium) was used during study days. At approximately 15 and 5 minutes predose, baseline FEV1 measurements were performed and calculated as the average of the 2 highest of 3 FEV1 measurements (within 5%). Post-dosing measurements were performed in duplicate of which the highest, technically satisfactory FEV1 was included into analysis.

METHACHOLINE BROMIDE (MCH) CHALLENGES A methacholine challenge determines the degree of non-specific bronchial reactivity and was used as a measure for bronchoprotective effects in this study. MCH challenges were performed as previously described [16] and according to a standardized protocol [17], at screening and 60 minutes after dosing in the groups of allergic asthmatics who received 0.003, 0.009 and 0.018 MG/KG RPL554. In short, three technically satisfactory FEV1 measurements within 5% were performed pre and post diluent; the mean of the 2 highest FEV1 values was included in analyses. The post diluent baseline had to be within 10% of the pre diluent baseline. Methacholine bromide (Janssen Pharmaceutica, Belgium) was aerosolized by a DeVilbiss 646 jet nebulizer (output 0.13 ML/MIN, Devilbiss Healthcare Inc., Somerset, USA) and inhaled during a 2 minute tidal breathing with the nose clipped shut. Consecutive MBR doses (0.0625-16 MG/ML) were inhaled at 5 minutes intervals and airway response was measured in duplicate following each dose (and expressed as percentage decline from post-diluent baseline FEV1). The procedure was discontinued after a fall in FEV1 of at least 20% from baseline occurred, or the highest permissible dose had been inhaled. Contrary to the routine procedure, rescue medication was not given at the end of the methacholine challenge at study days to enable estimation of the difference in FEV1 recovery following RPL554 versus placebo pretreatment.

NASAL ALLERGEN PROVOCATION TEST The nasal challenge, which investigates the pathophysiological response in patients with allergic rhinitis after provocation with an allergen, was performed as previously described [18] in allergic rhinitics and was conducted according to a standardized method [19;20]. In short, the choice of the provocative allergen (HDM, grass pollen, or cat dander) was based on the size of the wheal response obtained in the SPT in combination with the clinical symptoms and seasonal time. Before the allergen challenge, the allergen's diluent (phosphate-buffered saline containing human serum albumin 0.03% and benzoalkonium-chloride 0.05%, ALK Abelló) was sprayed into both nostrils (1 puff = 0.125 ML/nostril), and subsequently the allergen (in up to 3 consecutive doses of 100, 1,000 and 10,000 Biological Unites per ML) at 10 minutes intervals. The nasal response was quantified pre-challenge, 10 minutes after each dose and at regular intervals up to 24 hours afterwards, by the validated Lebel composite symptom scoring system [21]. The Lebel score describes and grades in allergen induced upper airway response, including: sneezing, rhinorrhoea, nasal blockage, nasal and ocular symptoms and pruritus, and consists of a linearly additive scale (0-3 points per symptom). Allergen dosing was discontinued if a Lebel score of 6 or more was reached. The nasal challenge was performed at screening and at 40 minutes after inhalation of the study treatments using the same cumulative doses of allergen that had been shown to evoke an allergen-induced early upper airway response at screening (I.E. a Lebel score of at least 6).

NASAL BRUSH In rhinitics, nasal brushes were obtained from the same nostril at the following times: baseline and 7 and 24 hours post-allergen, using a plastic nasal brush (Buccal Swab Brush, Biozym TC, the Netherlands). Immediately upon brushing, the brush was deposited in a 3 ML plastic tube containing 2 ML PBS (at room temperature). Subsequently, the cell suspension was centrifuged for 5 minutes at 390 G (1500 RPM) at room temperature, and mixed with of Trypan Blue and dried at room temperature. The differential counts were made by an experienced cytopathologist and expressed as a percentage of a sample of at least 250 nucleated non-squamous cells.

ENO In rhinitics ENO measurements were performed 10 MIN pre dose and 1.5 and 7 hours post dose and executed according to current guidelines [22] using the MINO (Aerocrine AB, Solna, Sweden). Subjects were sitting in upright position and wearing a nose clip. They inhaled NO-free air and subsequently exhaled for approximately 10 seconds. The first technically acceptable measurement was used for analysis. The results were expressed in parts per billion (PPB).

STATISTICAL ANALYSIS One rhinitis patient completed the first period only. Therefore, his results were included in the safety analysis but excluded from the pharmacokinetic and pharmacodynamic analysis. As the first asthma patient in each of the highest dose group (0.036 and 0.072 MG/KG), were treated with RPL554 (open label), their pharmacodynamic data were not included into the analysis.

PHARMACOKINETICS All pharmacokinetic parameters were calculated using non-compartmental analysis and using NCA, WinNonLinTM version 5.2, Model 200. CMAX and TMAX were determined from visual inspection of the plasma concentration-time profile. The area under the plasma concentration-time curve from zero to the time of the last quantifiable plasma concentration, AUC_{0-t}, was calculated using the linear and log trapezoidal rule for the upswing and downturn in plasma concentration respectively. The rate constant of the slowest disposition phase (λz) was calculated by log-linear regression of the terminal portion of the concentration-time profile. The plasma concentration-time curve from time zero to infinity (AUC) was determined by using λz to extrapolate AUC_{0-t} to infinity. Total apparent drug clearance (CL/F) and the volume of distribution (Vz/F) were estimated by dividing

the dose by AUC, and product of λz and AUC, respectively. The terminal half-life (T½) was derived from the equation ln(2)/λz. The exact dose nebulized by the subject was estimated by multiplying the MG/KG dose by the patient's weight.

PHARMACODYNAMICS The pharmacodynamic end point were PC20MCH and FEV1 in the groups of asthmatics who were receiving 0.009 and 0.018 MG/KG RPL554, FEV1 in the groups of asthmatics who were receiving 0.036 and 0.072 MG/KG, and cellular infiltration in the group of rhinitics.

In all stages, descriptive statistics (mean, median, standard deviation (SD), minimum and maximum) were used for numerical data, and counts and percentage of subjects in each category were used for qualitative data. FEV1, nasal brush eosinophils and neutrophils, exhaled NO and Lebel data were compared with a mixed model analysis of variance with fixed factors: treatment, period, time and treatment by time, random factors: subject, subject by treatment and subject by time and the average pre-value (average over baseline values before dosing and set at time=0) as covariate. eNO data was log transformed before analysis. PC20MCH doubling dose data were compared with a mixed model analysis of variance with fixed factors: treatment and period and random factor: subject. The contrast for RPL554 versus placebo was calculated and the data was presented as Least Square Means (LSMS) with 95% confidence intervals and the p-value of the contrast. All calculations were performed using SAS for windows V9.1.2 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

DEMOGRAPHICS A total of 65 subjects participated in the study, *Table 2* and 64 subjects completed the study. From the rhinitis patients, 7 out of 10 patients had concomitant clinically stable mild persistent asthma. All asthmatic subjects remained stable throughout the study (FEV1 within 10% of baseline value).

SAFETY AND TOLERANCE Inhaled RPL554 was well tolerated in healthy volunteers, allergic asthmatics and allergic rhinitics. The most frequently reported adverse event included nasal congestion (n = 11), headache (n = 8), somnolence (n = 7) and rhinorrhea (n = 4). These adverse events were reasonably equally distributed amongst RPL554 and placebo and amongst the different study populations. All adverse events reported in the course of the study were mild in severity, short lasting and self-limiting. In addition, no clinically relevant changes were observed in ECG, physical examination and laboratory safety assessment. RPL554 0.036 and 0.072 MG/KG increased the average heart rate compared to placebo with 3.1 beats per minute (BPM) (p = 0.45, 95%CI:-5.4; 11.5) and 8.9 BPM (p = 0.033, 95%CI: 0.8; 17.0) in asthmatics, respectively.

PHARMACOKINETICS In healthy volunteers, inhalation of a single dose of RPL554 0.003 MG/KG resulted in plasma concentrations below the lower limit of quantification in all 6 subjects and after 0.009 MG/KG RPL554, plasma concentrations could be measured in 4 out of 6 subjects for a short period after dosing, precluding detailed analysis. In asthmatics and rhinitics who received 0.018 MG/KG RPL554, plasma concentrations could be measured up to at least 8 hours, and at doses of 0.036 MG/KG and 0.072 MG/KG plasma concentrations of RPL554 were measured up to 24 hours. *Figure 1* shows the RPL554 plasma concentrations time graph, and pharmacokinetic parameters are listed in *Table 3*.

PHARMACODYNAMIC

BRONCHOPROTECTION In asthmatics, bronchoprotective action of inhaled RPL554 as measured by the PC20 methacholine challenge test (PC20MCH) showed an average increase of 1.9 and 1.3 doubling doses for RPL554 0.009 MG/KG and 0.018 MG/KG respectively, compared to baseline. Within each dose group, 2 out of 3 patients showed an increase in PC20MCH of at least 1.5 doubling doses. In view of the fact that a dose of 0.018 MG/KG RPL554 was also well-tolerated and raised no safety concerns, this dose was further evaluated in a placebo-controlled manner in allergic asthmatics. In these patients, this dose led to an increase of approximately 1.46 doubling doses change from baseline (p = 0.004, 95%CI: 0.63; 2.28) compared to placebo, *Figure 2*.

BRONCHODILATION Across all dose groups in asthmatic patients, progressive increase in FEV1 was observed starting shortly after the inhalation of RPL554 and reached a maximum after approximately 0.5-2 hour (*Figure 3*). In the group of asthmatics who received 0.018mg/KG RPL554, the maximum increase in FEV1 was reached at approximately 60 minutes after dosing at which a planned methacholine challenge influenced the FEV1 results from that point onwards. In the group of allergic rhinitics, in which no methacholine challenge was performed, bronchodilation was observed for a more extended period. In asthmatics who received 0.036 MG/KG and 0.072 MG/KG RPL554 and no concomitant methacholine challenge, bronchodilation was present up to 6 hours after dosing. The bronchodilation properties are provided in *Figure 4* and *Table 4*.

ANTI-INFLAMMATORY EFFECTS Although increase in eosinophils and neutrophils cell count induced by the allergen challenge at the 7 hour after dosing in allergic rhinitics was less under RPL554 (0.018mg/KG) compared to placebo (*Figure 5*), this did not reach statistical significance; an estimated difference between placebo and RPL554 of 7.1% (p > 0.1, 95% CI: -2.2; 16.4%) and of 9.7% (p = 0.3, 95%CI: -10.5; 29.8%) was observed for eosinophils and neutrophils cell counts respectively. While ENO values as measured in rhinitics appeared to be increased after RPL554 exposure at 24 hrs after the nasal allergen challenge (*Figure 5*), the estimated difference of 6.3% (p = 0.40, 95%CI: -9.3; 24,6%) between RPL554 and placebo did not reach statistical significance. Nasal allergen challenge in rhinitics with a relevant allergen, evoked an early nasal response on the Lebel score in all patients in the presence of RP554 or placebo. No statistical significance could be reached between placebo and RPL554 at any point in time after the nasal allergen challenge (*Figure 5*); the estimated difference for the change from baseline between the treatments over the entire observation was 0.2 points (p = 0.4, 95%CI: -0.4; 0.9).

DISCUSSION

This study showed that inhalation of the dual PDE3/4 inhibitor RPL554 is well tolerated up to a dose of 0.072 MG/KG. Mild and transient nasal congestion and rhinorrhea were observed, but these adverse events are likely to be related to the process of nasal inhalation of a nebulized substance and are not necessarily related to RPL554. The observed increase in heart rate during the administration of the highest RPL554 could be considered as a PDE-inhibitor-class-specific adverse event, as it possibly reflects a sympathetically mediated homeostatic reflex response occurring because of vascular smooth muscle relaxation, a well-known effect of PDE3 inhibition. In general, RPL554, administered by inhalation through the nose was well tolerated and did not raise any general or specific safety concerns. These findings contrast with previous studies that documented serious toxicities after administration of oral PDE4 inhibitors. Abdominal complaints such as nausea, emesis, loose stools, loss of appetite and weight loss are some of the most reported side effects of roflumilast and cilomilast noted in clinical trials involving asthma and COPD [5]. The serious (gastrointestinal) side-effects occurring after multiple dosing with MK0359, a highly selective PDE4 inhibitor, have resulted in cessation of its development [23]. The absence of gastro-intestinal side effect of RPL554 can be explained by the fact that the compound was administered via the nasal route and the compound's pharmacokinetic characteristics. The pharmacokinetics are characterized by rapid increase in plasma concentrations, a large apparent volume of distribution (exceeding 2500 L), a fast apparent clearance (exceeding 550 L/H) and a systemic terminal half-life of approximately 3 to 4 hours. Furthermore the mean CMAX plasma concentrations were in the range of 2-3 NG/ML for the higher dose groups. In the lowest dose groups RPL554 plasma concentration were even below the level of detection. These data suggest that systemic exposure after inhalation of RPL554 is low.

As expected the systemic exposure of nebulized RPL554 was low, but the exposure in the lungs resulted in substantial bronchodilation and bronchoprotection in allergic asthmatics and rhinitics with concomitant asthma. With regard to bronchoprotection, a dose of RPL554 of 0.018 MG/KG resulted in an increase of at least 1.5 doubling doses in PC20MCH allergic asthmatics, thereby reducing bronchial hyperresponsiveness to inhaled methacholine. This increase in PC20MCH seems superior to the bronchoprotective effects of the PDE 3 inhibitor cilostozal (100 MG), which is less than one doubling dose in PC20MCH after a single oral [24], and compares well to the protection obtained from 4 days B.I.D. inhaled salmeterol (50µg) and salbutamol (100µg) [25;26], demonstrating that a single dose of RPL554 shows potent bronchoprotection after inhalation.

Also the bronchodilation that we observed in allergic asthmatics was substantial; the maximum increase at approximately 1 hour after RPL554 nebulization (0.018 to 0.072 MG/KG) ranged from 310 to 520 ML. Increases of this magnitude can be classified as significant and are similar to reported bronchodilation effects of salbutamol [27;28]. Although the study design slightly differed among the three higher dosing groups, I.E. cross over in the RPL554 0.018 MG/KG group and a parallel design in the 0.036 and 0.073 MG/KG group, it might be that the maximum dose effect relationship at one hour after nebulization had already been reached at the 0.018 MG/KG RPL554 dose. Any conclusions about the duration of the bronchodilation effects after nebulizing 0.018 MG/KG of RPL554 are hard to establish in allergic asthmatics since a methacholine test was performed after 1 hour after RPL554 inhalation in this group. However, in the higher dose groups the bronchodilation effect lasted for at least 6 hours after inhalation of a single dose which compares favorably to the duration of inhaled salbutamol.

While the bronchoprotective and consistent bronchodilation properties in allergic asthmatics were obvious, the anti-inflammatory effects after nasal allergen challenge and effects on ENO as allergic rhinitics were less pronounced. We observed mitigated inflammatory response for RPL554 compared to placebo, but these effects did not reach statistical significance. Although Schmidt et al. suggested anti-inflammatory effects in allergic rhinitics (BM 2001) of PDE4 inhibitors [29], our results might be hampered by the small sample size, the fact that the study did not take

place in an allergen free season, and especially for the eosinophilic cell count the relatively large variability in the outcome measures. The findings may also be explained by the notice that anti-inflammatory effects may only occur after multiple dosing [29]. Thus, our data suggest anti-inflammatory effects of RPL554, but this property has to be investigated further.

The applied adaptive study design of the present study ascertained an efficient and swift early clinical evaluation of RPL554 which not only established its tolerability and pharmacokinetics, but also its pharmacodynamic properties. The advantage of the applied adaptive approach is the selection of a safe and potentially effective dose in a small number of subjects first, before administering the compound to a bigger group of patients, leading to an increased likelihood of finding a pharmacodynamic response in these groups. Although multiple doses of RPL554 were studied, the optimal dose selection remains uncertain. It appears that maximal bronchodilation effects of RPL554 were already reached at dose of 0.018 MG/KG while only limited dose effect information was obtained in lower dose ranges, possibly leading to the need of an additional dose finding study. It should be noted that although an increase in PC20MCH was established for RPL554, the bronchoprotective quality of RPL554 could be investigated further with E.G. an allergen challenge model, being a more robust method for the evaluation of anti-inflammatory properties and controller therapy potential [30]. Furthermore, the effect on tolerance and safety with multiple dosing of RPL554 should be investigated, ideally before any further investigations on RPL554 controller therapy potentials are initiated, as the present study involve single dosing only.

In conclusion, single doses of nasally inhaled RPL554 over a dose range of 0.003 to 0.072 MG/KG were shown to be safe in all subjects who enrolled in the study. The data show clinically relevant bronchodilative and bronchoprotective effects of RPL554 in allergic asthma. This study demonstrates a swift and efficient approach for the clinical development of this new PDE3/4 inhibitor. Further studies with multiple dosing and/or allergen challenge studies are warranted.

Table 1 Summary of study characteristics

RPL554 DOSE	0.003 mg/kg & 0.009 mg/kg	0.009 mg/kg & 0.018 mg/kg	0.018 mg/kg	0.036 mg/kg & 0.072 mg/kg
Population	Healthy volunteers	Allergic asthmatics	Allergic asthmatics & Allergic rhinitics***	Allergic asthmatics
Design	randomized		randomized	randomized
	2 cohorts, parallel	2 cohorts, parallel	cross over*	parallel
	placebo controlled		placebo controlled	placebo controlled**
	double blind	open label	double blind	double blind
Placebo/RPL554 ratio per dose group	3/6	NA	NA	3/7

* Cross over design including four week wash out periods; ** First patient was open label on RPL554 and therefore not used for comparison; *** Seven subjects with allergic rhinitis also had allergic asthma; NA: not applicable

Table 2 Summary of subject characteristics

RPL554 DOSE	0.003 mg/kg & 0.009 mg/kg	0.009 mg/kg & 0.018 mg/kg	0.018 mg/kg	0.036 mg/kg & 0.072 mg/kg
N. subjects	9 & 9	3 & 3	10 & 10	10 & 10
Population	Healthy volunteers	Allergic asthmatics	Allergic asthmatics & Allergic rhinitics**	Allergic asthmatics
Age (years, range)*	24 (18-41)	25 (22-29)	29 (19-50)	26 (19-38)
BMI (kg/m2) *	23 (3)	23 (3)	25 (3)	25(4)
FEV1 (l) *	4.81 (0.58)	4.28 (0.81)	4.33 (0.82)	4.12 (0.74)
FEV1 predicted (%)*	105 (10)	87 (15)	94 (11)	92 (14)
PC20MCH mg/ml*	NA	0.53 (0.87)	0.28 (0.46) &NA	1.60 (1.46)

All value are obtained at screening; * Mean (SD); PC20MCH was not measured in allergic rhinitics. Seven subjects out of ten with allergic rhinitis also had allergic asthma; NA: not applicable

Table 3 Pharmacokinetic parameters for RPL554

Parameter	RPL554 dose						
	0.003 mg/kg	0.009 mg/kg	0.009 mg/kg	0.018 mg/kg	0.018 mg/kg	0.036 mg/kg	0.072 mg/kg
n	6	6	3	13	10	7	7
TMAX (hours)	NQ	NQ	0.17	0.17	0.17	0.20	0.32
CMAX (ng/ml)	NQ	NQ	0.96	2.21	1.8	4.3	4.2
VZ/F (CV) (l)	NQ	NQ	1688 (10%)	3107 (39%)	2747 (38%)	2888 (29%)	3492 (45%)
CL/F (CV) (l/h)	NQ	NQ	553 (15%)	597 (41%)	626 (42%)	470 (23%)	586 (36%)
AUCINF (CV)(ng.h/ml)			1.16 (16%)	2.79 (35%)	2.74 (32%)	6.3 (21%)	10.8 (21%)
T ½ (CV) (hours)	NQ	NQ	2.1 (7.0%)	3.9 (36%)	3.29 (45%)	4.2 (13%)	4.1 (17%)

All values are mean values except for tmax for which median values are used; NQ: not quantifiable; CV: coefficient of variation expressed in percentage.

Table 4 Bronchodilation properties of RPL554

dose RPL554	0.018 mg/kg	0.018 mg/kg	0.036 mg/kg	0.072 mg/kg
Population	Asthmatics	Rhinitics**	Asthmatics	Asthmatics
FEV1 (ml)* RPL554 vs placebo at 1 hour post dose	520 ml p < 0.0001, 95%CI: 320; 720 ml	NA	420 ml p=0.002 95% CI: 170; 670 ml	310 ml p=0.016 95%CI: 60; 560 ml
FEV1 (ml)* RPL554 vs placebo over 24 hour post dose	200 ml P = 0.006 95%CI: 80; 320 ml	200 ml P = 0.004 95%CI: 90; 310 ml	150 ml P = 0.13 95%CI: -50; 360 ml	150 ml P = 0.13 95%CI: -50; 360 ml

* Measured by Least Square Means ; ** Seven out of ten rhinitic patients had concomitant asthma;
NA: Not applicable

Figure 1 RPL554 plasma concentrations up to two hours post dose, with SEM error bars for the two highest doses

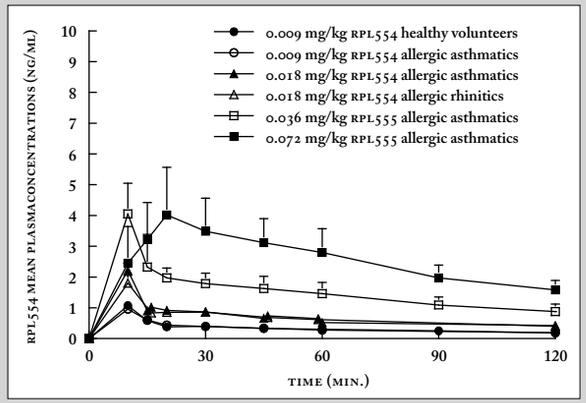


Figure 2 Allergic asthmatics: PC20MCH change in doubling dose with SEM, ✾ p= 0.004 versus placebo

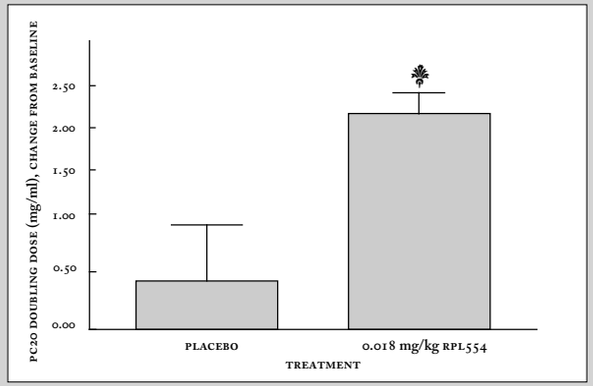


Figure 3 FEV1 (L) change from baseline as LSMs with SEMs error bars

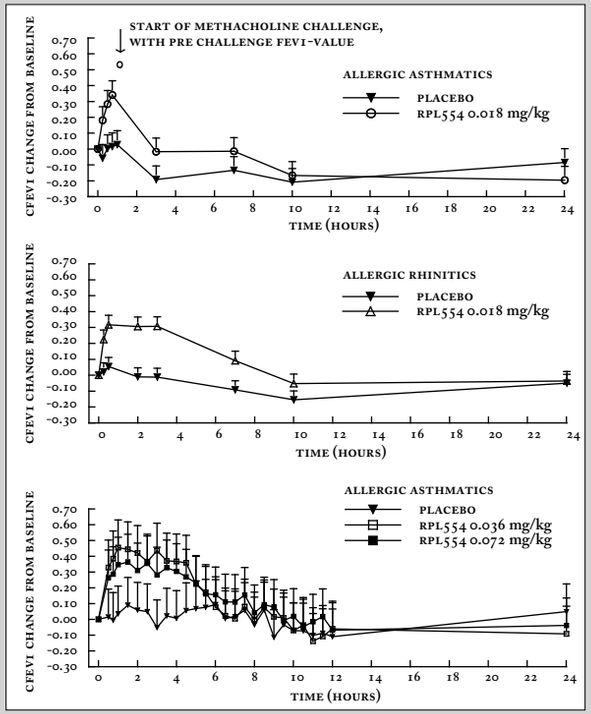


Figure 4 Box whisker plot for the maximum change in FEV1 from baseline (with median, inter quartile range, 5th and 95th percentile range) for different doses of RPL554 in allergic asthmatics. The 0.018 mg/kg RPL554 dosing group includes the 7 patients with allergic rhinitis who were also suffering from allergic asthma

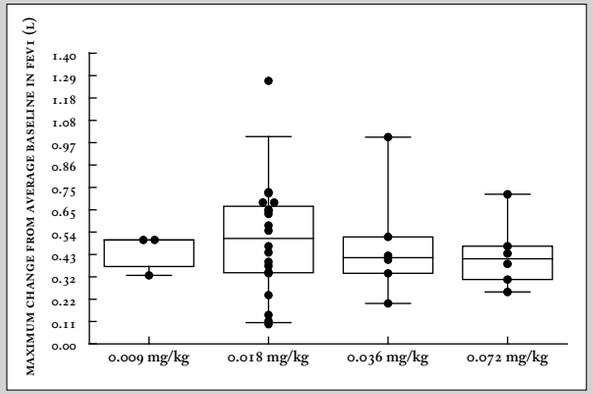
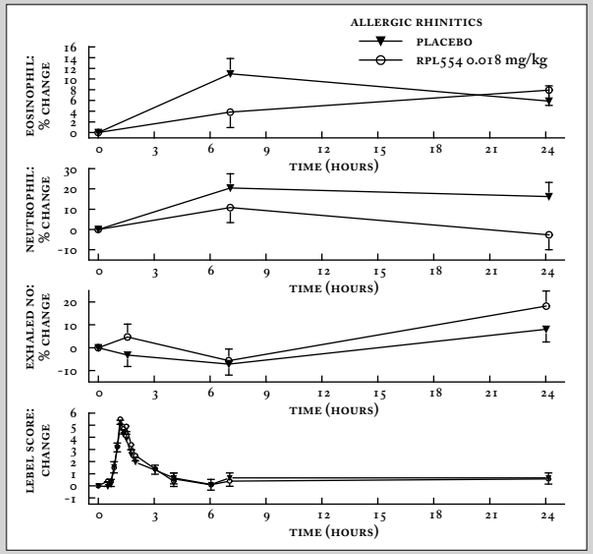


Figure 5 Inflammatory markers in allergic rhinitics, change from baseline as LSMs with SEMs error bars



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CHAPTER 7

REPEATED DOSING OF RPL554, A NOVEL INHALED PHOSPHODIESTERASE 3 /4 INHIBITOR, ELICITS SUSTAINED BRONCHODILATOR EFFECTS IN ALLERGIC ASTHMATICS

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Efficacy and safety of RPL554, a dual PDE3 and PDE4 inhibitor, in healthy volunteers and in patients with asthma or chronic obstructive pulmonary disease: findings from four clinical trials

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ABSTRACT

AIM To investigate the safety, tolerability and effect of repeated daily doses of nebulized RPL554, a dual phosphodiesterase 3/4 inhibitor.

METHOD Thirteen clinically stable allergic asthmatics received placebo on the first day and subsequently nebulized RPL554 (0.018 MG/KG) for 6 consecutive days in a single blind manner. FEV1 was measured during 6 hours after administration.

RESULT The mean estimated half-life of RPL554 in plasma was 7.4 hours. The median (range) value for accumulation on Day 6, based on AUCT ratio, was 1.17 (0.64 to 2.28). Repeated dosing of nebulized RPL554 caused consistent bronchodilation on all 6 consecutive days. An increase in mean FEV1 after RPL554 compared to placebo was 312.9, 300.5 and 275.4 ML on day 1, 3 and 6 respectively. The maximum increase in FEV1 compared to placebo on day 1, 3 and 6 was 555, 505 and 485 ML, respectively. Only a small decline in FEV1 of 38 ML (95%CI:-95.9; 20.9 ML) or 12% on day 6 compared to day 1 and corrected for placebo was observed. The most frequent reported adverse events were headache, irritation of the larynx and dryness of the throat. Heart rate increased with 5 BPM (95%CI: 3.6; 7.4) and diastolic blood pressure decreased with 3.3 MMHG (95%CI: -5.6; -1.1) on day 6 compared to placebo.

CONCLUSION Repeated daily doses of nebulized RPL554 0.018 MG/KG in allergic asthmatics for 6 days showed consistent bronchodilator effects and was generally well tolerated.

INTRODUCTION

Treatment of asthma currently consists of two distinct categories of drugs: relievers like β2 agonists which are used on an as needed basis and act quickly to reverse bronchoconstriction and relieve symptoms, and controllers like glucocorticosteriods which are taken daily on a long term basis to keep the asthma under clinical control [1]. A potentially new group of anti-asthmatics drugs is the selective phosphodiesterase (PDE) 3/4 inhibitor which may have both bronchodilation effects and anti-inflammatory properties. PDE3 inhibition produces bronchodilation [2;3], while PDE4 inhibition results in anti-inflammatory and disease modifying features as PDE4 regulates the function of several immune, inflammatory (neutrophils, eosinophils) and structural cells (E.G. airway smooth muscle) involved in the pathophysiology of chronic inflammatory diseases like asthma [4]. It has been suggested that PDE3 and PDE4 together act synergetic as each pathway affects the function of different cell types [5]. The dual PDE3/4 inhibitors studied so far in humans are all associated with unfavorable side effects [6;7], such as central nervous system (CNS) and gastrointestinal complaints.

To prevent these systemic side effects, RPL554, a novel dual PDE3/4 inhibitor to be administered by inhalation, was developed for the treatment of asthma. As RPL554 is administered directly into the airways, systemic exposure will to be low. RPL554 is a trequinsin analogue with human IC50 values for PDE3 and PDE4 of 400 PM and 1.5 μM respectively [8]. It has shown bronchodilation, bronchoprotective and anti-inflammatory properties in animal models of allergen-induced asthma and rhinitis. Safety and bronchodilation characteristics were investigated for the first time in humans at the Centre for Human Drug Research in Leiden, the Netherlands; RPL554 significantly produced bronchodilation at doses between 0.009 and 0.072 MG/KG with a mean maximal increase of 520 ML (95%CI: 320; 720 ML) in patients with mild to moderate asthma without CNS and gastrointestinal side effects. During methacholine challenge tests, RPL554 led to an increase of approximately 1.46 doubling doses change from baseline (95%CI: 0.63; 2.28) compared to placebo demonstrating both bronchoprotective and bronchodilative properties of RPL554 in asthmatic patients. The combination of both bronchoprotective and bronchodilatory properties with the absence of CNS and gastrointestinal side effects is promising for the further development of RPL554 for the treatment of allergic asthma.

Asthma therapy often starts at a young age and is given over many years. As continuous use of medication can be associated with a decline in drug effectiveness, longer term effectiveness of RPL554 might be a concern. Therefore, the next step in the development of RPL554 as a potential novel treatment for allergic asthma is to investigate RPL554's effectiveness after repeated administrations. In the present study, lung function is assessed after repeated daily dosing of RPL554 for 6 consecutive days, to investigate the sustainability of effect on FEV1.

METHODS

DESIGN The study consisted of a single arm active treatment, assessing the bronchodilator effects of 6 repeated inhaled daily doses of RPL554 (0.018 MG/KG) in 13 patients with allergic asthma in a single blind manner. Visits to the clinic occurred at screening and at study days: Day -1 for baseline assessments and placebo administration, Day 1 to 6 for daily dosing of RPL554, and Day 7 for follow-up.

Spirometry with frequent FEV1 measurements was performed on all study days, *Figure 1*. Subjects only proceeded to the next dosing day, when stable and no clinically relevant adverse events had occurred. The study protocol was approved by the medical ethical committee Stichting Beoordeling Ethiek Biomedisch Onderzoek (BEBO) in Assen, the Netherlands and conducted according to the principles of the "Declaration of Helsinki" and the pertaining Dutch law.

SUBJECTS Thirteen male subjects with mild to moderate persistent clinically stable allergic asthma participated in the study. All subjects were non-smoking or ex-smokers (> 6 months abstinence and < 10 pack years) and did not suffer from any other significant disease. Patients only used short acting β2-agonists pro re nata, had a FEV1 of > 70% of predicted, a positive provocation test to inhaled Methacholine with a fall in FEV1 of > 20% (PC20MCH) after < 8 MG/ML, methacholine, and a positive skin prick test (SPT > 3MM) for at least one allergen (grass or tree pollen, house dust mite, D. Farinae, cat, dog, or horse-dander, Aspergillus Fumigatus, A. Alternata or Artemisia Vulgaris). FEV1 values at screening and day -1 were within 10% of each other. Patients had a documented reversibility in lung function of > 12% or 0.2 L increase in FEV1 after 200 μg salbutamol and had no history of respiratory tract infections at least 3 weeks before enrollment and during the study. They had not, except for occasional inhaled short acting β2-agonists which was not allowed within 8 hours before start of the study days, used concomitant anti-asthma or anti-allergy medication in the weeks prior to the screening.

TREATMENT RPL554 is 9,10-Dimethoxy-2-(2,4,6-trimethylphenylimino)-3-(Ncarbamoyl-2-aminoethyl)-3,4,6,7-tetrahydro-2H-pyrimido[6,1-a]isoquinolin-4-one powder and was diluted in a citrate-phosphate buffer of pH 3.2. The same buffer was used for placebo. Drug administration took place by oral inhalation of nebulized RPL554 (0.018 MG/KG and based on an average weight of 65-70 KG) through a mouth piece connected to a calibrated electronic nebuliser (Clement-Clarke International AC4000, UK) while the nose was clipped. The drug solution was nebulized from a 0.6 MG/ML solution at a rate of 0.2 ML/minute during 10 minutes.

SAFETY Safety evaluation contained the recordings of adverse events, physical examinations, blood chemistry, haematology and coagulation (analysed at the Leiden University Medical Center), twelve lead ECG, blood pressure and heart rate measurements. Blood pressure and heart rate measurements were conducted daily by automated oscillometric equipment (BMK-1101K; Nihon Kohden, Japan, or Dash 4000; GE Healthcare, USA). Daily Twelve-lead ECG recordings were made using Electrocardiograph Marquette 5000/5500 (USA).

PHARMACOKINETICS Plasma samples, to assess the concentration of RPL554, were drawn at regular intervals (t = 0, 0.25, 0.5, 1, 2 and 4 hours post dose on days 1 up and till 6 and in addition at 6 hours on days 1, 3 and 6) in 4 ML EDTA tubes. The samples were centrifuged within one hour for 10 minutes at 4° C, 2000G, and stored until analysis at -70 °C. RPL554 plasma concentrations were determined with a validated HPLC method by ABL bv, Assen, The Netherlands.

PHARMACODYNAMICS PULMONARY FUNCTION TESTS Lung function tests were performed with the Vmax 20C pulmonary spirometer (SensorMedics, USA) at screening. A portable spirometer ZAN 100 (Accuramed, Belgium) was used during study days. At approximately 15 and 5 minutes predose, baseline FEV1 measurements were performed and calculated as the average of the 2 highest out of 3 FEV1 measurements (within 5%). Post-dosing measurements were performed in duplicate at regular intervals (t = 0, 0.25, 0.5, 1, 1.5 and 2 hours post dose on study days 1 up and till 6, and in addition on 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 hours on study days 1, 3 and 6) of which the highest, technically satisfactory FEV1 was included into analysis.

METHACHOLINE BROMIDE CHALLENGES (MCH) Methacholine challenge performed at screening was used to determine the degree of non-specific bronchial reactivity. MCH challenges were performed by tidal breathing method according to a standardized protocol [9;10]. In short, three technically satisfactory FEV1 measurements within 5% were performed pre and post diluent; the mean of the 2 highest FEV1 values was included in analyses. The post diluent baseline had to remain within 10% of the pre diluent baseline, or otherwise the subject would be excluded from the study. Methacholine bromide (MBR) (Janssen Pharmaceutica, Belgium) was aerosolized by a DeVilbiss 646 jet nebuliser (output 0.13 ML/minutes, Devilbiss Healthcare, inc, Somerset, USA) and inhaled during 2 minutes tidal breathing with the nose clipped. Consecutive MBR doses (0.0625-16 MG/ML) were inhaled at 5 minutes intervals and airway response was measured in duplicate following each dose (and expressed as percentage decline from post-diluent baseline FEV1). The procedure was discontinued after a fall in FEV1 of at least 20% from baseline occurred or the highest permissible dose had been inhaled.

PHARMACOKINETIC ANALYSIS The pharmacokinetic analyses were based on a non-compartmental analysis and calculated in Phoenix WinNonLin 6.2. CMAX and TMAX were determined from visual inspection of the plasma concentration-time profile. The area under the plasma concentration-time curve from zero to the time of the last quantifiable plasma concentration, AUC0-t, was calculated using the linear and log trapezoidal rule. AUCT was defined as the AUC from zero

to 24 hours post dose on each dosing day. The rate constant of the slowest disposition phase (λz) was calculated by log-linear regression of the terminal portion of the concentration-time profile. The terminal half-life (t½) was derived from the equation ln(2)/ λz. Total apparent drug clearance (CL/F) and volume of distribution (Vz/F) were estimated by dividing the dose by AUCt, and product of λz and AUC, respectively. The observed accumulation ratio (Rac) was estimated as follows: I) from the observed ratio (AUCt/ AUC0-24) where τ was 48 to 72 h (Day 3) and 120 to 144 h (Day 6), and II) from the observed ratio (Cmin, day n/Cmin,d2) where n = Day 3 and Day 6 respectively. The used Cmin reflects the pre-dose plasma concentration measured prior to dosing on the given day.

PHARMACODYNAMIC ANALYSIS To compare the FEV1 measures for 6 hours post dosing at day 1, day 3 and day 6, FEV1 results normalized for each individual to FEV1 at day -1 were analysed using a mixed model repeated measures analysis of variance with day, time, day by time as fixed factors and subject, subject by time and subject by day as random factors. The difference between two days (-1 and 1, -1 and 3, -1 and 6, 1 and 3, 1 and 6, 3 and 6) were used as contrasts and expressed as estimated least square mean difference in RPL554 effect, together with the corresponding estimated percentage difference, the 95% confidence interval and the p-value.

POWER CALCULATION A priori, the sample size of the study, as calculated with nQuery Advisor V5.0 (Statistical Solutions, Ltd, Cork, Ireland), was based upon intra individual variability in FEV1 obtained from previous studies with single doses of RPL554. Using a standard deviation of the differences of 180 mL, a decrease of 50% in treatment effect between day 1 and day 6 (corresponding to around 150 mL decrease in the drug response over 6 hours) could have been detected with a power of 80% and a significance level of 5% and 14 subjects. After 8 subjects the SD of the differences between days was estimated. This estimate revealed an SD of 97 mL leading to a subsequent recalculated sample size of 12 patients which would be an adequate number to detect a difference in treatment effect of 87 mL.

RESULTS

DEMOGRAPHICS A total of 13 patients with mild to moderate persistent clinically stable allergic asthma were recruited and received RPL554 of which 12 subjects completed the study, *Table 1*. One subject withdrew his consent on day 1. His results were included into safety and pharmacokinetic analysis but were excluded from the pharmacodynamic analysis. The reversibility test, using inhalation of 200 µg salbutamol, showed an average increase in FEV1 of 0.49 L or 12.7%.

SAFETY AND TOLERABILITY The most common adverse event were headache (n= 4), dizziness (n = 3) and larynx irritations (n=3). All events occurred as single events, were mild in intensity and transient. *Table 2* provides an overview of the most important vital signs and ECG parameters. Heart rate increased around 5 beats per minute and the diastolic blood pressure decreased with 3.3 mmHg on day 6 compared to placebo (day -1). Furthermore, a decrease in the QT interval compared to placebo emerges, which disappears after applying Frederica's correction method. In addition, there were no serious adverse events and no relevant changes observed in laboratory safety assessment and on the ECG parameters: RR-, PR-, QRS- and QT-intervals.

PHARMACOKINETICS Results of RPL554 plasma concentrations are depicted in *Figure 2* and the corresponding pharmacokinetic parameters are shown in *Table 3*.

PHARMACODYNAMIC Repeated dosing of nebulized RPL554 caused bronchodilation on all 6 consecutive days, *Figure 3* and *Figure 4*. The increase in mean FEV1 after RPL554 compared to placebo was 312.9, 300.5 and 275.4 mL on day 1, 3 and 6 respectively. The maximum increase in FEV1 compared to placebo on day 1, 3 and 6 was 555, 505 and 485 mL, respectively. The differences in the least square mean for the change in FEV1 corrected for placebo, between day 3 and day 1 was -4% or -12.4 mL, (p = 0.66, 95%CI:-70.9; 46.0 mL) and between day 6 and day 1 was -12 % or -37.5 mL (p = 0.20, 95%CI:-95.9; 20.9 mL). The corresponding estimated difference for the maximum FEV1, corrected for placebo, between day 3 and day 1 was -9.0% or -50.0 mL (p = 0.11, 95%CI: -113; 12.9 mL) and between day 6 and day 1 was -12.6% or -70 mL (p = 0.03; 95%CI:-133; -7.1 mL). On day 6, FEV1 results were characterised by a small decline in lung function at around 3 hours after dosing which was apparent in 10 of the 12 evaluated patients on day 6 and absent on other days of the study.

DISCUSSION

Previous studies with single doses of nebulized RPL554 showed strong bronchodilation and possible bronchoprotective effects in asthmatic patients, without significant side effects. These data are promising for the further development of RPL554 as a potential treatment of allergic asthma.

As asthma is a chronic condition, continuous drug use is often required. Tolerance for asthma medication has been described before for short-acting β2 adrenoceptor agonists, however it has generally been considered as non-clinically significant [11]. For the further development of RPL554 as a potential anti-asthma medication which inhibits PDE3/4, we investigated whether RPL554 did not lose its effectiveness, and studied especially the occurrence of acute tolerance, after repeated daily dosing. We report that six repeated daily doses of nebulized RPL554 (0.018 MG/KG) in allergic asthmatics was well tolerated and resulted in sustained bronchodilation.

Bronchodilation effects, expressed as mean and maximum increase in FEV1 compared to placebo, were sustained throughout the study. Nevertheless, a small decrease in FEV1 on day 3 and 6 and compared to day 1 was detected during the study period but felt within the observed standard deviation of the differences. The decrease in maximum FEV1 on day 6 compared to day 1 however was significant. We believe that during the time period of the study the decline in FEV1 did not influence the beneficial effects of RPL554 and was certainly too small to underpin the presence of acute tolerance, as the maximum increase in FEV1 of 485 ML on day six compared to placebo still matches up to the observed effects of salbutamol during the reversibility test.

It was observed that the FEV1 results on day 6 were characterised by a decline in lung function at around 3 hours after dosing. This decline was apparent in 10 of the 12 evaluated patients on day 6 and absent on other days of the study. However the observation was classified as spurious as no satisfactory explanation could be found; all procedures were identical during all study days, subjects had limited room to manoeuvre on a smoking free ward and lung function tests were performed by a team of experienced lung function technicians who worked in shifts in a random schedule throughout the study.

There was a minimal though variable accumulation of RPL554 which is reflected in the median ratio and range of RAC estimated using both AUCT and CMIN. The inter-patient variability noted in RAC was greater following estimation by CMIN ratios compared with the AUCT ratios. This observation likely reflects the variability inherent in a point estimate such as CMIN compared with the AUC parameter which represents PK behaviour over a period of time. The systemic exposure to RPL554, as reflected in low concentrations for CMAX and AUCT , can be regarded as little, which might explain the relative mild side effect profile. Indeed, only a few adverse events like mild headache and dizziness were reported. The observed decrease in diastolic blood pressure, the increase in heart rate and the subsequent decrease in QT-interval along the course of the study could be considered as a PDE-inhibitor-class-specific adverse event. The observed increase in heart rate possibly reflects a sympathetically mediated homeostatic reflex response to the observed tendency for a fall in blood pressure that occurs because of relaxation of vascular smooth muscle, a well-known effect of PDE3 inhibition (SMPC amrinone, milrone, olprinone and cilostazol). Furthermore, although the QT interval decreased as a result of heart rate increase, the QT interval as corrected with Fridericia's method remained stable, suggesting that RPL554 did not affect cardiac repolarization.

Although the design of the study provided the fundament to answer the primary objective on acute tolerance, the robustness of bronchodilation over longer period of time should be investigate in more extensive follow up studies, preferably with an active comparator. Given that duration of bronchodilation of a single dose of RPL554 lasts for several hours, a short acting β agonist would have been a reasonable contrast. However, the use of placebo on day -1 provided sufficient information to judge RPL554 on its own merits. The present study did not make use of a methacholine challenge as a measurement for tolerances, as the main outcome of this study was related to bronchodilation and not to bronchoprotection. However, as decreased protection against bronchoconstrictor stimuli like methacholine or histamine is a well-known effect of long term treatment with long acting β2 agonists [12;13], measuring bronchoprotection is something to consider in future studies with RPL554.

In conclusion, repeated daily doses of nebulised RPL554 0.018 MG/KG in allergic asthmatics for 6 days showed sustained bronchodilator effects during the study period and was generally well tolerated. The data suggest that acute tolerance to the effect of RPL554 does not develop during 6 days of continuous drug administration. Whether longer term treatment with RPL554 results in a clinically significant decreased effect, needs to be determined in subsequent clinical trials.

Table 1 Baseline Patient characteristics

Number of subjects	13
Age (years)	26 (19-52)
BMI (kg/m2)	23.0 (1.81)
FEV1 (l)	3.99 (0.66)
FEV1 PRED. (%)	88 (8.5)
FEV1/FVC (%)	73 (8.2)
PC20MCH (mg/ml)	1.68 (1.7)

All numbers are expressed in means (SD) expect age is expressed as mean (range)

Table 2 Vital signs and ECG parameters

	LMSS				Contrasts					
Parameter	Day -1	Day 1	Day 3	Day 6	Day 1 vs. Day -1	Day 3 vs. Day -1	Day -6 vs. Day -1	Day 3 vs. Day 1	Day 6 vs. Day 1	Day 6 vs. Day 3
Diastolic blood pressure (mmHg)	68.6	66.4	66.1	65.2	-2.1 (-4.4; 0.2) p=0.0670	-2.4 (-4.7; -0.2) p=0.0372	-3.3 (-5.6; -1.1) p=0.0052	-0.3 (-2.5; 1.9) p=0.7712	-1.2 (-3.4; 1.0) p=0.2572	-0.9 (-3.1; 1.3) p=0.3944
Systolic blood pressure (mmHg)	119.2	119.2	119.3	118.2	0.0 (-2.9; 2.9) p=0.9958	0.1 (-2.8; 3.0) p=0.9367	-1.0 (-3.9; 1.9) p=0.4962	0.1 (-2.7; 3.0) p=0.9391	-1.0 (-3.8; 1.9) p=0.4808	-1.1 (-3.9; 1.8) p=0.4355
Heart Rate: 12-lead ECG (bpm)	60.2	62.5	62.8	65.7	2.4 (0.4; 4.3) p=0.0180	2.6 (0.7; 4.5) p=0.0093	5.5 (3.6; 7.4) p=<.0001	0.3 (-1.0; 1.5) p=0.6807	3.2 (1.9; 4.4) p=<.0001	2.9 (1.6; 4.2) p=<.0001
QT interval (msec)	396.5	390.5	388.1	385.8	-6.1 (-11.8; -0.4) p=0.0379	-8.5 (-14.2; -2.8) p=0.0048	-10.7 (-16.4; -5.0) p=0.0005	-2.4 (-7.8; 3.0) p=0.3670	-4.7 (-10.1; 0.7) p=0.0870	-2.3 (-7.7; 3.2) p=0.3936
QTc Fridericia (msec)	394.8	393.5	392.0	395.7	-1.3 (-5.7; 3.1) p=0.5572	-2.8 (-7.2; 1.6) p=0.2072	0.9 (-3.5; 5.3) p=0.6770	-1.5 (-5.9; 3.0) p=0.4936	2.2 (-2.3; 6.6) p=0.3202	3.7 (-0.8; 8.1) p=0.1007

Contrast given as difference (95%CI) and p – value. LSM: least mean square

Table 3 Pharmacokinetic parameters of RPL554

	Day 1	Day 3	Day 6
	n = 13	n = 12	n = 12
CMAX (pg/ml)	2136 (1454)	2041 (1215)	2269 (1369)
CMIN (pg/ml)	NA	12 (7)	15 (12)
$T^1/_2$ (h)	NC	NC	7.4 (3.0)
AUCT (pg/ml)*	2111 (44%)	2056 (56%)	2730 (36%)
VZ/F* (L)	NC	NC	5117 (68%)
CL/F* (L/h)	NC	NC	498 (41) %
RAC using Cmin**	NA	1.26 (0.24-2.96)	1.18 (0.16-6.42)
RAC using AUC**	NA	0.90 (0.44-2.48)	1.27 (0.64-2.28)

Values given as mean (SD); *Values given as geometric mean (CV%) ; ** Values given as median (range);
AUCT : refers to post dose values, covering 0-24, 48-72 and 120-144 hours for day 1, 3 and 6 respectively.;
CMIN : refers to the pre dose concentration; VZ: apparent volume of distribution; CL: clearance; F: biological availability; RAC: accumulation ratio; NC: not calculated

Figure 1 Overview of study design. MCh: methacholine challenge. FEV1 was measured at frequent intervals during the study days

	SCREENING	DAY -1	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	FOLLOW UP
MCH	X								
REVERSIBILITY TEST	X								
FEV1	X	X	X	X	X	X	X	X	X
STUDY MEDICATION		PLACEBO	RPL554	RPL554	RPL554	RPL554	RPL554	RPL554	

Figure 2 RPL554 plasma concentrations with SD error bars

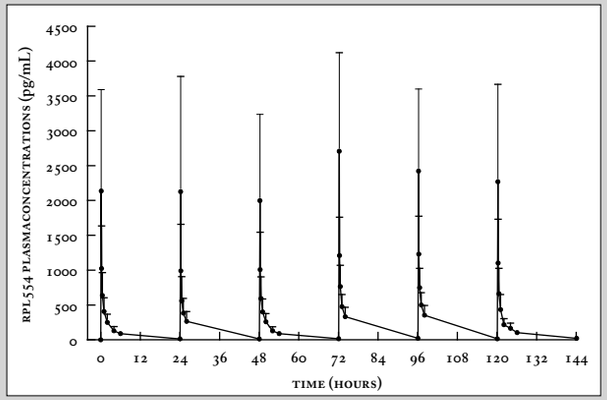


Figure 3 FEV1 time profile with SD error bars

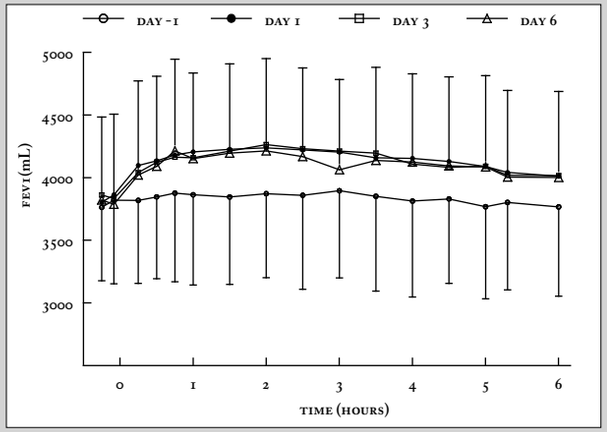
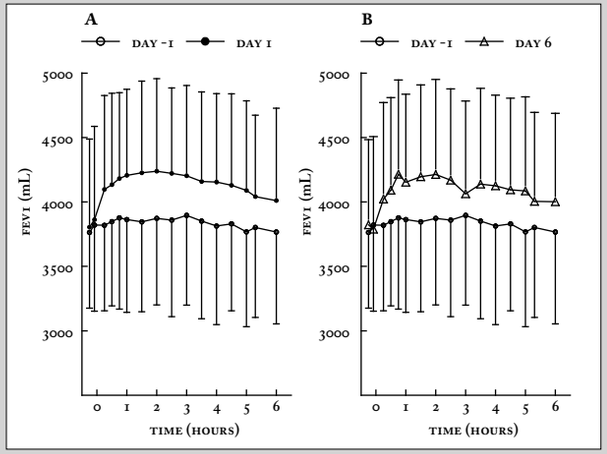


Figure 4A&B FEV1 time profile (with SD error bars) for placebo at day -1 and day 1 and for placebo at day -1 and day 6



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CHAPTER 8

GENERAL DISCUSSION AND IMPLICATIONS OF THE RESEARCH

This thesis consists of two parts; section 1 contains chapters on biomarker applicability; section 2 describes the early clinical development of a new anti-asthmatic drug.

SECTION 1

BIOMARKER DEVELOPMENT AND EVALUATION

CHAPTER 2 & 3 Inhalation of allergens has been effectively and safely applied as a tool to evaluate new drug treatments for asthma, including inhaled steroids, anti-leukotriene therapy, and biologics including anti-IgE and anti-TNF-α therapies [1]. The main application of these models in early drug development has been to estimate treatment changes in pulmonary function assessed by FEV1, bronchial hyper-responsiveness (BHR) assessed by methacholine challenge, and changes in inflammatory state as assessed by cell counts after inhaled allergens.
C*hapter 2* investigated an extension of the allergen challenge model with the ability to quantify changes of a broad range of specific TH2 cytokines in post allergen challenge collected sputum.

THE VALUE OF THIS STUDY FOR CURRENTLY DEVELOPED ASTHMATIC DRUGS ❦ The research pipeline for asthma contains a number of potentially new anti-inflammatory compounds and therapies with a need for PD airway biomarkers [2], *Table 1*. The study in chapter 2 describes the combined use of ultracentrifugation with multiplex quantification methods of sputum biomarkers following allergen challenge, reversed by fluticasone. This approach allows for non-invasive identification of pharmacodynamic targets for anti-asthma therapies. In the study, the most notable effects, in terms of reproducibility and the reversal effect of fluticasone, were observed for IL-5, IL-13, eotaxin-3 and TARC.

Interleukin 5 is a key inflammatory cell mediator in the pathogenesis of asthma as it promotes the proliferation, differentiation, recruitment and survival of eosinophils [15]. High eosinophil counts in sputum are associated with poor asthma control, can predict future exacerbations, and can help direct medication changes. These findings suggest that IL-5 may be an attractive target in allergic asthma. Mepolizumab is a monoclonal antibody treatment directed against IL-5, and Mepolizumab treatment has resulted in a decrease in asthma exacerbations in patients with severe eosinophilic asthma [12]. Benralizumab, a monoclonal antibody that targets α chain of the IL-5 receptor, has shown promising results in phase 2 trials [16].

Interleukin 4 and IL-13 are functionally and structurally related. Both induce B cells to produce IgE. Lebrikizumab is an IL-13 monoclonal antibody. Improvements were seen in patients with high levels of serum periostin, a protein produced by bronchial epithelial cells. This suggests that lebrikizumab may be useful for a specific group of patients with allergic asthma [13]. The compound is now in phase 3 trails. Dupilumab inhibits both IL-4 and IL-13 signaling and shows promising results in early clinical studies [14].

Eotaxin-3 and TARC are both chemokines for which no specific compound has been designed so far.

The allergen challenge model provides useful information on the ability of potential controller therapies to block the asthmatic response. The model also offers high negative predicting values for effectiveness. Indeed, many - but certainly not all - novel compounds with anti-inflammatory characteristics have been studied using the allergen challenge to assess the efficacy of the compound in allergic asthma with FEV1 as the main outcome parameter [14]. Few novel compounds however, have used the allergen challenge focusing on a panel of TH2 driven cytokines rather than individual TH2 cytokines, resulting in a more comprehensive insight in the pharmacodynamic effects of investigated compounds [17]. This is curious, since expectations are that this approach will result in a more robust predictive assessment of anti-inflammatory asthma medication in the early phase clinical drug development. The study described in chapter 2 contributes to this approach by determining the reproducibility and reversal effects of fluticasone of a wide range of cytokines and chemokines after allergen challenge in allergic asthmatic patients.

Chapter 3 presents the results from microarray analysis of sputum samples obtained from the study described in chapter 2. Little is known about microarray assessment of gene expression in induced sputum obtained after allergen challenge in allergic asthmatics. It was found that microarray technology yields reproducible levels of gene expression in induced sputum following inhaled allergen challenge. Moreover, a refined allergen induced gene signature was effectively reversed by fluticasone and individual signatures correlated with lung function and eosinophil measurements. It appears that this approach has the potential to further elucidate the complex interactions occurring during an inflammatory condition.

LIMITATIONS TO THE ALLERGEN CHALLENGE ❦ Asthma is a heterogeneous disorder consisting of several ill-defined endotypes. Nevertheless, adult asthmatic patients are likely to fall within 5 clusters, [18-20], *Table 2*.

The allergen challenge model triggers a TH2 pathway inflammation. Initially, mast cell fixed IgE leads to the immediate phase of the asthma attack. In the late phase, which is in essence an progressive inflammatory reaction, TH2 play a prominent role [21]. TH2 inflammation however, is not a hallmark for all clusters of asthma

limiting the generalizability of the allergen challenge model, *Table 2*. Other limitations include the need of experienced and well trained staff and the difficulty of recruiting eligible subjects.

CHAPTER 4 Several biomarkers sampling methods are often used within the same clinical trials, in the same individual on the same day and sometimes even repeatedly. Most of them are validated and have proven importance in drug development studies. But what if the complementary use of research tools causes them to interact with each other?
Two frequently used sampling methods in asthma drug development studies are sputum induction (SI) with hypertonic saline and fractional exhaled nitric oxide (FENO). Both methods assess the airway inflammation for the lower airways in response to E.G. new anti-inflammatory therapies. In *chapter 4* the effect of SI on FENO measurements was investigated in healthy chronic smoking and non-smoking subjects. The conclusion is that hypertonic saline decreased FENO levels in both groups. As a result of the observed interference of SI with FENO measurements, the latter should always be measured before the SI. There are several explanations for this observation: repeated spirometry measures as part of the SI are known to result in decreased FENO level [22]; mucus accumulation resulting from the SI procedure could inhibit NO diffusion from bronchial epithelial cells [23]; and osmotic changes in bronchial epithelium evoked by inhalation of hypertonic saline could have an effect on the inducible isoform of NO synthase (NOS) [24].

The effect of SI on FENO concentrations was already known; it was studied in healthy volunteers [25], in children [26] and in asthmatics [22]. Data demonstrating this effect in a group of chronic smokers was scarce. Therefore this study confirms and extends previous observations.

Besides the direct effect of SI procedures, smoking itself also has an effect on baseline FENO concentrations. Indeed, in our study the FENO levels were generally lower compared to the non-smokers group. In a more recent study, Hillas and colleagues showed that FENO levels were significantly lower in asthmatic smokers compared to non-smokers [27]. It would be interesting to assess the observed interaction between SI and FENO in a group of asthmatic smokers in a future study.

CHAPTER 5 One of the hallmarks for biomarkers is that they must be reliable and reproducible. *Chapter 5* evaluates the reproducibility of soluble markers obtained from sputum induction and serum in a group of chronic smokers and a group of non-smokers. Soluble markers in blood and sputum samples were obtained on 2 separate visits and analyzed with ELISA. The results were somewhat disappointing.

Although it was found that chronic smokers had higher concentrations of IL-8 in induced sputum and Pulmonary Surfactant Associated Protein D (SP-D) in serum, only SP-D out of a set of markers fulfilled the predefined criteria for reproducibility.

ASSESSMENT OF THE FEASIBILITY OF BLOCKING IL-8 ❦ Although this study was carried out in a group of healthy chronic smokers, smoking plays a prominent role in the pathophysiology of COPD, and is associated with ~80% of the cases. In smokers, the disease is characterized as an inflammation that involves increased numbers of CD8+ (cytotoxic) Tc lymphocytes. These cells, together with neutrophils and macrophages, release inflammatory mediators like IL-8. [28;29]. Increased concentrations of IL-8 have been found in sputum and broncho alveolar lavage (BAL) of patients with COPD. IL-8, also known as neutrophil chemotactic factor or CXCL8, is regarded as a pro-inflammatory factor. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. IL-8 also induces phagocytosis once they have arrived. Activation of the IL-8 receptor, CXCR1 and CXCR2, has similar effects. Despite the biological plausibility, several CXCR2 antagonists and IL-8 specific antibodies have been largely ineffective in clinical studies in patients with COPD. As an example, the CXCR2 antagonist SB-656933 reduces sputum neutrophils in patients with COPD. Larger studies however, have shown no clinical benefit [30;31].
It is difficult to pinpoint the reason for these failures. However, as is the case for asthma, numerous mediators are involved in the pathophysiology of COPD. It thus appears that blocking a single mediator is unlikely to provide a major clinical benefit, unless it is found to be solely responsible for orchestrating a cascade of inflammation. This also highlights that the pathophysiology of COPD is poorly understood. Therefore, it is unclear which cells could be promising therapeutical targets. It thus remains crucial to increase understanding of the underlying inflammatory mechanisms of COPD, and to identify biomarkers of disease activity that predict the clinical efficacy of anti-inflammatory treatments. In this respect, analysis of sputum and serum parameters as described in *chapter 5* may be useful.

ASSESSMENT OF THE FEASIBILITY OF SP-D ❦ The story is different for Surfactant Pulmonary Associated Protein D (SP-D) than for IL-8. The molecule is produced predominantly in the lungs and has been is recognized as an important regulator of innate immune system, capable of binding pathogens and facilitating phagocytosis [32]. It has microbicidal effect on certain bacteria and fungi and promotes the elimination of viruses [33-35]. The lower levels of SP-D found in broncho alveolar lavage fluids in smokers may thus be a cause of weakened lung immunity.

In addition, SPD is able to suppress inflammatory responses caused by lipopolysaccharide (LPS) [36]. SP-D is one of the few lung specific proteins that can be measured in serum, bypassing sometimes difficult and inconvenient sampling methods like BAL and sputum induction. Furthermore, COPD and COPD-exacerbations are associated with higher levels of serum SP-D, which due to some unknown mechanism is inversely correlated with BALF SP-D [37].

In our study we found that levels of serum SP-D in chronic smokers are up to twice as high compared to the levels of non-smokers. In addition, SPD proved to be the only marker of a set of biomarkers to be reproducible in both smokers and non-smokers. Moreover, it has been found that regular inhalation of salmeterol and fluticasone lowers serum SP-D levels in COPD patients [38].

Taken together, SP-D has an important role in the innate immunity, can be easily assessed in serum, is specific for COPD, levels change with severity and exacerbations, and last but not least it is sensitive to effective treatment. This does not necessary mean that a SP-D compound should be developed. However, it does seem that SP-D may be a potential candidate marker with characteristics of a validated biomarker for COPD.

LARGE SCALE PROTEIN DISCOVERY STUDIES ❦ The search for multiple disease specific protein biomarkers is sometimes called "a fishing expedition", an uncomplimentary term indicating that scientists have no idea what the catch will be. *Chapter 5* describes the results of identification of a broad array of analytes in sputum using ELISA technology. However, newer analytical technologies like multiplex analysis and peptidomics have exponentially increased the surface of the net allowing more fish to be caught. In fact these two technologies were also applied in the study, but not described in the manuscript.

In our study, Multiplexed analysis (Rules Based Medicine) was used to measure a total of 92 soluble markers in serum. The analysis showed increased levels of Marcophage Derived Chemokine (MDC), Carcino-Embryonic Antigen and Apolipoprotein H in smokers compared to non-smokers. All other biomarkers were not significantly different for the two study groups. Reproducibility was not assessed and therefore this analysis was not mentioned in the article.

For proteomics analysis, mass spectrometry MALDI-TOF-TOF was used to assess the feasibility for primary screening of yet to be discovered biomarkers. Once a peptide marker is identified and its sequence validated, the next step is to develop a standard ELISA assay. The ELISA can then be applied to the same clinical samples and deliver quantitative measures of the biomarker. In our study, proteomics analysis identified 147 proteins in the supernatant of induced sputum demonstrating the feasibility of the mass spectrometry MALDI-TOF-TOF as a tool for biomarker discovery in induced sputum and plasma.

What would the advantage of such technologies be? Although still in their infancy compared with other methodologies, large scale protein discovery studies could result in protein fingerprints which potentially could stratify patients for the treatment of COPD and may provide a tool to assess treatment response [39;40]. However it is unlikely that these findings can be used plausibly or reproducibly as markers for the activity of a new medicine unless they are properly validated and underpinned by a mechanistic understanding of the changes in these profiles. If this is not done the risk of statistically spurious findings is probably high.

SECTION 2

CLINICAL STUDIES IN HEALTHY VOLUNTEERS, ALLERGIC RHINITIS AND ALLERGIC ASTHMA; WITH USE OF BIOMARKER

In the second part of this thesis, the focus was on allergic asthma and the biomarker assisted development of the new anti-asthmatic drug RPL554.

Asthma and chronic obstructive pulmonary disease (COPD) are commonly treated with a combination of a bronchodilator and an anti-inflammatory drug, usually an anti-inflammatory glucocorticosteroid. A drug that combines clinically relevant symptom control with substantial anti-inflammatory activity in one molecule would therefore be very welcome.

Phosphodiesterases (PDE's) are a large family of 11 enzyme subtypes, modulating intracellular concentrations like CAMP. Increased levels of CAMP are related to airway smooth muscle relaxation and anti-inflammatory actions. PDE3 inhibitors cause airway smooth muscle relaxation in vitro [41;42], bronchodilation in vivo [43], and increases in forced expiratory volume in 1 second (FEV1) in patients with asthma [44]. PDE4 inhibitors inhibit the function of a wide range of inflammatory cells in vitro and have pronounced anti-inflammatory effects in patients with inflammatory airway disease [45;46]. Experimental data suggest that combined inhibition of PDE3 and PDE4 iso-enzymes may have synergistic effects [47]. RPL554 is a dual PDE3 and PDE4 inhibitor that has shown bronchodilator and anti-inflammatory effects in animals in vitro and in vivo [48].

CHAPTER 6 describes an experiment in which the effects of nebulized RPL554 were assessed in a series of adaptive proof of concept studies. Both its bronchodilator

and anti-inflammatory properties were investigated for the first time in humans. This single dose study was split into three stages. Safety of RPL554 was assessed in healthy individuals (stage 1), and safety and efficacy was assessed in otherwise healthy subjects with allergic asthma or allergic rhinitis (stage 2 & 3).

This experiment showed that RPL554 was well tolerated and had mild adverse events that were equal to placebo. In allergic asthmatic patients RPL554 at 0,018 MG/KG resulted in an increase in PC20Mch of around 1,5 doubling doses and substantial bronchodilation at doses between 0.009 and 0.072 MG/KG with a mean increase at 1 hour of 520 ML in FEV1. The latter effect is comparable to that of salbutamol, a widely used β-agonist. Increases in eosinophil count after the nasal allergen challenge were reduced by PRL554- compared to placebo (7.1%) although this did not reach statistical significance.

CHAPTER 7 In another study with RPL554, described in *chapter 7*, the reproducibility of the bronchodilator response to a daily dose of nebulized RPL554 at 0,018 MG/KG for 6 consecutive days in a placebo controlled study in 12 healthy men with allergic asthma was examined. This study showed that RPL554 had a similar maximum mean increase on day 1 (555mL) day 3 (505 ML) and day 6 (485 ML), suggesting that acute tolerance to the effect of RPL554 did not develop.

RPL554'S FURTHER DEVELOPMENT FOR ASTHMA ❋ As explained in the introduction of this thesis, the underlying pathophysiology of asthma is often – but not always - TH2 driven and a consequence of the contribution of numerous cells including the epithelium, dendritic cells, T lymphocytes, eosinophils, mast cells and airway smooth muscle. Many of these cells express PDE4 and inhibition of this enzyme could suppress the function of these cells [49].

When a new pharmaceutical agent for asthma is studied, its ability to block the early and late phase asthmatic response and subsequent airway hyperresponsiveness is important evidence for effectiveness [1]. So far, a few PDE4 inhibitors have been assessed for their effect on the acute allergen induced bronchoconstriction. An inhibition of the late phase response of around 30% was observed for CDP840 and roflumilast after several days of treatment [50;51]. In addition, the effect of GSK256066, an inhaled PDE4 inhibitor, was regarded as modest [52]. However, roflumilast showed a 30–50% reduction in sputum eosinophil number indicating anti-inflammatory activity in asthma. However, the effect of roflumilast on allergen induced airway hyperresponsiveness was limited; around 1 doubling dose of methacholine [53]. These effects of roflumilast are modest when compared to the effect of fluticason (*chapter 2*), and consistent with the lack of demonstrable action of PDE4 inhibition on mast cell [54].

Anti-inflammatory effects of RPL554 in the studies presented *in chapter 6 en 7* were difficult to establish. PC20MCH increased with 1,5 doubling dose compared to placebo. On the other hand, methacholine challenge only has moderate correlation with eosinophilic airway inflammation [55]. Also, RPL554 did not shown a significant effect on eosinophil cell count in patients with allergic rhinitis after a nasal allergen challenge, (*chapter 6*). Furthermore, RPL554 at a dose of 0,018 MG/KG continued to induce sustained bronchodilation in a group of mild allergic asthmatic volunteers, but it did not improve FEV1 values at baseline at each study day, (*chapter 7*).

In conclusion, the bronchodilator effects of RPL554 are promising. However, its anti-inflammatory effects in asthma based on the observations in our studies and based on the limited effects of PDE4 inhibitors on mast cells in general, will need to be determined in further clinical studies.

RPL 554 FOR COPD ❋ COPD is another inflammatory disease but the nature of the inflammatory response is different from asthma. COPD is characterized amongst other reactions, by activation of macrophages and cells recruitment of neutrophils in the lung. Lipopolysaccharide (LPS) challenges mimic the airway neutorophilia and have been used to evaluate the efficacy of new therapeutic agents for the treatment of COPD. The inhibiting effect of RPL554 (0,018 MG/KG) on LPS-induced sputum neutrophils was demonstrated in a placebo-controlled crossover trial with 21 healthy men [7], showing reduction in absolute numbers of neutrophils after 6 hours. The effect was similar to that observed with roflumilast in the sputum of patients with COPD [46].

In addition, an open-label, placebo controlled crossover trial, in 12 men with mild-to-moderate COPD showed a bronchodilator effect of RPL554 (0,018 MG/KG) with a mean maximum FEV1 increase of 17,2% [7]. These effect are comparable with peak effects reported in such patients with inhaled β2 agonists [56], and suggest potential therapeutic use of RPL554 in COPD.

OVERALL CONCLUSION

This thesis describes attempts to identify novel pathophysiology based biomarkers for chronic airway disease by applying newer detection techniques. It appears that such an approach may be worthwhile to pursue. Further, it shows that application of biomarkers allows effective development of new therapies in chronic airway disease.

Table 1 Summary of potential new therapies for asthma.

Ultra long acting β2-agonists	Effective for COPD, possible available in near future for asthma - especially when combined with ICS [3;4]
Long acting muscarine-antagonist	Effective for COPD, possible available in near future for asthma - especially when combined with ICS [5;6]
PDE-Inhibitors	Favorable early studies [7]
Bronchial thermoplasty	Effective but has possible immediate complications associated with the
TNF-α	Risk of side effects [9]
IL-9	Not effective [10]
IL-17	Not effective [11]
IL-5	Effective in asthmatic patients with high eosinophil levels [12]
IL-13	Effective in asthmatic patients with high periostin levels [13]
IL-4	Promising early studies [14]

Table 2 Clusters of asthma

Cluster 1	Early onset allergic asthma with Th2 signature: high levels of eosinophils, mast cells, IGE and FENO
Cluster 2	Adult onset non allergic asthma. Th2 signature with eosinophilia and high levels of IL-4, IL-5, IL-13, but absence of allergic disease
Cluster 3	Exercise induced asthma. Mast cells play an important role
Cluster 4	Prominent obesity and minimal Th2 response
Cluster 5	Sputum neutrophilia with Th17 response

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SAMENVATTING

Dit proefschrift bestaat uit twee delen. Sectie 1 beschrijft de ontwikkeling van biomarkers voor chronische aandoeningen van de luchtwegen. Sectie 2 beschrijft de toepassing van biomarkers bij de ontwikkeling van mogelijk nieuwe geneesmiddelen tegen astma.

SECTIE 1

DE ONTWIKKELING VAN BIOMARKERS IN ASTMA EN COPD

HOOFDSTUKKEN 2 & 3 Het uitlokken van een astma aanval bij proefpersonen door het geven van een allergeen (bijvoorbeeld graspollen) is een effectieve en veilige onderzoeksmethode om de werking van nieuwe middelen tegen astma te onderzoeken. Tijdens deze methode wordt veelal gekeken naar de effecten op de longfunctie, de gevoeligheid van de longen voor prikkelende stoffen en worden de ontstekingscellen in het sputum geanalyseerd. In *hoofdstuk 2* werd dit model verder uitgewerkt en werd onderzocht of het bepalen van een grote groep ontstekingseiwitten ook gebruikt kan worden als uitkomstmaat. De studie toonde aan dat het aantal door TH2-cellen geproduceerde eiwitten toeneemt na het ondergaan van een uitgelokte astma aanval, en ook dat deze toename in veel gevallen reproduceerbaar is. Behandeling met fluticason heeft een duidelijk verminderend effect op deze toename van eiwit concentratie. Deze resultaten kunnen goed gebruikt worden om de effecten van een nieuw te onderzoeken geneesmiddel tegen astma in kaart te brengen. *Hoofdstuk 3* beschrijft de RNA analyse op basis van dezelfde sputum monsters die in hoofdstuk 2 zijn beschreven. Er is nog maar weinig bekend van een dergelijke analyse in sputum afkomstig van patiënten met een uitgelokte astma aanval. Gevonden werd dat ook het RNA toeneemt na een astma aanval en dat deze resultaten reproduceerbaar zijn.

HOOFDSTUKKEN 4 & 5 Vaak worden in een klinisch onderzoek meerdere methoden gebruikt voor het verkrijgen van lichaamsmateriaal. De meeste van deze technieken zijn betrouwbaar en hebben hun waarde bewezen voor het gebruik in geneesmiddelen studies. Echter, gelijktijdig gebruik van twee of meerdere onderzoekstechnieken kan de uitkomst van de afzonderlijke technieken beïnvloeden. Twee methoden die frequent worden toegepast bij onderzoek naar nieuwe geneesmiddelen voor astma zijn sputum inductie met hypertoon zout en het bepalen van concentratie stikstofmonoxide in uitgeademde lucht. Met beide methoden kan de mate van ontsteking in de longen worden bepaald. In *hoofdstuk 4* wordt het effect van sputum inductie op de concentratie uitgeademde stikstofmonoxide bepaald bij een groep gezonde rokende en niet rokende manen en vrouwen. Er werd vastgesteld

dat sputum inductie een verlaging geeft van de concentratie uitgeademde stikstofmonoxide. Er zijn meerdere verklaringen mogelijk voor deze interactie. Allereerst is bekend dat meerdere opeenvolgende spirometrie metingen, die uitgevoerd worden bij sputum inductie, de stikstofmonoxide concentraties in uitgeademde lucht verlagen. Daarnaast zou het ophopen van slijm in de longen, als gevolg van de sputum inductie procedure, de diffusie van stikstofmonoxide beperken. Verder is bekend dat de hypertoon zoutoplossing een osmotische verandering teweeg kan brengen waardoor mogelijk de productie van stikstofmonoxide afneemt. Om deze interactie te vermijden verdient het aanbeveling om het bepalen van de stikstofmonoxide concentratie uit te voeren voorafgaand aan de sputum inductie.

Belangrijkste kenmerken van een biomarker zijn betrouwbaarheid en reproduceerbaarheid. Dat wil zeggen dat als het onderzoek onder dezelfde omstandigheden meerdere malen wordt uitgevoerd, de waarde van de biomarkers steeds hetzelfde blijft. Op basis van dezelfde studie als beschreven in *hoofdstuk 4* werd de reproduceerbaarheid van de concentratie markers in geïnduceerd sputum en serum onderzocht in een groep chronische rokers en een groep niet-rokers. De concentraties van de markers werden bepaald met een ELISA techniek. De resultaten van het onderzoek staan beschreven in *hoofdstuk 5*. Hoewel hogere concentraties in chronische rokers werden gevonden van IL-8 in sputum en Surfactant Pulmonary Associated Protein D (SP-D) in serum, bleek dat uit een set van markers alleen SP-D voldeed aan de vooraf bepaalde criteria voor reproduceerbaarheid.

SECTIE 2

KLINISCHE STUDIES IN GEZONDE VRIJWILLIGERS, PATIËNTEN MET ALLERGISCH ASTMA EN RHINITIS, MET GEBRUIK VAN BIOMARKERS

HOOFDSTUKKEN 6 & 7 In het tweede deel van dit proefschrift ligt de nadruk op allergisch astma en de klinische ontwikkeling van een nieuw anti-astma geneesmiddel (RPL554) waarbij gebruikt wordt gemaakt van biomarkers. Astma en chronisch obstructief longlijden (COPD) worden veelal behandeld met een combinatie van luchtwegverwijders en ontstekingsremmende geneesmiddelen. Een geneesmiddel dat tegelijkertijd luchtwegverwijding kan geven en ontstekingsremmend is, zou dus een aanvulling zijn op de bestaande behandelmogelijkheden. Fosfodiesterasen (PDE's) is een groep enzymen die wordt onderverdeeld in 11 subgroepen. Het gemeenschappelijk kenmerk van deze enzymen is dat ze concentratie cAMP in de cel verhogen door het remmen van de afbraak. Hogere concentraties cAMP leiden tot

verslapping van de gladde spieren in de long en tot ontstekingsremming. Binnen de groep PDE-remmers zorgen PDE3 remmers voor een verhoging van cAMP, wat leidt tot verwijding van luchtwegen waardoor het uitgeblazen volume in de eerste seconde van de longtest (FEV1) bij patiënten met astma toeneemt. Remmers van het iso-enzym PDE4 zorgen voor verminderde werking van een groot aantal ontstekingscellen en hebben een duidelijk ontstekingsremmend effect in patiënten met COPD. Experimenteel onderzoek heeft aangetoond dat een combinatie van PDE3 en PDE4 remming leidt tot synergie; het effect op de luchtwegen is groter dan de som van beide afzonderlijke effecten. RPL554 is een middel dat zowel PDE3 als PDE4 remt.

In *hoofdstuk 6* wordt beschreven hoe de verdraagbaarheid en effectiviteit van RPL554 over een brede reeks (0,03 tot 0,072 MG/KG) doseringen wordt onderzocht in een geneesmiddelenstudie. Zowel de bronchusverwijdende als de ontstekingsremmende effecten werden voor de eerste keer in mensen onderzocht. Het onderzoek werd opgedeeld in 3 deelonderzoeken, waarbij eerst opklimmende eenmalige doseringen van RPL554 werden gegeven aan gezonde vrijwilligers en patiënten met allergisch astma (1e deel), waarna een vaste dosering werd onderzocht in een groep patiënten met allergisch astma en allergisch rhinitis (2e en 3e deel).

De onderzoeksresultaten lieten zien dat RPL554 over het algemeen goed werd verdragen. De bijwerkingen waren mild en de frequentie was te vergelijken met die van de placebo groep. In patiënten met astma zorgde een dosis van 0,018 MG/KG RPL554 tijdens de PC20 methacholine test voor een toename van 1,5 verdubbelde dosis. Verder bleek dat doseringen tussen 0.009 en 0.072 MG/KG een aanhoudende luchtwegverwijding gaven met een gemiddelde toename in FEV1 op 1 uur na doseren van 520 mL. Dit laatstgenoemde effect is vergelijkbaar met het effect van de veel gebruikte β-agonist salbutamol. In de groep patiënten met allergische rhinitis werd ook een aanzienlijk bronchusverwijdend effect gemeten en waren er aanwijzingen voor een anti-inflammatoir effect. Het laatstgenoemde effect bereikte geen statistisch significantie, maar de observatie dat de toename in eosinofielen na een nasale allergeen challenge 7,1% lager was na RPL554 in vergelijking met placebo, suggereert dat RPL ook ontstekingsprojecten kan onderdrukken.

In een vervolgstudie, beschreven in *hoofdstuk 7*, werd onderzocht of de luchtwegverwijdende effecten ook aanhouden als patiënten met allergisch astma gedurende 6 achtereenvolgende dagen RPL554 gebruiken. Met dit onderzoek, dat werd uitgevoerd in 12 patiënten met astma, konden we laten zien dat RPL554 een aanhoudend luchtwegverwijdend effect gaf en dat het effect vergelijkbaar was met de effecten die optreden na een enkele dosering; een gemiddelde maximale toename in FEV1 op dag 1 van 555 mL, op dag 3 van 505 mL en op dag 6 van 485 mL. Het lijkt er dus op dat RPL554 geen acute tolerantie laat zien bij langduriger gebruik.

SAMENVATTING Dit proefschrift beschrijft het onderzoek naar nieuwe pathofysiologisch gebaseerde biomarkers voor chronische luchtweg aandoeningen met behulp van nieuwe detectie technieken. Een dergelijke aanpak lijkt geslaagd. Verder laat het proefschrift het gebruik van biomarkers zien bij de effectieve ontwikkeling van nieuwe therapieën voor chronische luchtweg aandoeningen.

CURRICULUM VITAE

Rob Zuiker was born on 6 April 1968 in Wognum, the Netherlands. After graduating from secondary school (Atheneum, Copernicus Scholengemeenschap, Hoorn) in 1988, he attended medical school at the University of Amsterdam. He obtained his MD in 1996 and subsequently worked as a physician for the Royal Dutch Navy. He obtained a master degree in business administration from Nijenrode University, Breukelen, the Netherlands in 2001. Thereafter he was employed as a medical advisor for Eli Lilly, the Netherlands. He joined Centre for Human Drug Research (CHDR), Leiden, the Netherlands in 2007, where the research described in this thesis was performed under the supervision of prof. dr. A.F. Cohen. Since 2012 he is a board certified clinical pharmacologist, and he currently holds a position as senior clinical scientist at CHDR.
Rob Zuiker is married to Saskia van der Geest, they have two daughters – Marijn and Renske – and a son – Jelmer.

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