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# BRONCHOPROVOCATION STUDIES TO DEFINE MECHANISMS IN ASTHMA AND AIRWAY INFLAMMATION

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# THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Evi, Christos and Stavros

# ABSTRACT

Bronchial provocations with a variety of stimuli have been widely used over the past 70 years in both asthma research as well as in daily clinical praxis in order to aid the physician to establish the asthma diagnosis. In research, the bronchoprovocation model represents an excellent tool to better understand asthma pathophysiology, and to assess the effects of different interventions and new investigational therapies. Most of the currently approved asthma therapeutic options have shown efficacy in previous studies using the bronchoprovocation setting.

In the current thesis, a range of bronchial provocations was performed prior to and after treatment with pharmacological interventions with different mode of actions. Responses were measured in the airways in the form of induced bronchoconstriction and airway inflammation (sputum cell counts), as well as in other matrices (skin, blood, urine).

For the first time, it was shown that treatment with the combination of budesonide-formoterol (bud/form) in a single inhaler taken three to four times per week provided the same magnitude of protection against exercise-induced bronchoconstriction (EIB) as to regular treatment with a low dose of budesonide. Moreover, subjects who received monotherapy with the short acting $\beta_2$ agonist (SABA) terbutaline had no protection against EIB over time. These results question the place of SABA monotherapy in asthma treatment even for subjects with mild asthma. It is recommended to replace SABA monotherapy with intermittent use of bud/form, which can also be an alternative option to regular treatment with low dose inhaled corticosteroids (ICS).

Furthermore, using an allergen bronchoprovocation model, it was demonstrated that treatment with the second-generation anti-IgE monoclonal antibody QGE031 (ligelizumab) elicited an inhibition of the early allergic response (EAR) that was three times greater than what was achieved by the currently approved anti-IgE treatment with omalizumab. In addition, the data showed that there were important differences in the allergen response in the airways compared to the skin during QGE031 therapy; the highest dose of QGE031 consistently supressed allergen induced skin test responses that persisted six weeks after the last dose was given, while there was a variable effect on the airway response that did not last six weeks after the last dose. These results elucidate the complexity of the IgE pathway and the different kinetics and tissue responses to anti-IgE therapy.

Finally, this thesis answered some important questions about the role of cysteinyl leukotrienes (CysLTs) and in particular leukotriene $E_4$ ($LTE_4$) in asthma. The data showed that treatment with the potent $CysLT_1$ receptor antagonist montelukast completely abolished the bronchoconstriction elicited by $LTE_4$ inhalation in subjects with mild asthma. Urine was collected during the $LTE_4$ provocations for analysis of lipid mediator excretion, which led to the serendipitous discovery of increased urinary excretion of metabolites of prostaglandin (PG) $D_2$, as well as other lipid mediators after $LTE_4$ inhalation. These novel findings add a new dimension, namely that $LTE_4$, in addition to a direct bronchoconstrictive action, can also activate both the mast cell as well as other cells to produce secondary responses that can amplify or modify its primary effect.

Thus, this thesis demonstrates that carefully planned and conducted studies using bronchial provocations in combination with various pharmacological interventions, can elucidate important mechanisms in asthma pathogenesis and reveal potential new targets for treatment.

# LIST OF SCIENTIFIC PAPERS

I. **Lazarinis N**, Jørgensen L, Ekström T, Bjermer L, Dahlén B, Pullerits T, Hedlin G, Carlsen K-H, Larsson K. Combination of budesonide/formoterol on demand improves asthma control by reducing exercise-induced bronchoconstriction. ***Thorax*** 2014; 69:130-136.

II. Gauvreau GM, Arm JP, Boulet LP, Leigh R, Cockcroft DW, Davis BE, Mayers I, FitzGerald JM, Dahlen B, Kilian KJ, Laviolette M, Carlsten C, **Lazarinis N**, Watson RM, Milot J, Swystun V, Bowen M, Hui L, Lantz AS, Meiser K, Maahs S, Lowe PJ, Skerjanec A, Drollmann A, O´Byrne PM. Efficacy and safety of multiple doses of QGE031 (ligelizumab) versus omalizumab and placebo in inhibiting allergen-induced early asthmatic responses. ***J Allergy Clin Immunol*** 2016; 138:1051-1059

III. **Lazarinis N**, Bood J, Gomez C, Kolmert J, Lantz AS, Gyllfors P, Davis A, Wheelock CE, Dahlén SE, Dahlén B. Leukotriene E4 induces airflow obstruction and mast cell activation through the cysteinyl leukotriene type 1 receptor. ***J Allergy Clin Immunol*** 2018; 142:1080-89.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACQ-5 | Asthma Control Questionnaire-5 |
| AHR | Airway hyperresponsiveness |
| $A_x$ | Area of reactance |
| AMP | Adenosine monophosphate |
| AERD | Aspirin-exacerbated respiratory disease |
| Allergen $PC_{15}$ | Provocative concentration of allergen causing a 15% decrease in $FEV_1$ |
| BAL | Bronchoalveolar lavage |
| BD | Bronchodilator reversibility test |
| Bud/form | Budesonide-formoterol |
| COPD | Chronic obstructive pulmonary disease |
| COX | Cyxlooxygenase |
| $CRTH_2$ | Chemoattractant receptor-homologous molecule |
| CysLT | Cysteinyl leukotriene |
| $CysLT_{1-2}$ | Cysteinyl leukotriene receptor 1-2 |
| DC | Dendritic cell |
| EAR | Early asthmatic response |
| EIA | Enzyme immunoassay |
| EIB | Exercise-induced bronchoconstriction |
| $EP_{1-4}$ | Prostaglandin E receptor 1-4 |
| EVH | Eucapnic voluntary hyperpnoea |
| FcεRI | High affinity IgE receptor |
| FcεRII/CD23 | Low affinity IgE receptor |
| FeNO | Fraction of exhaled nitric oxide |
| $FEV_1$ | Forced expiratory volume in 1 second |
| FVC | Forced vital capacity |
| ICS | Inhaled corticosteroids |
| IgE | Immunoglobulin E |
| ILC2 | Group 2 innate lymphoid cells |
| IOS | Impulse oscillometry |

| | |
|---|---|
| LABA | Long acting $\beta_2$ agonist |
| LAR | Late asthmatic response |
| LT | Leukotriene |
| $LTE_4PD_{20}$ | Provocative dose of $LTE_4$ causing a 20% decrease in $FEV_1$ |
| LTRAs | Leukotriene receptor antagonists |
| Methacholine $PC_{20}/PD_{20}$ | Provocative concentration/dose of methacholine causing a 20% decrease in $FEV_1$ |
| PG | Prostaglandin |
| $R_5$ | Resistance at 5Hz |
| $R_{20}$ | Resistance at 20Hz |
| $R_5$-$R_{20}$ | Frequency dependent resistance |
| SABA | Short acting $\beta_2$ agonist |
| TP | Thromboxane prostanoid receptor |
| TX | Thromboxane |
| UPLC-MS/MS | Ultra performance liquid chromatography tandem mass spectrometry |

# 1  INTRODUCTION

Asthma is a common, chronic respiratory disease, which is characterized by a history of symptoms of wheeze, shortness of breath, chest tightness and/or cough that vary over time and in intensity together with variable expiratory airflow limitation (GINA report 2018). These symptoms can be triggered by factors such as exercise, allergen, and change in weather or viral respiratory infections. Asthma is usually associated with airway hyperresponsiveness (AHR) to exogenous stimuli (Hargreave, Ryan et al. 1981) and with chronic inflammation. The disease is heterogeneous with different underlying mechanisms and continues to increase. It is estimated to affect more than 300 million individuals worldwide (Global Burden of Disease Study 2015) and about 800,000 persons in Sweden (Socialstyrelsen 2015). More than half of asthma patients have a mild disease where symptoms and airflow limitation may resolve spontaneously or in response to medication. On the other hand, some patients will experience episodic flare-ups (exacerbations) that may be life threatening and carry a significant burden for the community. Over the past 15-20 years asthma mortality has declined due to progress that has been made not only in asthma therapy but also in establishing a correct diagnosis and better assessing the disease; however, there is evidence that overdiagnosis of asthma occurs (Aaron, Vandemheen et al. 2008), which probably is also true for underdiagnosis. This problem is due to the lack of measurement and documentation of typical physiological asthma features such as variable airway obstruction and AHR. According to both international (British Thoracic and Scottish Intercollegiate Guidelines 2014, GINA report 2018) and Swedish asthma guidelines (Socialstyrelsen 2015), although a diagnosis of asthma could be suspected on the basis of patient reported symptoms, it should be supported by objective measures of variable airway obstruction or AHR.

Spirometry is the preferred method to measure airway obstruction and a reduced $FEV_1$ with a low ratio of $FEV_1$ to FVC indicates its presence. A positive bronchodilator reversibility test (BD) in adults usually defined as an increase in $FEV_1$ of 12% or more with at least a 200 ml change after administration of a SABA (GINA report 2018), suggests the presence of asthma when other diseases have been excluded. Asthma is a variable disease and airway calibre can change over time, such that patients when assessed for suspected asthma might have normal pulmonary function or non-significant reversibility after a BD test, and thus fail to have the correct diagnosis. In these patients bronchoprovocation tests can be performed to confirm the presence of AHR.

Almost all asthmatic patients will respond to either direct bronchoprovocation challenges, acting directly on airway smooth muscle, such as methacholine or indirect acting through activation of inflammatory cells such as exercise, eucapnic voluntary hyperpnoea (EVH), hypertonic saline, allergen, adenosine monophosphate (AMP) or mannitol inhalation (Cockcroft 2001, Cockcroft 2003). The degree of AHR may not directly correlate with asthma severity in all patients, though in general indirect challenges are considered good markers of airway inflammation, they correlate with airway eosinophilia (Polosa, Ciamarra et al. 2000, Van Den Berge, Meijer et al. 2001) and AHR to indirect stimuli decreases with anti-

inflammatory therapy (van den Berge, Kerstjens et al. 2001). The latter makes them useful not only for establishing the diagnosis but also for treatment monitoring (Sont, Willems et al. 1999) and AHR to inhaled mannitol is known to normalize with adequate therapy (Brannan, Koskela et al. 2007). In addition, mannitol AHR has been used as a method of ICS titration in persistent asthma (Lipworth, Short et al. 2012). Thus it is evident that bronchoprovocations are important tools for everyday clinical praxis, and are recommended in most asthma guidelines.

Bronchoprovocations can with the same utility be used for asthma research not only to establish the diagnosis but also to trigger a standardized, reproducible airway reaction and thereby obtain integrated *in vivo* information on inflammatory and functional responses in subjects with asthma. Asthma is a complex and heterogeneous disease with a range of subgroups or phenotypes emerging during the last years (Wenzel 2012) with different underlying pathophysiological mechanisms. Most of these mechanisms can be explored using bronchoprovocation studies with a goal to target specific receptors or cells/signalling molecules and in the end define which patients will benefit most from each therapeutic intervention. The latter is a major focus of asthma research today.

# 2 AIMS OF THE THESIS

The overall objective of this thesis was to use different bronchoprovocations in order to elucidate distinct underlying mechanisms in asthma and airway inflammation. Bronchoprovocations were performed prior to as well as after pharmacological interventions with both currently approved and new investigational drugs. In this way by blocking one or several steps in the response induced by the various bronchoconstrictive stimuli, we can gain further knowledge about certain pathophysiological mechanisms and eventually establish new targets for treatment of asthma.

This project included a series of different bronchoprovocations/challenges, namely exercise, allergen and inhalation of $LTE_4$ in subjects with asthma. The effect of both the challenges and the specific interventions were used to answer the following questions:

1. Does on-demand treatment with the combination of budesonide-formoterol (bud/form) improve asthma control as assessed by exercise induced bronchoconstriction (EIB)?
2. Is treatment with the new investigational anti-IgE antibody QGE031 (ligelizumab) superior to omalizumab in inhibiting the early asthmatic response (EAR) following an allergen inhalation challenge?
3. Does treatment with the potent $CysLT_1$ antagonist montelukast block both the bronchoconstriction and airway inflammation, as assessed by sputum eosinophils, induced by inhalation of $LTE_4$ in asthmatic subjects? Which lipid mediators are released after a $LTE_4$ challenge and how is this release affected by treatment with montelukast?

# 3 BACKGROUND

## 3.1 BRONCHIAL PROVOCATIONS

Airway hyperresponsiveness (AHR) is an abnormal increase in airflow limitation following exposure to exogenous stimuli (Lotvall, Inman et al. 1998). Bronchoprovocation challenges using allergen aerosols were widely used over the first half of the 20th century much more than non-allergic challenges, after the classic work of Blackley (CH. 1873) who in the 1870s used provocation tests with whole pollen to show that grass pollen was a cause of allergic rhinitis and asthma. Over the years with the spread of the bronchial provocations, AHR has progressed from a concept to a physiological measurement. Already in 1945 Tiffeneau and Beauvallet reported, "bronchodilation and bronchoprovocation challenges should be helpful in the assessment of patients with lung disease" (Tiffeneau R 1945). In the 1950s, Herxheimer performed the first provocation study using various stimuli (Herxheimer 1951) and was also the first to describe in the lab the late asthmatic reaction (LAR) following an allergen challenge (Herxheimer 1952). In the following years, the first bronchoprovocation studies using a pharmacological intervention with sodium cromoglycate in order to block the elicited response were performed (Pepys, Hargreave et al. 1968, Booij-Noord, Orie et al. 1970, Pelikan, Snoek et al. 1970, Booij-Noord, Orie et al. 1971). In fact in recent years most of the currently approved asthma therapies have shown efficacy in inhibiting the response elicited by either inhaled allergen or exercise; this includes ICS (Vathenen, Knox et al. 1991, Gauvreau, Doctor et al. 1996), combination of ICS/LABA (Weiler, Nathan et al. 2005, Dahlen, Lantz et al. 2009) and leukotriene antagonists (Dahlen, Zetterstrom et al. 1994, Leff, Busse et al. 1998, Diamant, Grootendorst et al. 1999).

AHR to a certain stimulus during a bronchial challenge is reflected both by the magnitude and the ease of the induced bronchoconstriction (Woolcock, Salome et al. 1984). An increase in the magnitude of the reaction is defined by a progressive elevation of the plateau response in the dose-response curve and in the ease of bronchoconstriction by a leftward shift of the curve. The leftward shift can be further defined by the reduced provocation concentration or dose producing a 20% fall in $FEV_1$, called the $PC_{20}$ or $PD_{20}$ respectively. Even if the magnitude of the bronchoconstriction is an important parameter, traditionally AHR is defined by the $PC_{20}/PD_{20}$.

Bronchial provocations are usually categorized into direct and indirect depending on the agent that is used and the mechanism of action (Figure 1) (Pauwels, Joos et al. 1988). Direct agents act directly on airway smooth muscle receptors (e.g., muscarinic receptors for methacholine, $H_1$ receptors for histamine, $CysLT_1$ receptors for cysteinyl leukotrienes) but also in mucous glands and on airway microvasculature without involving intermediate pathways. Direct challenges, for example methacholine, are widely used and are highly sensitive with a negative predictive value approaching 100% provided symptoms are clinically current (Hopp, Bewtra et al. 1984, Cockcroft, Murdock et al. 1992, Crapo, Casaburi et al. 2000). An exception to that are high-intensity or elite athletes who may have EIB with a negative methacholine challenge (Holzer, Anderson et al. 2002). Specificity on the other hand of methacholine is low and a positive test is not diagnostic of asthma especially in the absence of typical symptoms, though it may indicate

airway dysfunction. The positive predictive value (PPV) of a histamine challenge $PC_{20}$ below 8mg/ml in a random population was less than 50% (Cockcroft, Murdock et al. 1992), which improved with an increased pre-test probability (increased asthma probability based on symptoms) and was even higher if the provocation induced symptoms which mimicked the symptoms that the patient reported (Crapo, Casaburi et al. 2000). When using a lower threshold for the test (1mg/ml), PPV increased almost to 100% with a much lower sensitivity of course. It must also be noted that direct AHR reflects airway smooth muscle function and airway calibre and is influenced by remodelling, which can explain why some COPD patients might also have a positive methacholine test. AHR in these patients is possibly caused by a geometric phenomenon related to the airway lumen obstruction (Ramsdell, Nachtwey et al. 1982, Ramsdale, Morris et al. 1984). Thus it is difficult to interpret a positive methacholine test for asthma diagnosis in patients with resting airflow obstruction.

**Indirect**

**Aspirin, AMP**
**Allergen, Exercise**
**EVH, Mannitol**
**Hypertonic saline**

**Direct**

**Histamine**
**Methacholine**
**Leukotriene $D_4$**

Direct – hyperreactivity
Indirect – inflammation (+ hyperreactivity)
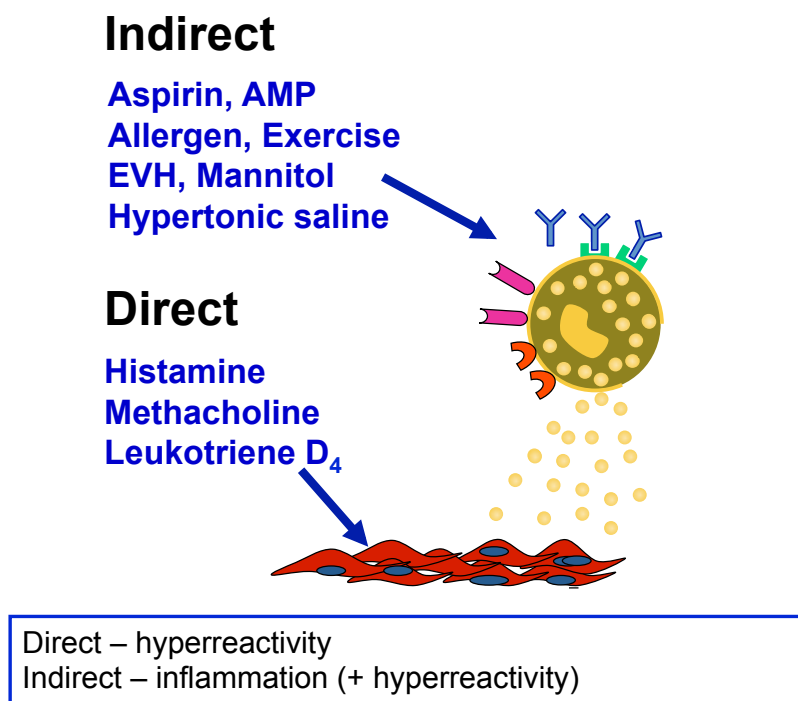
**Figure 1: Categorization of bronchial provocations according to their mode of action**

In contrast to direct challenges, indirect stimuli act through different intermediate pathways mostly through inflammatory (predominantly mast cells) and neuronal cells, which then release mediators and cytokines to cause bronchoconstriction. These stimuli are osmotic (exercise, EVH, mannitol, hypertonic saline) or non osmotic (AMP, propranolol, allergen, aspirin) and because the responses to these challenges are modified or inhibited by ICS they are considered to reflect closer active airway inflammation and to be more clinically relevant for asthma (Joos, O'Connor et al. 2003). A wide spectrum of mediators such as histamine, leukotrienes, prostaglandins, neuropeptides are involved in the bronchoconstriction induced by indirect stimuli (Van Schoor, Joos et al. 2000). Data from previous studies have shown that compared to direct challenges (methacholine), indirect provocations can differentiate better between asthma and COPD (Joos, O'Connor et al. 2003, Hassan, Hargreave et al. 2010), correlate better with airway eosinophilia and improve more with ICS treatment (Taylor, Jensen et al. 1999, Hofstra,

Neijens et al. 2000). After the completion of an indirect challenge tachyphylaxis to a second indirect stimulus is frequently observed (cross refractoriness) (Van Schoor, Joos et al. 2000, Larsson, Perry et al. 2011) a phenomenon, which is not so evident after repeated challenges with inhaled histamine or methacholine (Laprise and Boulet 1996). Another characteristic of the indirect tests is that they can be inhibited by pre-treatment with single doses of cromones (sodium cromoglycate, nedocromil), which are known mast cell stabilizing agents and thus indicating that mast cells are activated during these challenges (Van Schoor, Joos et al. 2000, Cockcroft and Davis 2009).

## 3.2 AIRWAY RESPONSE TO CHALLENGES AND HOW TO MEASURE IT

Over the past years, spirometry with measurement of $FEV_1$ has been the method of choice in order to measure bronchoconstriction following an airway challenge. Already in 1968, Pepys et al used repeated measurements of $FEV_1$ in order to describe the effect of treatment with sodium cromoglycate on both the EAR and LAR following an allergen inhalation challenge. In the following years, some bronchoprovocation studies used a 35% fall in specific airway conductance (SGaw) as outcome for calculation of $PC_{35}$ and not $PC_{20}$ $FEV_1$ (Arm, O'Hickey et al. 1990). A comparison of the two methods by Cockcroft and colleagues showed that the $PC_{35}$ SGaw was consistently approximately four-fold lower than the $PC_{20}$ $FEV_1$ both in mildly hyperreactive asthmatics as well as moderately hyperreactive asthmatics and concluded that $FEV_1$ may be preferable to SGaw because of better separation of asthmatics from other groups (Cockcroft and Berscheid 1983). Thus, $FEV_1$ is now considered the method of choice to detect changes in lung function in the bronchoprovocation setting, although it has its own pitfalls. The main problem is that in many subjects it is difficult to obtain good quality, highly reproducible flow-volume curves, which are essential when performing an airway challenge, because spirometry requires effort-dependent expiratory manoeuvres. This becomes more evident in preschool children and the elderly who might not be able to perform spirometry at all. In addition, it is known that $FEV_1$ reflects mainly changes in the larger airways (Annesi, Oryszczyn et al. 1992), without providing any information about the smaller airways that have been a major focus in asthma research in recent years.

### 3.2.1 Small airways impairment following a bronchial challenge

Small airways (less than 2 mm luminal diameter) have been a major focus of respiratory research over the past few years. It is now evident that airway inflammation involves also the peripheral airways (Wenzel, Szefler et al. 1997, Gelfand and Kraft 2009, Hyde, Hamid et al. 2009). This has resulted in the development of inhalers containing extra-fine particles of ICS alone or in combination with LABA, that demonstrate better deposition in the peripheral airways compared to conventional devices (De Backer, Devolder et al. 2010). A major step towards this development in order to be able to understand better the pathophysiology of the small airways was the introduction of impulse oscillometry (IOS) as an alternative technique to assess lung function. IOS is one type of forced oscillation technique (FOT), that was first developed by Dubois et al (Dubois, Brody et al. 1956) over 50 years ago. The method uses the superimposition of pressure fluctuations in the airway over the subject`s normal breathing. IOS

delivers a regular square wave of pressure 5 times per second with the advantage of providing more detailed characterization of respiratory function compared to other FOT techniques (Smith HJ 2005). IOS is non-invasive, requires only quiet, tidal breathing and provides information about both lung resistance (Rrs) as well as reactance (Xrs) in various frequencies, which together reflect total pulmonary impedance (Zrs). Measurements are under one minute long, thereby pressure and flow volume tracings are produced giving the investigator the possibility to choose the best tracings afterwards. IOS has been widely used to measure lung function in children (Marotta, Klinnert et al. 2003), as well as evaluate AHR after bronchial challenge with methacholine (Aronsson, Tufvesson et al. 2008), mannitol (Horsman, Duke et al. 2009) and exercise (Lee, Lee et al. 2010). Furthermore, it has been reported that significant increases in Rrs can be seen earlier than a $FEV_1$ response during a methacholine challenge, suggesting that IOS is more sensitive than spirometry (Schulze, Smith et al. 2012).

## 3.3 MAST CELLS AND THEIR ROLE IN BRONCHIAL CHALLENGES

Although discovered for more than hundred years ago, the role of mast cells in human pathophysiology is not clearly outlined. They are usually located around blood vessels and mucosal surfaces that are more exposed to the external environment, such as the skin or the lungs. They normally contribute to tissue homeostasis and defence against bacterial infections (Echtenacher, Mannel et al. 1996) as well as tissue damage and revascularization (Weller, Foitzik et al. 2006). Mast cells are central effectors in asthma and allergies, both in acute reactions but also in sustaining the chronic inflammation in the airways (Bradding, Walls et al. 2006). They can be activated through a variety of stimuli although the classical and best-studied mechanism which is also implicated in the EAR following an allergen challenge is through the high affinity IgE receptor FcεRI, causing mast cell degranulation. The mediators released upon activation had been stored (e.g. histamine, heparin, tryptase), *de novo* synthetized upon activation (e.g. $PGD_2$, leukotrienes) or both (e.g.TNF-a, interleukins). In asthma compared to healthy airways, mast cells are not increased in number (Laitinen, Laitinen et al. 1993a), although they infiltrate some of the most important structures in the airways i.e. the airway epithelium, the mucosal glands and the airway smooth muscle (Carroll, Mutavdzic et al. 2002, Begueret, Berger et al. 2007, Dougherty, Sidhu et al. 2010). Mast cells play a key role for EIB pathogenesis as will be discussed later on as well as for both the EAR and LAR after exposure to allergen. Previous studies have shown a rapid increase in the concentration of the specific mast cell marker tryptase in bronchoalveolar lavage (BAL) fluid within minutes following a local bronchial allergen challenge (Wenzel, Fowler et al. 1988, Salomonsson, Gronneberg et al. 1992). In recent years, non-invasive methods have been developed in order to confirm mast cell activation in asthma and in the bronchoprovocation setting, namely the measurement of mast cell derived lipid mediators in body fluids.

### 3.3.1 Mast cell derived lipid mediators and their receptors

Mast cell activation and degranulation leads to the release of preformed mediators, such as histamine and tryptase but also *de novo* synthesis and release of several lipid-derived molecules. These substances are derived from arachidonic acid (AA) that is cleaved from the cell membrane

8

by phospholipase $A_2$ and the products are called eicosanoids because they contain 20 carbon atoms. These include prostaglandins (PG), leukotrienes (LT), thromboxanes (TX), lipoxins (LX) as well as hydroxyl and hydroperoxy fatty acids (HETE and HPETE).

The synthesis of PGs begins with cyclooxygenase (COX) catalysed reactions. AA is first oxygenated to $PGG_2$, which is a highly unstable metabolite. $PGG_2$ is then further metabolised to $PGH_2$ through a peroxidase reaction. $PGH_2$ is then subsequently metabolised to the individual prostanoids depending on which specific synthase that is expressed by the individual cells. The PGs are divided into two classes; the stimulatory PGs namely $PGD_2$, $PGF_{2\alpha}$ and $TXA_2$ that have bronchoconstrictive effects and inhibitory PGs such as $PGE_2$ that can inhibit bronchoconstrictive responses and release of bronchoconstrictive mediators (Claar, Hartert et al. 2015). The PGs are metabolised further primarily through 15-hydroxy dehydrogenation by 15-PGDH and $\Delta^{13}$ reduction by 13-15 ketoprostoglandin reductase (13-PGR). Oxidation of $TXB_2$ follows another pathway catalysed by $11\text{-}TXB_2DH$. The final metabolites are formed after subsequent β- and ω-oxidation and excreted in the urine.

All prostanoids act through distinct transmembrane, G-protein coupled receptors (GPCRs). The biological effects of $PGD_2$ are mediated through $DP_1$, $DP_2$ that is also known as $CRTH_2$ and TP receptors (Pettipher, Hansel et al. 2007). The bronchoconstrictive effects of $PGD_2$ are mediated through the TP receptors (Coleman and Sheldrick 1989). $PGD_2$ also has a chemotactic effect for eosinophils and $TH_2$ lymphocytes, as well as a modulatory effect of $TH_2$ cytokine production, which are mediated by $PGD_2$ stimulation of the $CRTH_2$ receptor (Hirai, Tanaka et al. 2001). The inhibitory prostanoid $PGE_2$ acts through four GPCRs that are termed EP receptors 1 through 4. All four are expressed in the lungs and activation of $EP_2$ and $EP_4$ by $PGE_2$ increased intracellular cAMP concentrations resulting in smooth muscle relaxation (Coleman, Smith et al. 1994). Stimulation of the $EP_2$ receptor by $PGE_2$ inhibits mast cell mediator release (Safholm, Manson et al. 2015)

The synthesis of LTs begins when activated 5-lipoxygenase (5-LOX) translocate near the nuclear membrane where it interacts with 5-lipoxygenase-activating protein (FLAP). Although FLAP has no enzymatic activity, it enhances the ability of 5-LOX to convert AA to the unstable metabolite 5-hydroxyperoxyeicosatetranoic acid (5-HPETE). 5-HPETE can then be converted to 5-HETE or to leukotriene $A_4$ ($LTA_4$) by 5-LOX. Depending on the type of cell, $LTA_4$ can be then converted to leukotriene $B_4$ ($LTB_4$) (e.g. in neutrophils) or it can be conjugated with glutathione by leukotriene $C_4$ ($LTC_4$) synthase (e.g. in mast cells and eosinophils) in order to convert to $LTC_4$. $LTC_4$, which is the first of the family of the cysteinyl leukotrienes (CysLTs) is then exported from the cell. The released $LTC_4$ is rapidly converted to leukotriene $D_4$ and subsequently to leukotriene $E_4$ ($LTE_4$) by sequential amino acid hydrolysis. The largest part of $LTE_4$ is eliminated through the faecal route, while about 20% is excreted into the urine within 24 hours (Maltby, Taylor et al. 1990).

There is well-documented pharmacological evidence confirming the existence of two receptor subtypes for the CysLTs, namely $CysLT_1$ and $CysLT_2$. The nomenclature was based on the fact that the $CysLT_1$ receptor was sensitive to inhibition by classical antagonists like zafirlukast,

pranlukast and montelukast, whereas the effects mediated by the $CysLT_2$ receptor were not inhibited by these antagonists (Back, Dahlen et al. 2011). They belong to the rhodopsin family of the GPCR gene and are located on the outer plasma membrane of various structural and inflammatory cells (e.g. mast cells, eosinophils, neutrophils, smooth muscle cells). When CysLTs are ligated to the receptor, there is an increase in intracellular calcium and reductions in intracellular cAMP that activates kinase cascades downstream. The $CysLT_1$ receptor has an important role in asthma pathophysiology and upon activation mediates bronchoconstriction, mucous secretion and airway oedema. The $CysLT_2$ receptor is not involved in bronchoconstriction but may contribute to increased inflammation, vascular permeability and tissue fibrosis (Back, Dahlen et al. 2011).
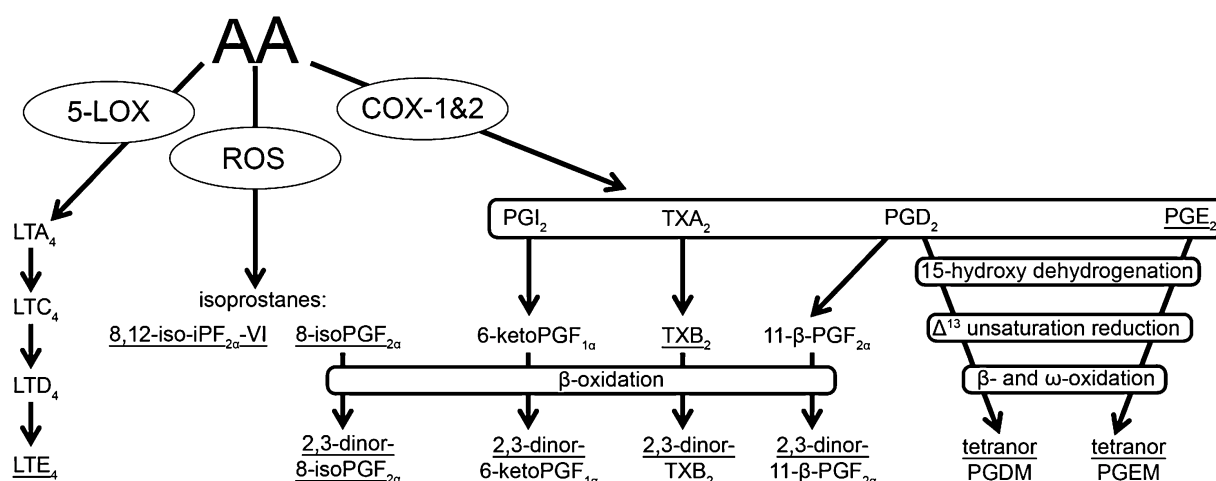


**Figure 2: Simplified overview of eicosanoid metabolism. Underlined metabolites can be measured in urine. Reproduced with permission from Balgoma et al 2013.**

### 3.3.2   Urinary excretion of lipid mediators

Measurement of eicosanoids is a challenge due to the fact that they are very rapidly metabolised and removed from the circulation (Samuelsson, Granstrom et al. 1975). In addition, it has been shown that it is difficult to measure them in the blood because some eicosanoids like $TXB_2$ can be produced *ex vivo* when withdrawing the blood sample (Patrono, Ciabattoni et al. 1986). Hence, measurement of the different eicosanoid metabolites in the urine has been used as alternative method. Primary $PGD_2$ is not detectable in urine, instead a variety of metabolites have been discovered. The earliest metabolite was $11\beta$-$PGF_{2\alpha}$ that has some biological activity and is further metabolised to $2,3$-dinor,$11\beta$-$PGF_{2\alpha}$. The most abundant $PGD_2$ metabolite in the urine is tetranor-PGDM. For $PGE_2$ it is demonstrated that a very small part of the primary mediator is measured in the urine and that the main measured metabolite is tetranor-PGEM. Likewise, $TXB_2$ was measured in small amounts and 11-DH-$TXB_2$ and 2,3-dinor-$TXB_2$ are the major metabolites. The terminal product of the CysLT metabolism, namely $LTE_4$ is also measured in the urine. The method of measuring lipid mediator metabolites in the urine is now widely accepted and has been used for many years in the bronchoprovocation setting in order to investigate important pathophysiological mechanisms in asthma (Kumlin, Dahlen et al. 1992, Dahlen and Kumlin 1998, Brannan, Gulliksson et al. 2003, Bood, Sundblad et al. 2015). In

10

particular, measurement of $PGD_2$ metabolites in the urine following an airway challenge is considered a sign of mast cell activation because $PGD_2$ is released predominately by mast cells and to a very small proportion by macrophages.

## 3.4 RATIONALE FOR THE PERFORMED STUDIES

### 3.4.1 Exercise induced bronchoconstriction (EIB)

Exercise induced bronchoconstriction (EIB) is defined as a syndrome, where airflow limitation lasting 30 to 90 minutes in the absence of treatment is triggered from a brief period of vigorous exercise. It is known that EIB is a common feature in asthma, with much higher prevalence up to 70% among elite-athletes in some studies, depending on the implemented methods (Parsons, Hallstrand et al. 2013). Exercise-related symptoms have a substantial impact on daily life and about 45% of adults with asthma reported that they avoid physical activities due to symptoms (Parsons, Craig et al. 2011).

#### 3.4.1.1 Pathogenesis of EIB

It is useful to think of EIB as an asthma component and not an isolated disorder; because exercise itself does not cause asthma, the often-used term "exercise-induced asthma" should be avoided. It is well established that a similar reaction in the airways can be triggered by increase in ventilation or by using hypertonic aerosols like mannitol, that activate the same pathways. The amount of ventilation, as well as the water content and temperature of the inspired air are critical factors for the development of EIB. At tidal breathing at rest, minimal conditioning of the inspired air takes place beyond the upper airways and the trachea, while during periods of high ventilation large amounts of incompletely conditioned air penetrate the lower airways over a short period, leading to water loss from the airway surface with resulting stress to the epithelium (Anderson and Schoeffel 1982, Zawadski, Lenner et al. 1988, Gilbert and McFadden 1992). As water is moved from the airways, the osmotically sensitive epithelium rapidly corrects the osmolarity of the airway surface liquid through water movement (Tarran 2004). Though this hyperosmolarity through evaporative water loss is transient (Kotaru, Hejal et al. 2003), it serves as the initial stimulus to the epithelium for subsequent release of adenosine tripshosphate (ATP), which through specific GPCRs activates chloride channels and increases intracellular calcium (Tarran 2004). This epithelial stress/injury is demonstrated by an increased concentration of columnar epithelial cells in induced sputum in asthmatics with EIB compared to asthmatics without EIB (Hallstrand, Moody et al. 2005a). Not only epithelial cells, but also mast cells are activated from hyperosmolar stimuli. It is known that after an exercise challenge histamine and the mast cell protease tryptase are released in the airways (Hallstrand, Moody et al. 2005b). Release of bronchoconstrictive eicosanoids such as CysLTs and $PGD_2$ is also increased after exercise challenge (O'Sullivan, Roquet et al. 1998) and pre-treatment with the mast cell stabilizing drug sodium cromoglycate has a protective effect against mannitol-induced bronchoconstriction (Anderson, Brannan et al. 2010), thus supporting the key role of mast cells in the pathogenesis of EIB.

*3.4.1.2 Diagnosis and treatment of EIB*

The diagnosis of EIB should be made by changes in lung function provoked by exercise and should not be based only on exercise related symptoms, which are neither sensitive nor specific in order to identify subjects with EIB (Parsons, Hallstrand et al. 2013). It is recommended that an indirect challenge (e.g. exercise with dry air or a surrogate challenge) should be performed to establish EIB diagnosis instead of a direct challenge (e.g. methacholine), due to the higher sensitivity to detect EIB (Parsons, Hallstrand et al. 2013, Weiler, Brannan et al. 2016). Surrogate challenges include EVH, inhalation of dry powder mannitol or hyperosmolar aerosols of 4.5 % saline and are considered easier to perform compared to the exercise challenge.

Inhalation of a $\beta_2$ agonist, SABA or LABA, prior to exercise or after EIB has occurred is effective in reducing or even abolishing EIB (Jones, Wharton et al. 1963, Anderson, Caillaud et al. 2006). Nonetheless, it is known that with regular treatment, the bronchoprotective effect of $\beta_2$ agonists diminishes over time and does not offer the same degree of protection as after the first dose (Cheung, Timmers et al. 1992, O'Connor, Aikman et al. 1992). In subjects with insufficient control, despite using $\beta_2$ agonists prior to exercise, the addition of controller medications, such as ICS or LTRAs is recommended (Parsons, Hallstrand et al. 2013). Although both ICS and LTRAs can reduce the degree and severity of EIB, they do not completely abolish it, meaning that subjects still need to rely on their SABA for symptom relief. If it is taken in mind that these subjects have predominately mild asthma with no other or minimal asthma symptoms, besides the ones that are triggered by exercise, this treatment option would lead inevitably to poor adherence to the therapy. An alternative treatment option, which is investigated in **Paper I** is the use of a fixed combination of ICS with a LABA that has a rapid onset of action, like budesonide/formoterol (bud/form) taken on demand i.e. before exercise and for symptom relief.

## 3.4.2 Asthmatic response to allergen and anti-IgE therapy

*3.4.2.1 Early and late asthmatic response to inhaled allergen*

In the airways sensitization begins when antigen-presenting cells and dendritic cells (DCs) detect the inhaled allergen through their FcεRI receptors and present it to naïve T-cells that stimulate the development of Th2 cells. These release a variety of type-2 cytokines (IL-4, IL-5, IL-9 and IL-13) that promote IgE production, eosinophil production and maturation, mast cell development as well as goblet cell hyperplasia and increase in AHR (Gauvreau, El-Gammal et al. 2015). These cytokines can be also released from other cells, e.g. type II innate lymphoid cells (ILC2). The response to inhaled allergen is initiated when IgE that is bound to FcεRI receptors on the surface of mast cells and basophils cross-links to the allergen leading to mast cell degranulation and release of preformed mediators, such as histamine and chemotactic factors, as well as activation of the eicosanoid pathways and release of newly formed mediators, such as CysLTs and PGD$_2$. These mediators are potent bronchoconstrictors and are responsible for the EAR, which is observed shortly after an allergen challenge, reaches max at 10 to 20 minutes or slightly later and resolves spontaneously by 2 to 3 hours (O'Byrne, Dolovich et al. 1987). EAR slightly resembles exercise in its time course although it is more prolonged.

Approximately 30 to 50% of subjects challenged with inhaled allergen will also develop a late asthmatic response (LAR). The LAR consists of an episode of airway obstruction that appears after the spontaneous resolution of the EAR, which is usually about four to five hours but can be measured up to 8 to 12 hours after an allergen challenge (Diamant, Gauvreau et al. 2013). The type of the allergen used for the challenge seems to have importance for the development of LAR, with house dust mites inducing a greater response than animal allergens and pollens (Boulet, Gauvreau et al. 2015). The bronchoconstriction observed during the LAR is also caused by release of histamine and CysLTs and treatment with a combination of antihistamine and antileukotriene predominately inhibited both responses to inhaled allergen (Roquet, Dahlen et al. 1997, Davis, Illamperuma et al. 2009). Nonetheless, mast cell activation and degranulation is not the only important mechanism involved in the airway response to allergen; it is known that LAR is related to eosinophilic airway inflammation measured in BAL (Djukanovic, Feather et al. 1996), as well as in sputum (Gauvreau, Watson et al. 1999) and release of type-2 cytokines. It has been shown that asthmatics that develop isolated EAR have a smaller increase in sputum eosinophils compared to those that develop LAR with the same degree of bronchoconstriction (Imaoka, Gauvreau et al. 2011) and that exercise, which also activates the mast cell does not cause eosinophilic inflammation (Gauvreau, Ronnen et al. 2000). Another important mechanism involved in the allergen-induced responses is the role of DCs, which are the most potent antigen-presenting cells in the airways and are also involved in the regulation of the allergen response in the airways. In fact, it is shown that after an allergen challenge in asthmatics, there is a rapid reduction of circulating DCs in the blood (Upham, Denburg et al. 2002) with a subsequent increase in the bronchial mucosa (Moller, Overbeek et al. 1996) and in induced sputum 24 hours after the challenge (Dua, Watson et al. 2010).

### 3.4.2.2 Treatment with anti-IgE

Thus, it is evident that the interaction of IgE with its receptors is crucial for the development of both the EAR and the LAR after allergen inhalation. This has led to the approach of developing antibodies that are directed against the region of the IgE molecule that binds to the IgE receptors and hence interrupts the allergic cascade by preventing IgE binding with FcεRI receptors on mast cells, basophils, DCs and other inflammatory cells. Omalizumab is a humanized anti-IgE monoclonal antibody directed against an epitope on the constant region (Cε3) of the IgE molecule, which is the region that binds to the IgE receptors. Omalizumab does not interact with the variable allergen-specific region of IgE and that is why it inhibits the allergic response regardless of allergen specificity. Moreover, omalizumab does not bind to cell-bound IgE because in those IgE the epitope is already attached to the receptors and thus avoids FcεRI crosslinking that could increase the anaphylaxis risk (Holgate, Casale et al. 2005). This binding of IgE by omalizumab leads to a rapid decrease in serum free IgE, that in turn causes a downregulation of FcεRI surface expression on effector cells, such as mast cells, basophils as well as DCs (MacGlashan, Bochner et al. 1997, Prussin, Griffith et al. 2003, Beck, Marcotte et al. 2004). This reduction of FcεRI expression on mast cells contributes to further dampening of the effector cell response to allergen, as well as reduced facilitated allergen presentation by DCs (Oliver, Tarleton et al. 2010, Kuhl and Hanania 2012).

Omalizumab has been effective in inhibiting both the EAR and the LAR following an allergen inhalation challenge in previous studies (Boulet, Chapman et al. 1997, Fahy, Fleming et al. 1997, Zielen, Lieb et al. 2013). In addition, treatment with omalizumab has shown efficacy in reducing the allergen-induced responses on the skin. Using an intradermal allergen challenge model, it was shown that omalizumab given at the approved dose suppressed both the early and late phase allergen induced cutaneous response (Ong, Menzies-Gow et al. 2005). In the study by Corren et al, treatment with high doses of omalizumab given intravenously (i.v.) suppressed the allergen-induced wheal and flare reactions on skin prick tests, although this effect returned to baseline after discontinuation of treatment (Corren, Shapiro et al. 2008). Beyond the provocation setting, there are several studies that have investigated the clinical efficacy of omalizumab in subjects with moderate-to-severe allergic asthma. A pooled analysis of data from seven clinical trials with omalizumab (two open label and five double-blind, placebo controlled) showed that omalizumab reduced the rate of asthma exacerbations by 38 % and the rate of total emergency visits by 47 % (Bousquet, Cabrera et al. 2005). Moreover, omalizumab treatment reduced asthma symptoms and was associated with improvements in asthma-related quality of life as measured by the asthma quality of life questionnaire (AQLQ) in subjects with severe persistent asthma (Humbert, Beasley et al. 2005).

However, omalizumab therapy has its caveats and some patients will not respond to treatment. In a 2-year real world effectiveness study with 943 patients with uncontrolled persistent asthma treated with omalizumab, 30.1 % were assessed as non-responders to treatment according to physicians global evaluation of treatment effectiveness (GETE) (Braunstahl, Chen et al. 2013). It is unclear why some subjects do not respond to omalizumab treatment, as it is also difficult to predict responders. Interestingly, Johansson et al (Johansson, Oman et al. 2006) reported that specific IgE antibodies to allergens, such as cat and house dust mite, consisted a much higher fraction of total IgE in patients with low serum IgE levels (30-74 kU/L) compared to patients with higher levels. Using a mathematical model the authors concluded that despite suppression of serum IgE to 10 kU/L or lower, 25 % of this population would still have enough concentrations of specific IgE in order to elicit an allergic response. Because omalizumab dosing depends on bodyweight and the amount of total IgE, these results indicate that the recommended dose of omalizumab would be insufficient for those patients and higher doses would be necessary. An alternative approach in this group of patients would be to use the basophil allergen threshold sensitivity test (CD-sens), as a means to monitor omalizumab treatment more effective (Nopp, Johansson et al. 2006, Dahlen, Nopp et al. 2011).

Nonetheless, it is evident that there is a need for better and more effective anti-IgE treatment. Correlations between free IgE levels and asthma symptoms indicate that more effective IgE suppression leads to better asthma control and more clinical benefits (Lowe, Tannenbaum et al. 2009). QGE031 (ligelizumab) is a fully humanized monoclonal antibody that binds to the Cε3 domain of IgE with higher affinity than omalizumab and is designed to suppress free IgE and IgE bound to mast cells and basophils in greater extent than omalizumab (Arm, Bottoli et al. 2014). In **Paper II** the efficacy of QGE031 to suppress the EAR after an allergen inhalation challenge was compared to omalizumab and placebo.

14

### 3.4.3 What is known about the role of $LTE_4$ in asthma

The CysLTs are potent bronchoconstrictors of human airways (100 to 1000 times more potent than histamine) with equal potency of $LTC_4$ and $LTD_4$ (Adelroth, Morris et al. 1986). $LTE_4$ is the most stable and abundant CysLT *in vivo* and $LTE_4$ has been detected in BAL fluid and urine in patients with severe asthma, acute asthma exacerbations as well as aspirin exacerbated respiratory disease (AERD) (Christie, Tagari et al. 1991, Kumlin, Dahlen et al. 1992, Vachier, Kumlin et al. 2003, Green, Malice et al. 2004, Austen, Maekawa et al. 2009). $LTE_4$ is also a potent bronchoconstrictor in human subjects (Davidson, Lee et al. 1987), although its potency as agonist of the two known CysLT receptors is variable (Lynch, O'Neill et al. 1999, Heise, O'Dowd et al. 2000). Although $LTE_4$ is similar to $LTC_4$ and $LTD_4$ in its bronchoconstrictor activity, it has been reported to have a unique relationship with one of the cardinal features of asthma, namely AHR. Previous studies suggest that airway responsiveness to inhaled $LTC_4$ and $LTD_4$ is relatively greater in healthy than in asthmatic subjects (Holroyde, Altounyan et al. 1981, Weiss, Drazen et al. 1982, Griffin, Weiss et al. 1983), whereas subjects with asthma are relatively more sensitive to $LTE_4$. Accordingly, asthmatic subjects were 26 times more sensitive to inhaled $LTE_4$ than healthy subjects, whereas they were only 7 times more sensitive to methacholine and histamine, suggesting that asthmatic airways selectively might be hyperresponsive to $LTE_4$ in contrast to other CysLTs (O'Hickey, Arm et al. 1988). This observation was confirmed and extended in a subsequent study by the same investigators in which the relative potencies of $LTC_4$, $LTD_4$ and $LTE_4$ were directly compared with those of histamine and methacholine in the same healthy and asthmatic subjects. Compared with healthy subjects, the airways of the asthmatic subjects were on average only 14-fold more responsive to inhaled histamine, 16-fold more responsive to methacholine, 6-fold more responsive to $LTC_4$ and 9-fold more responsive to $LTD_4$ but 219-fold more responsive to $LTE_4$ supporting that the mechanism of the bronchoconstriction induced by $LTE_4$ might be distinct from that produced by $LTC_4$ and $LTD_4$, and possibly reflecting functionally important CysLT receptor heterogeneity (Arm, O'Hickey et al. 1990).

In addition to bronchoconstriction there seem to exist also differences in the pro-inflammatory effects of CysLTs; inhalation of $LTE_4$ increased the number of eosinophils in both sputum and airway mucosa in patients with mild asthma (Laitinen, Laitinen et al. 1993a, Gauvreau, Parameswaran et al. 2001, Laitinen, Lindqvist et al. 2005), whereas $LTD_4$ was not as effective (Mulder, Gauvreau et al. 1999). These observations has led to the theory that there might be a third separate receptor that could be controlling the pro-inflammatory effects and the eosinophilic response to $LTE_4$. Experiments with $CysLT_1$ and $CysLT_2$ receptor double knockout mice have shown the existence of a pro-inflammatory pathway independent of these two receptors having ligand specificity for $LTE_4$ (Maekawa, Kanaoka et al. 2008). Further experimental studies on mice have tried to identify such a receptor with the ADP-reactive platelet $P2Y_{12}$ receptor being one candidate (Paruchuri, Tashimo et al. 2009). Moreover, studies in genetically modified mice have identified GPR99 as a predominantly epithelial receptor that is distinctly sensitive to $LTE_4$; mice lacking all three receptors i.e. $CysLT_1$, $CysLT_2$ and GPR99 lost all ability to respond to CysLTs, including $LTE_4$ (Kanaoka, Maekawa et al. 2013). Similarly,

in experiments in mice it was shown that GPR99 was expressed in respiratory epithelial cells and mediated mucin release in response to $LTE_4$ (Bankova, Lai et al. 2016). This has however not been confirmed in human subjects; current LTRAs such as montelukast target only the $CysLT_1$ receptor and may thus not provide sufficient anti-inflammatory effect against inhaled $LTE_4$. All in all, if the hypothesis of a functionally important and distinct $LTE_4$ receptor is correct, there will be a scientific rationale to develop broader antagonists of CysLTs that also block such a receptor. In **Paper III** an inhalation challenge with $LTE_4$ in subjects with mild asthma was performed prior to and after treatment with montelukast, in order to further elucidate the role of $LTE_4$ in asthma and airway inflammation.

# 4 MATERIAL AND METHODS

## 4.1 SUBJECTS AND STUDY DESIGN

**Paper I**



| Visit 1 | Visit 2 | Visit 3 | Visit 4 | Visit 5 | Visit 6 |
|---------|---------|---------|---------|---------|---------|
| -21-8 days | -6-2 days | 0 days | 21 days ±4 | 42 days ±4 | 42 days +2-5 |

**A** = budesonid 400µg x1 + terbutaline 0.5mg before exercise and as needed

**B** = placebo x1 + terbutaline 0.5 mg before exercise and as needed

**C** = placebo x1 + budesonid/formoterol 200/6µg before exercise and as needed
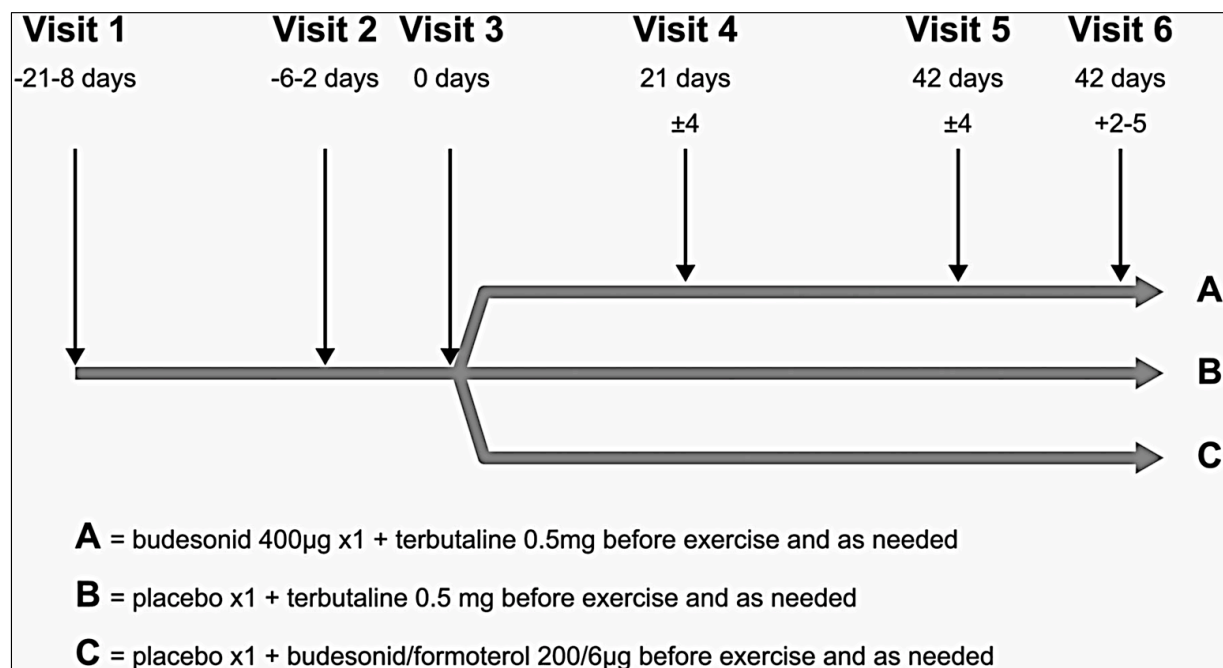
**Figure 3. Study design Paper I**

The aim of **Paper I** was to evaluate the magnitude of the bronchoprotective effect of three different pharmacological treatments on EIB in adults and adolescents with mild intermittent asthma. Regular daily treatment with budesonide and terbutaline on demand (arm A) was compared with terbutaline inhaled on demand (arm B), which still is the currently recommended treatment, and with a fixed combination of bud/form inhaled only on demand (arm C). On demand means that subjects inhaled the medication before exercise and for symptom relief. The hypothesis was that treatment with the fixed combination on demand would be superior to monotherapy with terbutaline on demand and non-inferior to regular budesonide treatment with terbutaline on demand regarding protection against EIB.

Sixty-six subjects with mild asthma according to the guidelines at the time of the study (Bateman, Hurd et al. 2008) were recruited from 10 study sites in Sweden and Norway. Subjects performed physical exercise three to four times per week and had a history of EIB using a reliever medication also for prevention up to four times per week. Adolescents were $\geq$ 12 years of age and all subjects had a $FEV_1$ greater than 80% of the predicted normal value (Solymar, Aronsson et al. 1980, Quanjer, Tammeling et al. 1993). The study was registered at ClinicalTrials.gov (NCT00989833).

On the first screening day spirometry, a standard skin prick test, physical examination and a maximal exercise test on a treadmill were performed while breathing ambient air in order to calculate the maximal aerobic capacity. Control of vital signs, medical history, concomitant

medications was also conducted and all subjects filled out a shortened version of the Asthma Control Questionnaire ACQ5 (Juniper, Svensson et al. 2005). The ACQ5 was also filled at visits 3, 4 and 6.

On the second screening day, a six-minute standardized exercise test on a treadmill at 90% of maximal aerobic capacity was performed, while subjects were breathing dry air. $FEV_1$ was measured up to 30 min after exercise cessation. If a fall of $\geq 10\%$ compared to pre-exercise value was recorded, the subject was included. At the third visit a mannitol bronchial challenge was performed, and subjects were randomized to one of the three treatment arms. They also gained access to an electronic diary for daily recording of physical exercise, asthma symptoms, and use of as needed medication. They were instructed to perform physical exercise three to four times per week, and use the as needed medication for prevention and treatment of asthma symptoms during exercise. The six-minute standardized exercise test was repeated three weeks (visit 4) and six weeks (visit 5) after randomization to treatment. The mannitol bronchial challenge was repeated at visit 6.

All subjects abstained from asthma medications 24 hours prior to all exercise tests, thus the aim was not to study the direct effect of treatment on EIB but the long-term bronchoprotective effect of the medication given.
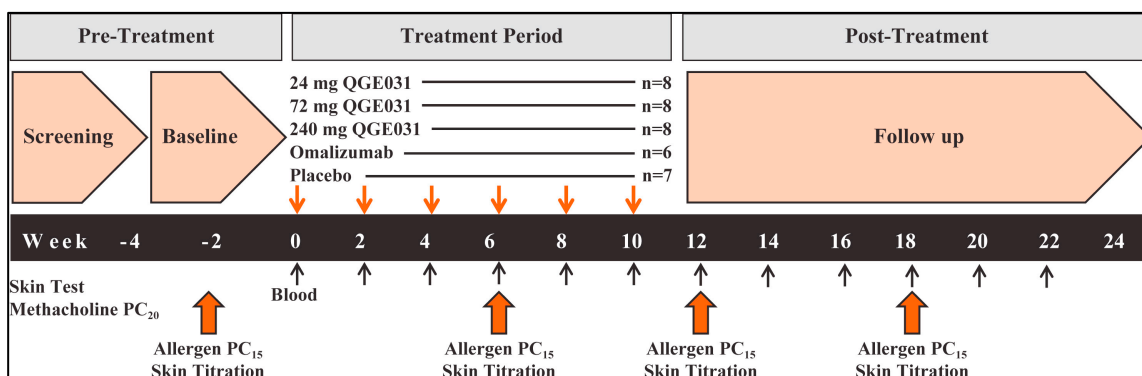
**Paper II**



**Figure 4. Study design Paper II**

The aim of **Paper II** was to compare the potency of a new anti IgE drug QGE031 (ligelizumab) with omalizumab and placebo in inhibiting the early asthmatic response (EAR) to inhaled allergen. The efficacy of three doses of QGE031 in inhibiting EAR as well as the pharmacokinetics, pharmacodynamic effects of QGE031 on total IgE, basophil bound IgE, basophil FcɛRI levels and skin prick test responses to allergen were also assessed. The hypothesis was that treatment with the highest dose of QGE031 (240mg) would be more effective in inhibiting EAR compared to omalizumab at the end of the treatment period.

Thirty-seven subjects with mild allergic asthma were recruited from 7 study sites in Canada and Sweden, of which thirty-five completed the study. All subjects had at the time of the investigation well-controlled or asymptomatic asthma with a $FEV_1 \geq 70\%$ of predicted and only use of rescue SABAs no more than twice a week, with the exception of in conjunction with

exercise. Atopy was documented by the presence of a positive skin prick test to one or more common airborne allergens. The study was registered at ClinicalTrials.gov (NCT01703312).

The study consisted of a 28-day screening period, a baseline evaluation, a treatment period of 10 weeks, a follow up period of 12 weeks and a study completion evaluation (Figure 4). At screening, a standard skin prick test as well as skin prick titrations and a methacholine inhalation challenge were performed. For inclusion in the study, the provocative concentration of inhaled methacholine to reduce $FEV_1$ by 20% (methacholine $PC_{20}$) should be 16mg/ml or less. At baseline, an allergen inhalation challenge was performed where subjects should demonstrate a decrease in $FEV_1 \geq 15\%$ from baseline within two hours of the challenge. The allergen $PC_{15}$ was calculated. Eligible subjects were then randomized to receive one of three subcutaneous doses of QGE031 (24, 72 or 240 mg), omalizumab or matching placebo for 10 weeks. QGE031 was administered every two weeks (six doses) and omalizumab every two or four weeks (three or six doses) depending on bodyweight and screening IgE levels. An allergen inhalation challenge as well as skin titrations were performed 6, 12 and 18 weeks after the first dose and allergen $PC_{15}$ was calculated. Blood samples were collected throughout the study for measurements of serum total QGE031, total IgE, basophil bound IgE and FcεRI levels on basophils and DCs.

**Paper III**
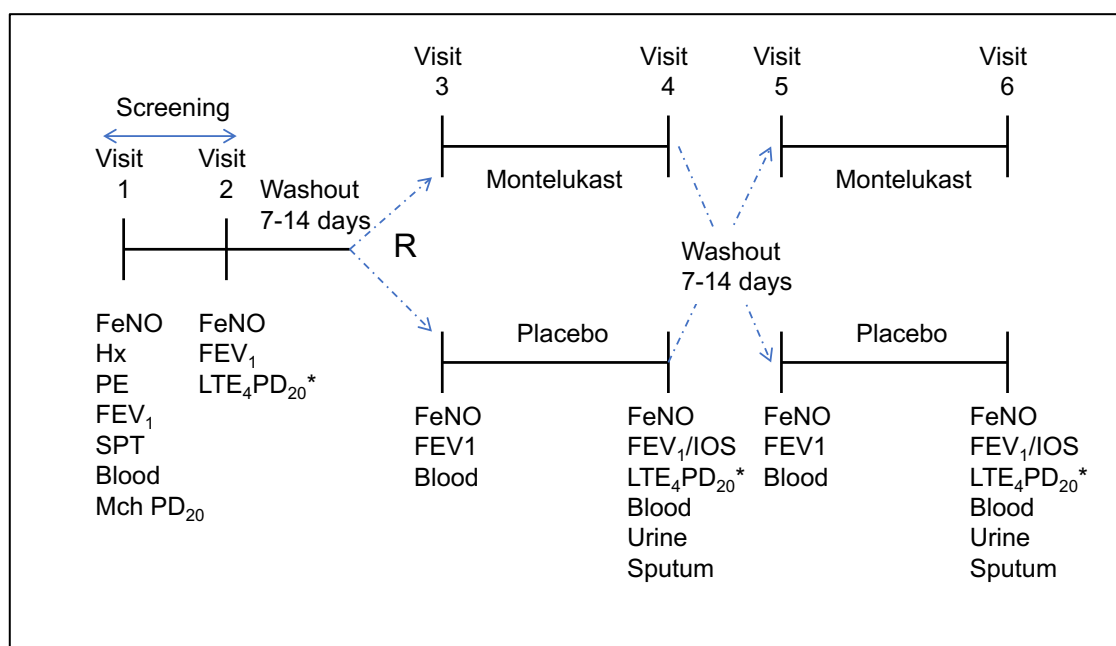


**Figure 5. Study design Paper III.** *$LTE_4$ challenge.

The aim of **Paper III** was to investigate whether the effect of inhaled $LTE_4$ in asthmatics is mediated solely via the $CysLT_1$ receptor or if there is possibly another receptor involved as indicated by previous animal studies. In order to answer that question a double blind, randomized, placebo-controlled study with a crossover design was performed, where asthmatic subjects inhaled $LTE_4$ before and after treatment with the potent $CysLT_1$ receptor antagonist montelukast or matching placebo (Figure 5). The hypothesis was that if there were a functionally

important and distinct $LTE_4$ receptor for chemotaxis, treatment with montelukast would block the bronchoconstriction but not the cellular response induced by inhaled $LTE_4$.

Fourteen subjects with mild intermittent asthma according to current Global Initiative for Asthma guidelines (GINA report 2015) and two subjects with aspirin-exacerbated respiratory disease (AERD) with a history of unequivocal severe bronchoconstriction after intake of aspirin-like drugs were included. All subjects had well-controlled asthma using only rescue SABAs no more than twice a week with the exception of the two AERD patients who were on regular treatment with a fixed combination of ICS/LABA. Subjects were 18-55 years of age with $FEV_1$ at screening more than 70% of predicted. The study was registered at ClinicalTrials.gov (NCT01841164).

Spirometry for measurement of baseline lung function as well as measurement of $F_ENO$ was performed on all study days. On the first screening day a standard skin prick test, physical examination and a methacholine inhalation challenge were performed during which eligible subjects would display a fall in $FEV_1$ of 20% or more from baseline after inhalation of $\leq 3621$ µg cumulated dose of methacholine (methacholine $PD_{20}$). Blood samples for routine laboratory tests were also collected. Subjects were then scheduled for the next screening day after at least 24 hours, when the first inhalation challenge with $LTE_4$ was performed. The provocation was terminated when $FEV_1$ had fallen at least by 20% from baseline value or the maximum dose of $LTE_4$ was reached. Blood samples were collected five minutes after inhalation of the last dose of $LTE_4$ for analysis of circulating white blood cells.

After a washout period of one to two weeks subjects continued in the crossover phase where they were randomized to treatment with montelukast or placebo for five to seven days. Inhalation challenge with $LTE_4$ was then repeated on the last day of each treatment period. Impulse oscillometry (MS-IOS) was conducted prior to spirometry in order to examine the effect of inhaled $LTE_4$ on small airways.

Urine samples were collected before, during and after the end of the challenge at hourly intervals for up to four hours for measurement of lipid mediator excretion. Sputum induction was performed four hours after the end of the challenge for calculation of sputum cell counts. In order to validate the results regarding excretion of lipid mediators in the urine, we also analysed urine samples from one of our previous bronchoprovocation studies with $LTD_4$ (Gyllfors, Kumlin et al. 2005), that included asthmatics with mild asthma using ICS (n=10), asthmatics using only rescue SABAs (n=10) as well as healthy individuals (n=10). These samples had been biobanked at -20 °C for 12 years.

## 4.2 ETHICS

All studies were approved from the local Ethics Committees (Dnrs 2009/920-31/2, 2012/2180-31/2 and 2011/1016-31/1). All participants gave their written informed consent prior to inclusion.

## 4.3 BRONCHIAL PROVOCATIONS

As already mentioned, airway hyperresponsiveness (AHR) is a key asthma characteristic that can be used in the research lab to study a controlled asthma attack with the use of different bronchial provocations. This temporary flare up of the disease gives us unique possibilities to study asthma pathophysiology, cellular interactions and mediator release. In my thesis bronchial provocations have been in the heart of every performed study and the methodology that was used is described in detail below.

### 4.3.1 Exercise challenge

The exercise challenge was performed in **Paper I** and was used to identify and quantify EIB. It is standardized according to the American Thoracic Society´s guidelines (ATS) (Crapo, Casaburi et al. 2000). The type, duration, intensity of exercise, as well as the temperature of the water content of the inhaled air, are important factors that determine the airway response, as established by previous studies (Bar-Or, Neuman et al. 1977, Chen and Horton 1977, Anderson, Daviskas et al. 1979, Anderson, Schoeffel et al. 1982). The challenge can be conducted using a treadmill or a cycle ergometer, although the rapid increase in ventilation during treadmill running makes it the preferable test. The patient should wear nose clips during the challenge because nasal breathing decreases water loss from the airways (Shturman-Ellstein, Zeballos et al. 1978, Mangla and Menon 1981). Most protocols recommend that the inspired air is dry (<10 mg $H_2O$/L) and less than 25$^o$C. The system delivers dry air through a mouthpiece and a two-way valve from a talc-free meteorological balloon filled with medical-grade compressed air (Eggleston, Rosenthal et al. 1979). Treadmill speed and inclination are progressively increased during the first two to three minutes of exercise until target ventilation is achieved. The target should be at least 17.5 times $FEV_1$ and preferably greater than 21 times $FEV_1$ (Anderson, Lambert et al. 2001). This load should be maintained for four to six minutes (ERS 1997). Another important factor is that subjects must abstain from exercise before the challenge, because some become refractory to another exercise stimulus for up to 4 hours (Anderson and Schoeffel 1982, Haverkamp, Dempsey et al. 2005). The lowest $FEV_1$ value within 30 minutes after exercise is recorded and the difference between this value and the pre-exercise $FEV_1$ value is expressed as a per cent of the pre-exercise value. The cut-off for the per cent fall in $FEV_1$ for a positive challenge is ≥10% in most guidelines (Sterk, Fabbri et al. 1993, Crapo, Casaburi et al. 2000, Carlsen, Anderson et al. 2008). The ≥10% fall in $FEV_1$ is based on the mean plus two standard deviations (SDs) of the per cent fall in normal healthy subjects without a family history of asthma, atopy or recent respiratory track infection (Custovic, Arifhodzic et al. 1994, Anderson, Pearlman et al. 2010). The severity of EIB can be graded as mild if the per cent $FEV_1$ fall is ≥10% but <25%, moderate if it is greater than 25% but <50% and severe if the fall is ≥50% (Anderson and Brannan 2003).

All subjects in **Paper I** during screening performed a maximal exercise test on a treadmill, while breathing ambient air according to a predefined protocol starting at 50 Watts (W), which was then increased by 10 or 15 W (depending on body weight) every minute. Heart rate was monitored by ECG and registered during the last 15 seconds of each workload level. Dyspnoea

and leg fatigue were registered according to the Borg CentiMax scale (CR100) at every second workload (Borg and Kaijser 2006). Maximal heart rate and the maximal aerobic capacity (workload) were registered. The workload at which subjects stopped was used to calculate the workload for the standardised exercise challenges. They were also performed on a treadmill while breathing through a tube connected to a gas cylinder containing dry air as recommended in the guidelines (Aiolos Astmatest, Aiolos Medical AB, Karlstad, Sweden). The workload was gradually increased from 60% to 90% of max during the first two minutes and then sustained at 90% for the remaining four minutes of the test that lasted in total six minutes. The target ventilation was set to 26 times $FEV_1$. During the challenge, patients used nose clips and were encouraged to cover the mouthpiece with their mouth and keep the balloon inflated at all times. $FEV_1$ was measured before exercise and 1, 5, 10, 15 and 30 min afterwards. No asthma medication was allowed 24 hours prior to exercise, nor any physical exercise.

### 4.3.2 Methacholine challenge

Methacholine, which is a muscarinic agonist activating $M_3$ receptors in the airways, is used to help assess the severity of AHR in subjects with symptoms consistent with asthma. It is the most commonly used direct bronchial challenge and it has almost replaced histamine, which is associated with more systemic adverse events such as headache and flushing (Scott and Braun 1991). All challenge protocols use a progressive dosing regimen and focus mostly on standardized dose delivery. The American Thoracic Society (ATS) published in 2000 details on two frequently used methacholine challenge methods (Crapo, Casaburi et al. 2000).

In **Paper II** the challenge was performed according to Clinical Investigators Collaborative (CIC) protocol using a Wright nebulizer (Roxon Medi-Tech, Montreal, Quebec, Canada) directly connected to a Hans Rudolph two-way valve in order to generate aerosols (Cockcroft 1985). The air source used for this purpose was wall unit hospital air supply. With this method the dose of aerosol deposited in the lung could be altered by the nebulizer output and the duration of inhalation. Each concentration of methacholine (0.031 up to 32 mg/mL) was inhaled during tidal breathing for two minutes. $FEV_1$ was measured 30 and 90 seconds post inhalation and the per cent fall was calculated from the lowest baseline $FEV_1$ value after diluent inhalation. The challenge was terminated when a $FEV_1$ fall of at least 20% occurred or the highest concentration of methacholine was administered. The result was expressed as $PC_{20}$ representing the concentration of methacholine that causes a fall in $FEV_1$ of 20% and it was calculated from linear interpolation.

In **Paper III** a dosimeter-controlled jet-nebulizer (Spira Elektro 2, Medela, Medical AB, Täby, Sweden) was used for inhalation of methacholine according to a slight modification of a protocol previously published (Nieminen, Lahdensuo et al. 1988). By changing the number of breaths and using different methacholine solutions, doubling increments of each dose were administered (Table 1). Methacholine was inhaled every third minute and a single $FEV_1$ measurement was performed at 2.5 minutes after each dose. The result was expressed as $PD_{20}$ representing the provocative dose causing a 20% fall from baseline post-diluent $FEV_1$ and it was derived from linear interpolation between the two last doses.

22

Subjects abstained from using SABAs for at least six to eight hours and did not ingest any caffeine-containing beverages for four hours prior to the methacholine test.

| Methacholine concentration mg/mL | No. Breaths | Dose μg | Log dose | Cumulated dose |
|---|---|---|---|---|
| 1 | 2 | 14.2 | 1.15 | 14.2 |
| 1 | 4 | 28.4 | 1.45 | 42.6 |
| 1 | 8 | 56.7 | 1.75 | 99.3 |
| 8 | 2 | 114 | 2.06 | 213.3 |
| 8 | 4 | 227 | 2.36 | 440.3 |
| 8 | 8 | 454 | 2.66 | 894.3 |
| 64 | 2 | 909 | 2.96 | 1803 |
| 64 | 4 | 1818 | 3.26 | 3621 |
| 64 | 8 | 3635 | 3.98 | 7256 |

**Table 1. Protocol for dosing of methacholine in Paper III (molar weight 160.24)**

### 4.3.3  Mannitol challenge

Mannitol is a sugar alcohol and the inhalation of cumulative doses of mannitol has been developed as a standardized bronchoprovocation test (Anderson, Brannan et al. 1997), that is most often used as a surrogate measure of EIB (Brannan, Koskela et al. 1998, Brannan, Anderson et al. 2005). This is based on the osmotic theory of EIB pathogenesis i.e. that the hyperventilation that occurs during exercise leads to a hyperosmolarity of the airway lining fluid due to water evaporation, which in its turn causes bronchoconstriction due to mast cell activation (Anderson and Daviskas 2000, Brannan, Gulliksson et al. 2006). In **Paper I** the mannitol bronchial challenge was performed using the Aridol[®] provocation kit (Pharmaxis Ltd, Frenchs Forest, Sydney, Australia). Subjects were seated comfortably with a nose clip applied and were encouraged to maintain good posture in order to assist a better delivery of mannitol to the lungs. Mannitol was provided in capsules with different doses (0, 5, 10, 20, 40 mg) with a single capsule inserted in the Osmohaler™, one at a time. The higher doses of mannitol were given as multiples of smaller individual doses according to a protocol provided by the manufacturer. $FEV_1$ was measured in duplicate to obtain reproducibility one minute after each step and the highest value recorded after inhalation of the first capsule (0 mg) was used as baseline $FEV_1$. The challenge was considered as positive if there was a fall in $FEV_1$ more than 15% from baseline and the $PD_{15}$ was calculated or if a 10% fall occurred in-between consecutive doses. If the maximal cumulative dose of 635 mg mannitol was attained without reaching any of these endpoints, the challenge was considered as negative. The airway response to mannitol was

measured prior to (visit 3) as well as after six weeks of treatment (visit 6) and was used as an explorative variable in the study where the primary outcomes were published **(Paper I)**.

### 4.3.4  Allergen challenge

Bronchoprovocation challenges using allergen aerosols have been used widely over the past 60 years to examine both the early (EAR) as well as the late asthmatic reaction (LAR). In **Paper II** subjects inhaled doubling concentrations of aqueous allergen extracts (Aquagen SQ$^®$ ALK Nordic, Copenhagen, Denmark) using a Wright nebulizer according to CIC`s protocol (O'Byrne, Dolovich et al. 1987). Nebulizer output was the same as the one used for methacholine with duration of inhalation of 2 minutes under tidal breathing. The allergen chosen depended on the greatest reaction recorded on the skin prick test and the individual reported symptoms to previous exposure. Skin prick titration with two-fold increasing concentrations of the chosen allergen extract were performed in duplicate on the volar surface of one or both forearms in order to define the skin prick test endpoint (SS) i.e. the lowest titration of allergen that causes a skin wheal at least 2 x 2 mm in size. The predicted $PC_{20}$ allergen was then calculated from the methacholine $PC_{20}$ and the SS by simple linear regression (Cockcroft, Murdock et al. 1987, Cockcroft, Davis et al. 2005). The starting concentration of allergen extract for inhalation at baseline would be two to four doubling concentrations below the predicted $PC_{20}$ allergen.

$FEV_1$ was measured twice (30 and 60 sec intervals) at 10 minutes after each inhalation of allergen. If $FEV_1$ had fallen less than 10% from baseline, the next concentration was given. If the fall was between 10 and 15% a new measurement was done 10 minutes later. If it remained the same or if $FEV_1$ started to rise, the next concentration of allergen was administered. The test was stopped when $FEV_1$ had fallen by 15% or more and the allergen $PC_{15}$ was calculated using linear interpolation. If $FEV_1$ at the highest concentration of allergen had fallen by 8% to 14% then $PC_{15}$ was extrapolated using the following formula $PC_{15}$=concentration x (15/% fall in $FEV_1$). If $FEV_1$ at the highest concentration of allergen had fallen by 0% to 7% the next doubling concentration was used.

### 4.3.5  Leukotriene E$_4$ challenge

Challenges with CysLTs are used almost exclusively in research due to the high cost of their production. Early studies confirmed that $CysLT_1$ receptor antagonists could inhibit the bronchoconstriction induced by inhalation of $LTD_4$ (De Lepeleire, Reiss et al. 1997). Formal evaluations of the repeatability and sample size requirements for leukotriene inhalation challenges have not been published. There is considerable and unique experience with these challenges in our lab (Kumlin, Dahlen et al. 1992, Gyllfors, Kumlin et al. 2005, Gyllfors, Dahlen et al. 2006), to support that the leukotriene challenge is highly repeatable and considerable less variable than the exercise challenge. Moreover, a rising dose challenge is more repeatable than a fixed dose challenge (Kumlin and Dahlen 2000).

In **Paper III,** GMP grade $LTE_4$ was purchased from Cayman Chemicals Corp (USA, Ann Arbor Michigan) in color-coded vials with ten fold increasing concentrations from 0.042 to 4200 µmol/L dissolved in ethanol-water (Figure 6). The leukotriene was administered for inhalation

after nebulization by a dosimeter-controlled jet-nebulizer (Spira Elektro 2, Respiratory Care Center, Hameenlina, Finland). By using five or sometimes six different solutions of $LTE_4$ and by variations in the number of tidal breaths (normally 1 to 7), stepwise increments of the cumulative dose of $LTE_4$ were administered (Table 2). The $LTE_4$ solution was inhaled every 10 minutes with approximately half-log dose increments administered at every step. $FEV_1$ was obtained at 5 and 10 minutes after each dose increment. The provocation was terminated when $FEV_1$ had fallen 20% from baseline or the maximum dose of $LTE_4$ had been reached. If $FEV_1$ was reduced between 15 and 19%, the investigator waited 5-10 minutes and reassessed the $FEV_1$ before further increases in the dose of $LTE_4$. If the drop in $FEV_1$ remained just below 20%, the responsible physician would make a decision as to whether or not the next dose should be given. In subjects with a steep dose-response curve for $LTE_4$ it was recommended to repeat the previous dose once more before giving the next dose in the protocol. After a positive reaction, $FEV_1$ was followed every 15 minutes during the first hour, thereafter at hourly intervals according to the protocol. Dose-response relations for $LTE_4$ were constructed and used for calculation of the $LTE_4PD_{20}$. The lowest $FEV_1$ measurement at 5 or 10 minutes after each dose was plotted against the log-cumulated dose of $LTE_4$. The $PD_{20}$ value was derived from linear interpolation between the two last doses.
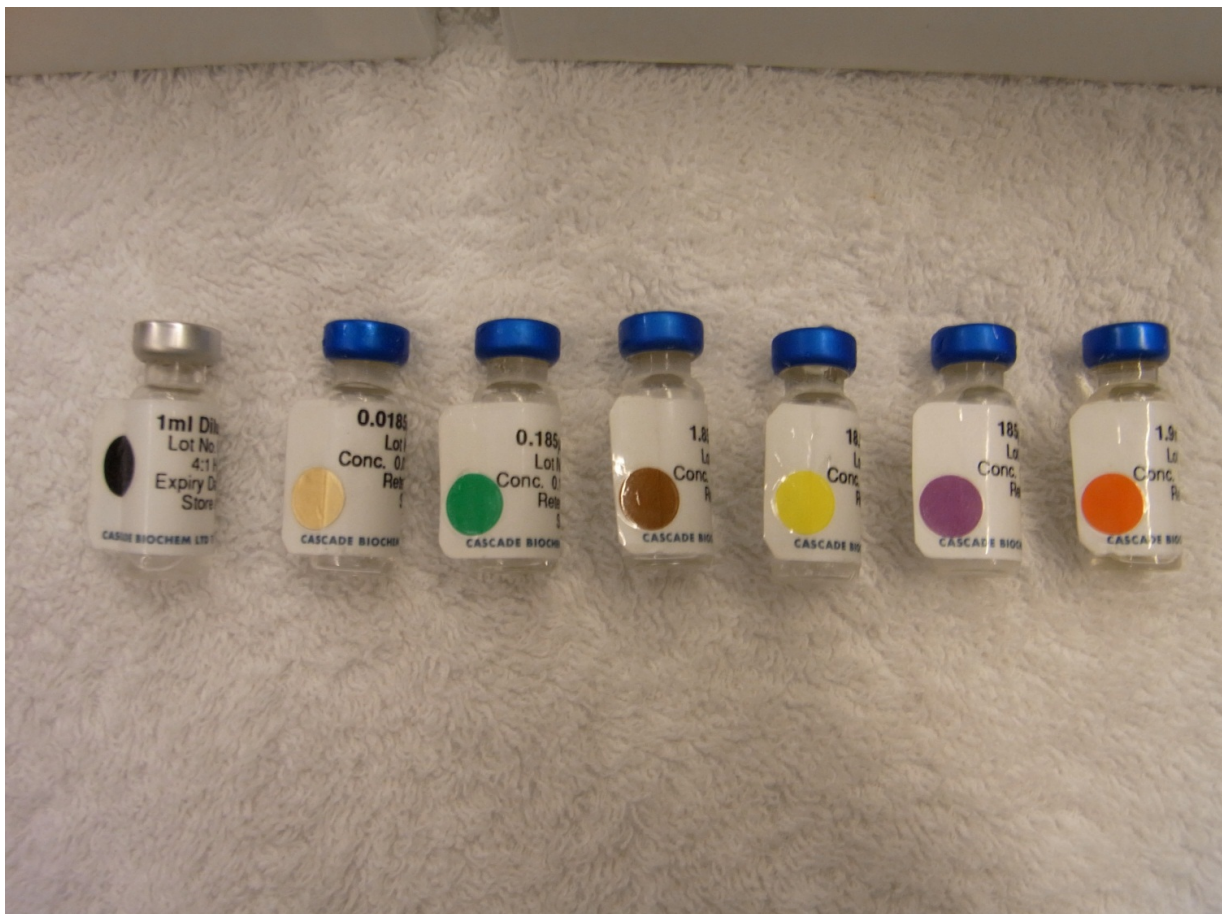


**Figure 6. Color-coded vials with increasing concentrations of $LTE_4$**

| Conc. LTE$_4$ in nebuliser μM | No. Of breaths | Delivered dose pmol | Cumulated dose pmol |
|---|---|---|---|
| **FOR EXTREMELY SENSITIVE SUBJECTS ONLY** | | | |
| Yellow: | | | |
| 0.042 | 1 | 0.3 | 0.3 |
| 0.042 | 2 | 0.6 | 1 |
| 0.042 | 7 | 2.3 | 3 |
| **NORMAL START LEVEL at screening** | | | |
| Green: | | | |
| 0.42 | 1 | 3.4 | 3 |
| 0.42 | 2 | 6.7 | 10 |
| 0.42 | 7 | 23.5 | 34 |
| ED   *0.42* | *14* | *47* | |
| Turquoise: | | | |
| 4.2 | 2 | 67.5 | 100 |
| 4.2 | 7 | 235 | 336 |
| ED   *4.2* | *14* | *473* | |
| Blue: | | | |
| 42 | 2 | 672 | 1,008 |
| 42 | 7 | 2,350 | 3,360 |
| ED   *42* | *14* | *4,700* | |
| Lilac: | | | |
| 420 | 2 | 6,720 | 10,080 |
| 420 | 7 | 23,500 | 33,580 |
| ED   *420* | *14* | *47,000* | |
| Red solution: | | | |
| 4,200 | 2 | 67,200 | 107,800 |
| 4,200 | 7 | 235,000 | 335,780 |
| ED   *4,200* | *14* | *470,000* | |
| ED   *4,200* | *21* | *705,000* | |

**Table 2. Protocol for dosing of LTE$_4$. ED: extra dose of LTE$_4$ used if FEV$_1$ decreased between 15% and 20% from baseline.**

## 4.4   LUNG FUNCTION MEASUREMENTS

In order to monitor how lung function was affected by the various challenges, spirometry was used. In particular, impulse oscillometry was included to determine the effect on small airways.

### 4.4.1   Spirometry

Spirometry was performed before, during and after each challenge, as well as on most of the study days according to the European Respiratory Society/American Thoracic Society guidelines (ATS 1995). Following a maximal inspiration subjects performed a fast and powerful exhalation manoeuvre whilst connected to a mouthpiece and wearing a nose clip. The forced expiratory volume in one second ($FEV_1$), the forced vital capacity (FVC), the forced expiratory flow between 25% and 75% of FVC (FEF25-75) and the peak expiratory flow rate (PEFR) were recorded.

### 4.4.2   Impulse oscillometry

In **Paper III,** impulse oscillometry (MS-IOS, Jaeger, Friedberg, Germany) was performed according to current guidelines (Oostveen, MacLeod et al. 2003, Smith HJ 2005). Subjects were sitting upright with their hands supporting the cheeks wearing a nose clip. One-minute measurements were obtained during tidal breathing and airway resistance at 5 Hz ($R_5$) and 20 Hz ($R_{20}$), reactance at 5 Hz ($X_5$), frequency dependence of resistance (FDR; $R_5$-$R_{20}$), area of reactance (Ax) and resonance frequency (Fres) were calculated. At least two technically acceptable recordings were performed on each subject, sequences with possible artefacts due to subject swallowing were excluded from analysis.



**Figure 7. Impulse oscillometry system used in Paper III**

## 4.5   SKIN PRICK TESTING

In all three papers skin prick testing was performed at screening to assess allergy; in **Paper II** a skin prick titration was also done over a range of dilutions just for the allergen extract that was chosen for the inhalation challenge. Standardized extracts of 10 common allergens (ALK Abello Soluprick SQ Allergen solution, Copenhagen, Denmark) were used with histamine (10mg/ml) as a positive, and the diluent as a negative control. Positive response was specified as a wheel diameter $\geq 3$ mm.

## 4.6   MEASUREMENT OF $F_E$NO

Nitric oxide (NO) is produced by airway epithelial cells mostly through inducible NO synthase (iNOS) which is upregulated in asthmatic inflammation and suppressed by corticosteroid treatment (Dweik, Boggs et al. 2011). Measurement of the fraction of exhaled NO ($F_E$NO) at a fixed flow rate is a non-invasive test and is widely considered as a surrogate marker of eosinophilic inflammation (Barnes, Dweik et al. 2010, Bjermer, Alving et al. 2014). In **Paper III** $F_E$NO measurements (NIOX analyzer, Aerocrine AB, Solna, Sweden) were performed at a flow rate of 50 mL/s according to American Thoracic Society guidelines (Dweik, Boggs et al. 2011) on all study days.

## 4.7   SPUTUM INDUCTION AND PROCESSING

Sputum is a safe, non-invasive and reproducible method of sampling airway secretions and associated cells from proximal airways (Pizzichini, Pizzichini et al. 1997, Szefler, Wenzel et al. 2012). The processing of sputum allows for the investigation of the cellular and fluid space components to assess the current inflammatory state of the airways. The non-invasive nature of sputum induction gives the technique several advantages over other methods, such as bronchoalveolar lavage (BAL) and bronchial biopsy that involve bronchoscopy. It allows for repeated assessment of large number of subjects with varying degrees of airway obstruction. Sputum induction was performed in **Paper III** four hours after the end of the $LTE_4$ challenge. Although relatively mild, sputum induction is de facto a bronchoprovocation procedure. Therefore subjects inhaled 0.2 mg of salbutamol prior to inhalation of an aerosol containing increasing concentrations of saline (3%, 4% and 5%) for 7 minutes through an ultrasonic nebulizer (De VilBiss Ultraneb 3000, Dolema AB, Täby, Sweden). Baseline $FEV_1$ should be $\geq$ 70% of predicted prior to the test. $FEV_1$ was measured after each concentration and the induction stopped if $FEV_1$ fell by 20%. Sputum plugs were identified and extracted and processed within 2 hours as described previously (Pizzichini, Pizzichini et al. 1996). Tryptan blue solution (0.4%) was then used to assess cell viability and the cells were classified as viable, nonviable and squamous. Samples with high squamous cell contamination ($\geq 20\%$ of all cells) were rejected (Efthimiadis, Spanevello et al. 2002). Cytospins were stained with May-Grünvald-Giemsa solution and differential non-squamous cell counts were performed.

## 4.8 URINE SAMPLING AND ANALYSIS

Urine was collected in **Paper III** in order to investigate lipid mediator release from the lungs in response to inhaled $LTE_4$. The platform measured the main urinary metabolites of prostaglandins (PG), thromboxanes (TX), isoprostanes and the CysLTs. Urine collection was performed prior to, during and after bronchial challenge with $LTE_4$; collected samples were distributed into smaller plastic tubes, stored at $-70^{o}C$ until time of analysis. To normalize for changes in urine production, creatinine was measured in all samples using a colorimetric assay. In order to analyse several lipid mediators simultaneously in the same sample, an in-house and validated ultra performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) method was used (Balgoma, Larsson et al. 2013). Enzyme immunoassay (EIA) was also applied to measure the $PGD_2$ metabolite $11\beta$-$PGF_{2a}$ (Cayman Chemicals, Ann Arbor, Mi, USA) as described previously (Bood, Sundblad et al. 2015).

## 4.9 MEASUREMENTS IN BLOOD

In **Paper II,** blood samples were collected before dosing and every other week up to 24 weeks after the first dose. QGE031 concentrations were measured with ELISA and total IgE was measured using the ImmunoCAP® total IgE assay (Phadia AB, Uppsala, Sweden). Free IgE in serum was calculated using a sandwich ELISA and flow cytometry was used to measure FcεRI on basophils and dendritic cells, CD23 on B cells and bound IgE on basophils and B cells.

In **Paper III,** routine blood samples were collected during the screening visit to exclude comorbidities that might eventually influence the study results. Blood samples were also collected at study visits 4 and 6 prior to $LTE_4$ inhalation, as well as 5 minutes after peak fall in $FEV_1$ or following the last dose of $LTE_4$ (20 ml each occasion) for analysis of circulating white blood cells.

## 4.10 RECORDING OF ASTHMA SYMPTOMS

In **Paper I,** at the third visit enrolled subjects gained access to an electronic diary in order to record their physical activity, asthma symptoms as well as daily use of on demand medication. Asthma symptoms were rated on a scale from 0 to 4, with higher scores indicating worse asthma control. ACQ5 was completed at visits 1, 3, 4 and 6.

## 4.11 DRUGS AND INTERVENTIONS

In all the papers included in this thesis, therapeutic interventions were performed and compared with either current recommended treatments and/or matching placebo. All studies were randomized and double blind; subjects, investigators, and study centre staff were blind to the identity of study treatments. Randomization data were kept strictly confidential until the time of unblinding and all packaging, labelling, appearance and schedule of administration of the study drugs were identical.

### 4.11.1 Budesonide and Formoterol

In **Paper I,** a combination of budesonide an inhaled corticosteroid (ICS) and formoterol (formoterol fumarate dehydrate), a long acting $\beta_2$ agonist (LABA) delivered via a dry-powder inhaler (DPI) (SYMBICORT® TURBUHALER® Astra Zeneca) inhaled on demand was tested as an alternative treatment of EIB. The drug is approved since 2007 as both maintenance and reliever therapy in persistent asthma in many countries all over the world. The device is an inspiratory-flow-driven, multi dose DPI that accurately dispenses very small amounts of dry powder when the subject inhales through the mouthpiece; thus there is no need to coordinate dose actuation with inhalation.

Budesonide is an efficacious glucocorticosteroid with high affinity for the glucocorticosteroid receptor displaying a wide variety of anti-inflammatory effects in pharmacological studies (Szefler 1999). A unique property of budesonide is its reversible esterification with fatty acids (Tunek, Sjodin et al. 1997), which prolongs binding of the drug in the airways and possibly contributes to high airway activity and selectivity (Miller-Larsson, Jansson et al. 2000). The drug undergoes a 90% hepatic first-pass biotransformation into metabolites of low glucocorticosteroid potency, which results in a favourable profile regarding systemic steroid side effects, such as adrenal suppression, growth inhibition and catabolic changes of the skeleton and connective tissues (Szefler, Lyzell et al. 2004).

Formoterol is a potent and selective $\beta_2$ adrenoreceptor agonist with a rapid onset of action and a long duration of effect when inhaled (Lofdahl and Svedmyr 1989, Bartow and Brogden 1998, Lotvall 2002). The primary pharmacological effect of formoterol is smooth muscle relaxation, although it also inhibits the release of inflammatory mediators from mast cells, microvascular leakage as well as mucosal plasma exudation (Erjefalt and Persson 1991, Lindberg, Khan et al. 1995, Barnes 2002). Systemic side effects include tachycardia, hypokalaemia, muscle tremor and hyperglycaemia as for all $\beta_2$ adrenoreceptor agonists.

### 4.11.2  QGE031 (ligelizumab)

In **Paper II,** treatment with a new humanized $IgG_1$ monoclonal antibody against IgE was compared to currently recommended treatment with omalizumab, and placebo. QGE031 binds to the C$\varepsilon$3 domain of IgE and efficiently blocks its interaction with both the high affinity IgE receptor (Fc$\varepsilon$RI) and the low affinity (Fc$\varepsilon$RII/CD23). The drug is designed to achieve higher IgE suppression compared to omalizumab with low equilibrium dissociation constant (Kp= 139 pM) that could lead to better clinical efficacy. Preclinical experiments have demonstrated that the antibody inhibits completely the release of histamine from mast cells and basophils induced by human recombinant IgE linking by preventing its binding to Fc$\varepsilon$RI. QGE031 also inhibited IgE-dependent activation and subsequent degranulation of human cord blood derived mast cells at IgE concentrations up to 19 times higher than those inhibited by omalizumab. In a rhesus monkey local passive cutaneous anaphylaxis model, QGE031 showed an at least 25 times higher specific activity than omalizumab.

30

QGE031 has been administered in clinical studies both intravenously in single doses ranging from 0.1 to 10 mg/kg and subcutaneously in doses between 0.2 mg/kg x 2 to 4.0 mg/kg x 4 given at two-week intervals and before this study up to 196 patients were exposed to the drug. QGE031 suppressed the levels of free circulating IgE below the lower limit of detection when administered both intravenously and subcutaneously. The extent and duration of suppression of free IgE, basophil FcεRI expression, basophil IgE and skin prick test response to allergen was greater than after administration of omalizumab. Safety assessments showed good tolerability with six severe adverse events reported although none of them were considered to be related to treatment. Urticarial events were reported after intravenous administration that appeared to be dose dependent, although no such events were seen in the subjects that received biweekly subcutaneous doses of 4.0 mg/kg QGE031.

### 4.11.3 Montelukast

In **Paper III** an intervention with the potent $CysLT_1$ receptor antagonist montelukast (Singulair™; Merck Sharp & Dohme, Stockholm, Sweden) was performed in a cross over manner with a placebo comparator. Montelukast belongs to the "lukast" family of leukotriene antagonists and is a highly selective pharmacological antagonist of the $CysLT_1$ receptor (Jones, Labelle et al. 1995). The recommended daily dose for asthma treatment in adults is 10 mg once a day. As it is a competitive receptor antagonist, higher doses provide greater antagonism, as thoroughly documented *in vitro* (Back, Dahlen et al. 2011). Accordingly, bronchoprovocation studies have documented that doses higher than 10 mg cause progressively increasing inhibition of the bronchoconstriction induced by inhalation of $LTD_4$ (De Lepeleire, Reiss et al. 1997). In the early clinical development up to 250 mg of montelukast were given to subjects with asthma for several weeks without reports of significant adverse events. Taken together for this study the daily dose of 40 mg was selected to get significant antagonism on the $CysLT_1$ receptor, thereby optimizing the testing of the overall study hypothesis that some effects of $LTE_4$ may be resistant to $CysLT_1$ antagonism.

Montelukast was purchased from the Karolinska University Hospital Pharmacy (KUH) and the matching placebo from Recipharm Pharmaceutical Development AB, Stockholm, Sweden. The drugs were kept under appropriate storage conditions at ≤ 30°C with tightened cover in the Clinical Research Unit. The dispensation and coding was done by the KUH pharmacy and kept in sealed envelope in the Clinical Research Unit.

### 4.12 PHARMACOKINETICS MODELING AND SIMULATIONS

In **Paper II,** a pharmacokinetics/pharmacodynamics mathematical model descriptive of drug pharmacokinetics (either omalizumab or QGE031), as well as total circulating IgE, basophil FcεRI and surface IgE levels was implemented as previously described (Lowe 2015). Simulations from the applied model were performed for approximately 1000 virtual patients with the same demographics as the subjects studied in the actual paper.

## 4.13 STATISTICAL ANALYSIS

In **Paper I,** results were presented as mean ± standard deviation (SD) or 95% confidence intervals (CI). Analysis of covariance was performed for the analysis of the primary endpoint with treatment as a factor and the baseline value of the post-exercise $FEV_1$ before treatment as a covariate. Most of the secondary endpoints were analysed similarly while some using descriptive methods. For the non-inferiority part of the study regarding comparison between the budesonide/formoterol group with the regular budesonide group, non-inferiority was defined as a difference in post exercise $FEV_1$ fall < 7.28% and a SD of 7.13 estimated from a previous study (Jonasson, Carlsen et al. 2000). A sample size of 66 randomized subjects provided 80% power with 5% significance level was calculated to be enough for the non-inferiority analysis.

In **Paper II,** the primary statistical analysis focused on the comparison of QGE031 240 mg to omalizumab and to obtain 95% CI of the difference. The difference to baseline $log_2$ transformed allergen $PC_{15}$ was assumed to be normally distributed and was analysed using a linear mixed effect model for repeated measurements. The model included effects for baseline (last measurement prior to first dose of study drug), treatment (dose of QGE031, omalizumab, placebo), time, and treatment by time interaction and baseline by time interaction. A covariance matrix was used to model the correlation of $log_2 PC_{15}$ measured on the same patient. Treatment contrasts were estimated separately for each study day, 95% CI were provided and the contrasts tested for statistical significance at the one-sided 2.5% alpha level. The estimated differences and CIs were back transformed to obtain geometric means. The sample size was selected to provide adequate power for the statistical tests of the primary contrasts in $log_2 PC_{15}$. Based on a previous study (Boulet, Chapman et al. 1997), the SD of $log_2 PC_{15}$ was assumed to be 1.5. Then six subjects per group provide 80% power to detect a treatment difference of 2.7 doubling solutions, the reported difference between placebo and omalizumab treated subjects, using a one sided test at 2.5% alpha level. Assuming that no more than two subjects in a treatment group would drop out, 40 subjects (8 each group) were planned to be randomized. Enrolment stopped after 37 patients because drop out rate was smaller.

In **Paper III,** for the primary endpoint $LTE_4PD_{20}$ values were logarithmically transformed and results presented as geometric means and ranges; repeated measures ANOVA was performed. Correlations were also performed on log-transformed data. Values from IOS measurements and sputum cell counts were presented as median with ranges and the Wilcoxon signed rank test was used for analysis. Paired t tests were used to calculate differences in blood cell counts and changes in the levels of urinary metabolites. For a two period crossover treatment study, a minimum of 9 patients is required to demonstrate a 50% reduction of the airway response to inhaled allergen (Gauvreau, Watson et al. 1999), whereas a minimum of 16 subjects would be required when using an exercise challenge (Dahlen, O'Byrne et al. 2001). Formal evaluations of the repeatability and sample size requirements for leukotriene inhalation challenges are not available but there is considerable experience with these challenges in our lab (Kumlin, Dahlen et al. 1992, Gyllfors, Kumlin et al. 2005, Gyllfors, Dahlen et al. 2006) to support that the leukotriene challenge is highly repeatable and less variable than the exercise challenge. Moreover a rising dose challenge is more repeatable than a fixed dose challenge (Kumlin and

Dahlen 2000). Therefore we assumed that a sample size of 14 subjects would be sufficient to detect a significant shift in the dose response curve to inhaled $LTE_4$.

# 5  RESULTS AND DISCUSSION

## 5.1  DEMOGRAPHICS AND BASELINE CHARACTERISTICS

The subjects that where recruited for all three studies had mild intermittent asthma that was stable at the time of the investigations. They only used rescue SABA medications with the exception of the two AERD subjects in **Paper III** who were on regular treatment with a combination of ICS/LABA.

### 5.1.1  Paper I

189 subjects went through screening (visits 1 and 2) of which 66 were eligible and were randomized to study treatment. The most common reasons for exclusion was a negative exercise challenge test at visit 2 ($FEV_1$ fall < 10%), low baseline lung function ($FEV_1$ < 80% of predicted), significant comorbidities, or obvious indication for regular treatment with ICS according to the investigator´s judgment. Subject's baseline characteristics are shown in Table 3.

By chance, most of the ex-smokers (had quit > 1 year prior to inclusion) were randomized to the budesonide/formoterol (bud/form) group, although their baseline lung function did not differ from never-smokers. More than half of the subjects in each group were atopic with a positive skin prick test to at least one aeroallergen, and all of them had normal lung function. During the maximal exercise test, subjects that were randomized to the terbutaline group on demand had slightly more symptoms and achieved lower max workload compared to the other to two groups although the difference was not significant. There were more women (n=16) randomized to that group compared to the other two. All treatments groups had a higher maximal dyspnoea Borg score than maximal leg fatigue score, which indicated that breathlessness and not leg fatigue was in most cases the main reason for ceasing the test.

Results for the primary outcome were obtained at visit 5 in 59 of 66 subjects. For the 7 subjects that had missing data from visit 5, the last observation carry-forward principle was performed and data from visit 4 were used in the full analysis set. Four of the subjects were excluded from the per protocol analysis set (three in the budesonide group due to low compliance to treatment and one in the bud/form group due to excessive training/medication).

| Characteristics | Regular placebo once daily + budesonide/formoterol on demand N=23 | Regular placebo once daily + terbutaline on demand N=22 | Regular budesonide once daily + terbutaline on demand N=21 |
|---|---|---|---|
| Age (years) | 31±12 | 28±12 | 26±10 |
| Female/male | 9/14 | 16/6 | 11/10 |
| BMI (kg/m$^2$) | 25.0±4.0 | 23.4±3.6 | 24.6±3.6 |
| Ex-Smokers (n) | 8 | 1 | 1 |
| Smoking cessation (years) | 7.1±5.6 | 3.4±0.0 | 7.2±0.0 |
| FEV$_1$. baseline (liters) | 3.8±0.8 | 3.4±0.6 | 3.8±0.8 |
| FEV$_1$. % predicted value | 100±12 | 97±10 | 99±9 |
| Positive skin prick test. n (%) | 14 (61) | 16 (73) | 15 (71) |
| | | | |
| **Maximal exercise test** | | | |
| Max work load (W) | 251±66 | 232±57 | 255±63 |
| Max dyspnea (Borg CR100) | 86.5±20.6 | 95.9±21.2 | 85.3±25.5 |
| Max leg fatigue (Borg CR100) | 77.0±25.0 | 87.3±23.2 | 79.1±26.5 |
| Max heart rate (beats/min) | 186±9 | 189±12 | 181±25 |

**Table 3. Baseline characteristics at visit 1. Data are presented as mean ± SD.**

## 5.1.2  Paper II

Thirty-seven subjects were randomized to treatment of whom thirty-five completed the study. One subject in the QGE031 24 mg treatment group was excluded due to difficulties to complete the whole study and one due to the decision of the sponsor (dosing error). The baseline characteristics of all subjects are shown in Table 4. All had normal lung function and there were no significant differences in demographics between the treatment cohorts.

| Parameters | QGE031 24mg (N=8) | QGE031 72mg (N=8) | QGE031 240mg (N=8) | Omalizumab (N=6) | Placebo (N=7) |
|---|---|---|---|---|---|
| Age, years (range) | 26.3 (24 – 29) | 32.3 (22 – 59) | 34.4 (18 – 55) | 33.0 (22 – 48) | 30.1 (18 - 50) |
| Male, n (%) | 2 (25.0) | 3 (37.5) | 4 (50.0) | 2 (33.3) | 1 (14.3) |
| Race, n (%) | | | | | |
| Caucasian | 3 (37.5) | 8 (100.0) | 7 (87.5) | 5 (83.3) | 7 (100.0) |
| Asian | 4 (50.0) | 0 | 0 | 0 | 0 |
| Other | 1 (12.5) | 0 | 1 (12.5) | 1 (16.7) | 0 |
| Weight, kg | 64.0 (14.60) | 67.8 (18.20) | 82.6 (18.64) | 77.8 (16.43) | 68.4 (14.73) |
| BMI, kg/m$^2$ | 23.2 (4.45) | 23.6 (3.91) | 28.6 (6.22) | 26.2 (4.17) | 24.4 (3.17) |
| FVC, L | 3.93 (0.93) | 4.28 (1.09) | 4.43 (1.28) | 4.50 (1.10) | 4.06 (0.93) |
| FEV$_1$, L | 3.06 (0.62) | 2.98 (0.62) | 3.33 (1.00) | 3.55 (0.88) | 3.18 (0.96) |
| % FEV$_1$ predicted | 87.63 (7.96) | 83.31 (9.93) | 90.45 (17.57) | 95.17 (11.34) | 87.07 (10.75) |
| Screening total IgE (IU/mL) Median (range) | 163.00 (83.7, 695.0) | 117.00 (37.0, 336.0) | 98.90 (77.1, 521.0) | 97.95 (41.6, 604.0) | 97.10 (67.7, 295.0) |

**Table 4. Baseline characteristics at visit 1. Data are presented as mean ± SD unless specified.**

### 5.1.3  Paper III

Thirty-one subjects went through the screening phase of the study of which sixteen subjects (fourteen subjects with mild asthma and two AERD subjects) were randomized to treatment. The most common reason for exclusion was a negative methacholine challenge at visit 1; four subjects did not display sufficient sensitivity to inhaled LTE$_4$, and one subject withdrew consent due to incapacity to complete all study visits. Demographics as well as bronchial responsiveness to inhaled methacholine and LTE$_4$ at screening are shown in Table 5. All subjects had normal lung function with a geometric mean F$_E$NO value at screening (24.2 ppb) within normal range (< 25 ppb) including the two AERD subjects, indicating that they had a stable asthma at the time of the investigation. The mean ratio between PD$_{20}$ methacholine and PD$_{20}$ for LTE$_4$ at screening was 76.9 meaning that LTE$_4$ was about 75 times more potent on a molar basis than methacholine as bronchoconstrictor. Eleven subjects were sensitized to one or more aeroallergens according to skin prick test results at visit 1 (data not shown).

| Patient | Age (y) | M/F | FeNO (ppb) | FEV$_1$ (L) | FEV$_1$ % predicted | Mch PD$_{20}$ (nmol) | Screening LTE$_4$ PD$_{20}$ (nmol) | Ratio MCh PD$_{20}$ / LTE$_4$ PD$_{20}$ |
|---|---|---|---|---|---|---|---|---|
| 1 | 45 | F | 8 | 2.1 | 79 | 4986 | 3.8 | 1326 |
| 2 | 24 | M | 68 | 4.2 | 86 | 1654 | 54.9 | 30.2 |
| 3 | 37 | F | 50 | 2.9 | 114 | 499 | 39.4 | 12.7 |
| 4 | 28 | F | 13 | 2.8 | 81 | 2565 | 30.8 | 83.4 |
| 5 | 38 | M | 116 | 4.3 | 106 | 916 | 70.2 | 13 |
| 6 | 29 | M | 24 | 4.3 | 90 | 2378 | 19.8 | 120.6 |
| 7 | 46 | F | 10 | 2.7 | 99 | 3532 | 49.4 | 71.5 |
| 8 | 20 | F | 11 | 3.5 | 95 | 2153 | 31.9 | 67.4 |
| 9 | 52 | F | 14 | 2.6 | 101 | 12188 | 43.2 | 282 |
| 10 | 45 | F | 36 | 2.4 | 96 | 924 | 8.6 | 107.3 |
| 11 | 25 | F | 30 | 2.9 | 92 | 899 | 11.3 | 79.5 |
| 12 | 44 | F | 52 | 4.1 | 129 | 4281 | 22.7 | 188.4 |
| 13 | 38 | F | 11 | 2.5 | 103 | 2197 | 12.6 | 173.8 |
| 14 | 28 | F | 48 | 2.6 | 93 | 649 | 2.9 | 224 |
| AERD 1 | 48 | M | 14 | 3.8 | 97 | 17206 | 87.4 | 197 |
| AERD 2 | 35 | M | 21 | 3.8 | 79 | 2528 | 55.7 | 45.4 |
| **Mean** | **36.4** | | **24.2**[¶] | **3.2** | **96.3** | **1815**[¶] | **23.6**[¶] | **76.9** |
| Range | 20-52 | | 8-116 | 2.1-4.3 | 79-129 | 499-17206 | 2.9-54.9 | 12.7-1326 |

**Table 5. Patient characteristics and airway sensitivity to methacholine and LTE$_4$ at screening.** [¶] **Geometric mean.**

## 5.2 AIRWAY RESPONSE TO CHALLENGES AND THE EFFECT OF INTERVENTIONS

In all three papers, an airway challenge was performed at screening and after treatment with the interventional drug and/or placebo. The airway response was measured using spirometry and IOS (only in **Paper III**), and the bronchoprotective effect was compared to screening for each treatment arm as well as in between treatment arms. The nature of the stimulus and the challenge methodology defined the differences with respect to how the data was analysed and presented. Exercise challenge differs in as much that it uses a maximal stimulus for a short period of time, where spirometry can be performed continuously only after the end of the challenge and data are plotted against time. Unlike exercise, mannitol, allergen and inhaled LTE$_4$ challenges are cumulative with a number of dose steps, where spirometry can be performed in between and the challenge is terminated when a predefined threshold of FEV$_1$ fall is reached (15 or 20% from baseline) or the maximal dose is delivered. This allows for determination of a shift in the dose-response curve from the screening provocation as well as assessment of group differences in

sensitivities to the respective challenging agents between the treatments. Methacholine challenge was used as a screening tool without any related intervention. That is why the results from this challenge are not going to be presented here.

### 5.2.1 Exercise challenge and EIB treatment

The exercise challenge described above (see section 4.3.1) was performed at screening (visit 2), as well as after 3 weeks (visit 4) and 6 weeks (visit 5) of treatment. No asthma medication or physical exercise was allowed 24 hours prior to the challenges. The mean max post exercise $FEV_1$ fall from pre-exercise values was less than 20% in all treatment groups at baseline i.e. before treatment (Table 6), which indicates that the majority of subjects had mild EIB. Treatment with bud/form on demand for 6 weeks resulted in a mean max post exercise $FEV_1$ fall that was 5.4% smaller than the post exercise $FEV_1$ fall recorded at baseline (95% CI: -8.9 to -1.8). For the group receiving regular budesonide the mean max post exercise $FEV_1$ at 6 weeks was 6.6% smaller than at baseline (95% CI: -10.3 to -3.0), whereas the response was 1.5% greater (95% CI: -2.1 to + 5.1) for the terbutaline on demand group. This translates to a 28.5% reduction in EIB for the bud/form on demand group compared to a 38.6% reduction for the group that received budesonide regularly. In contrast there was an 8.9% increase in EIB for the group that was treated only with terbutaline on demand, see also Table 6. When compared to the terbutaline treatment group both the bud/form group and the regular budesonide group were accordingly significantly better in inhibiting EIB after six weeks of treatment (p= 0.017 and p= 0.0026 respectively).

After three weeks of treatment there was a small reduction in EIB in both the bud/form (18.7%) and the regular budesonide group (26.4%), whereas the response in the terbutaline group remained almost unchanged (1.6%). The difference after three weeks of treatment in the protective effect against EIB between the terbutaline group and the bud/form group was however not significant (p=0.113), although it almost reached statistical significance for the regular budesonide group (p=0.051).

| | | Baseline | 3 weeks | 6 weeks |
|---|---|---|---|---|
| **Regular placebo and terbutaline on demand** | Max post-exercise $FEV_1$-fall | 17.7 ± 8.9 | 16.9 ± 8.5 | 18.8 ± 10.9 |
| | Relative mean change (%) | - | -1.6 | +8.9 |
| **Regular budesonide and terbutaline on demand** | Max post-exercise $FEV_1$-fall | 15.3 ± 5.7 | 11.3 ± 7.2 | 9.6 ± 7.0 |
| | Relative mean change (%) | - | -26.4 | -38.6 |
| **Regular placebo and budesonide/formoterol on demand** | Max post-exercise $FEV_1$-fall | 16.4 ± 7.5 | 12.6 ± 7.2 | 11.1 ± 8.2 |
| | Relative mean change (%) | - | -18.7 | -28.5 |

**Table 6. Maximal post-exercise $FEV_1$ fall at baseline (randomization) as well as after 3 and 6 weeks of treatment for all groups. The relative mean change indicates the relative change in EIB compared after 3 and 6 weeks of treatment. Data are presented as mean ±SD.**

When comparing the bud/form with the regular budesonide group there was no significant difference regarding their bronchoprotective effect against EIB at three weeks (p=0.697) or at

six weeks of treatment (p=0.582). The reduction of maximal post-exercise $FEV_1$ fall after 6 weeks of treatment was 1.2 % in favour of regular budesonide treatment compared with the group who received bud/form on demand. The lower limit of the 97.5% confidence interval was -3.47%. Thus non-inferiority for the treatment with the combination of budesonide and formoterol compared with regular budesonide treatment was proven for the main outcome variable, see Figure 8.
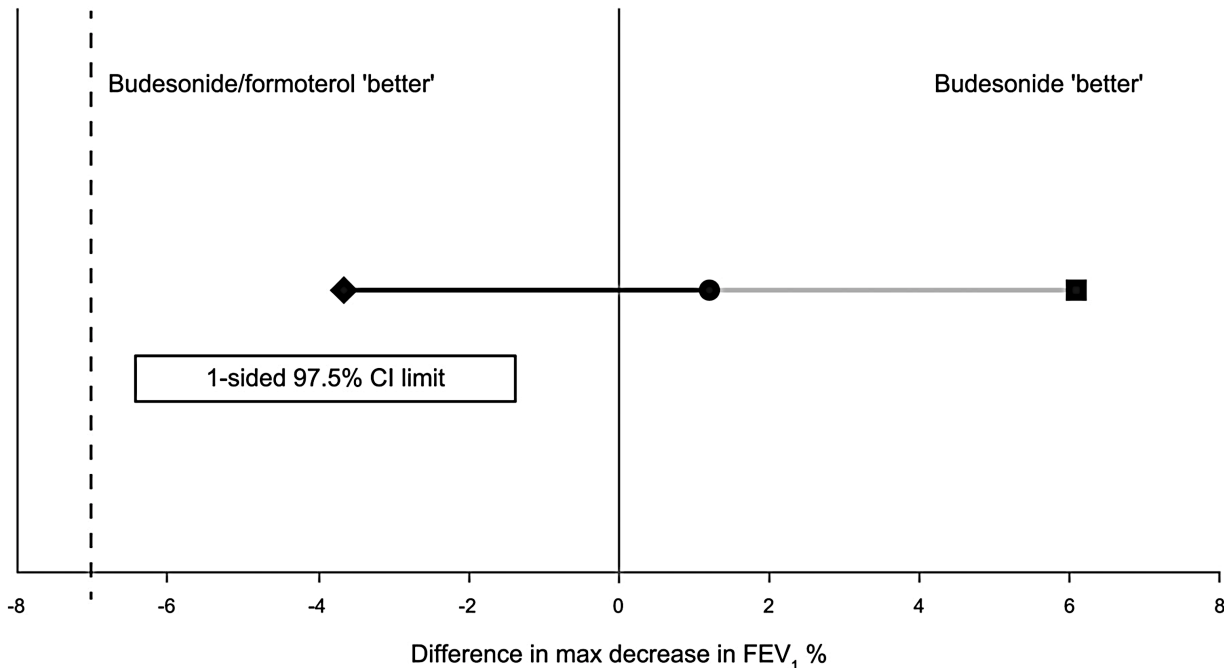


**Figure 8. Demonstration of non-inferiority between regular budesonide and budesonide/ formoterol on demand for the primary outcome.**

Lung function measurements ($FEV_1$) were performed for up to 30 minutes after the end of the challenge at which point a SABA was inhaled and lung function recovered to baseline. This enabled us to plot the post exercise $FEV_1$ fall against time, as done in a previous exercise challenge study (Fogel, Rosario et al. 2010). Figure 9 visualizes the post exercise $FEV_1$ fall up to 30 minutes after the end of the exercise challenge at baseline i.e. before treatment as well as after six weeks of treatment for all three groups.
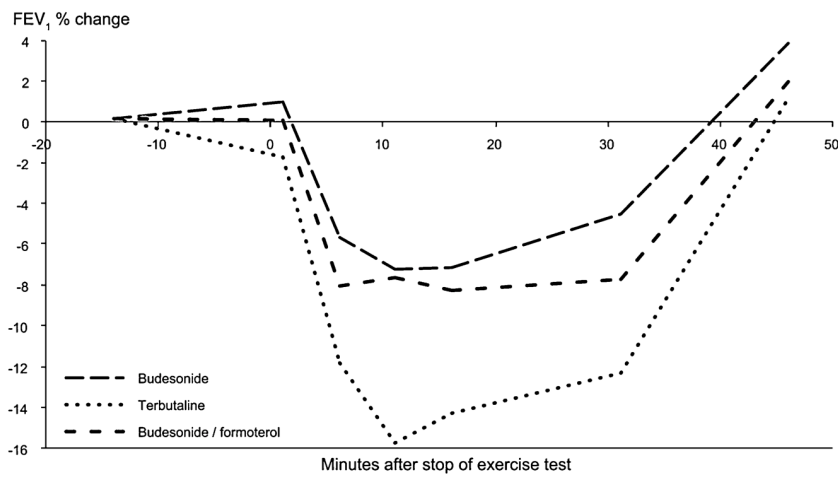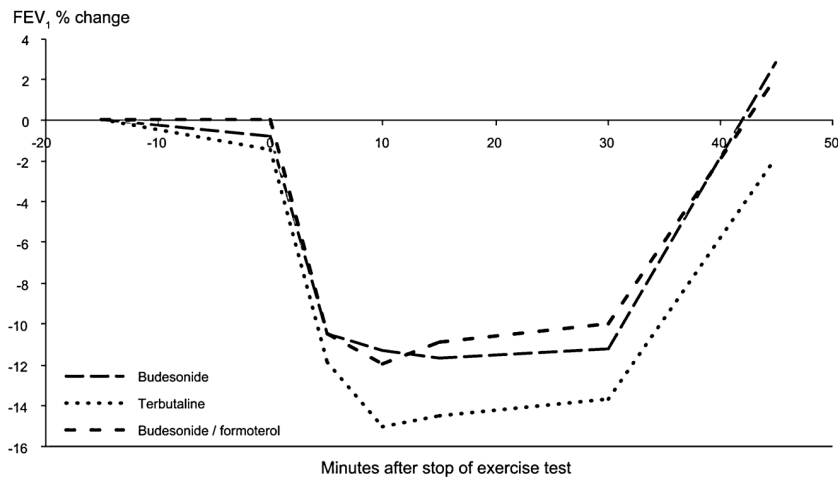
**Figure 9: FEV$_1$ before and after an exercise challenge at baseline i.e. before treatment (upper panel) as well as after six weeks of treatment (lower panel) for all groups.**

The nadir in FEV$_1$ for all treatment groups occurred within 15 minutes after the end of exercise both at baseline as well as after treatment, which happens in most cases after an exercise challenge (Brudno, Wagner et al. 1994). At baseline, the post exercise FEV$_1$ fall for all groups reached a plateau with very limited spontaneous recovery of lung function until subjects received SABAs 30 minutes after the end of exercise. At the end of study (six weeks) there was a better spontaneous recovery of FEV$_1$ for the regular budesonide group that almost reached -5% of pre-exercise value. The bud/form on demand group had a plateau shaped curve similar to the baseline response (Figure 9).

During the 6-week treatment period, the daily dose of inhaled budesonide was on average 393 μg in the group that inhaled budesonide on a regular basis and 163 μg in the group that inhaled the combination on demand, see Table 7. The average total need for daily extra medication was 0.93 ± 0.54 inhalations in the terbutaline group, 0.81 ± 0.50 inhalations in the bud/form group and 0.77 ± 0.67 inhalations in the regular budesonide group. The inhalations taken for symptom relief, i.e. not prior to training sessions, did not differ between the groups and was 0.15 ± 0.24 inhalations per day in the terbutaline group, 0.07 ± 0.09 inhalations per day in the bud/form group and 0.14 ± 0.55 inhalations per day in the regular budesonide group (Table 7).

40

| | Placebo once daily and budesonide + formoterol on demand N=23 | Regular placebo once daily + terbutaline on demand N=22 | Regular budesonide once daily + terbutaline on demand N=21 |
|---|---|---|---|
| Budesonide, metered dose 400 µg/inhalation (µg /day) | 0 | 0 | 393 ± 12 |
| Budesonide, metered dose 200 µg/inhalation (µg /day) | 163 ± 80 | 0 | 0 |
| Terbutaline (µg/day) | 0 | 372 ± 216 | 309 ± 266 |
| Formoterol (µg/day) | 3.7 ± 2.3 | 0 | 0 |
| Maintenance, number of inhalations/day | 0.96 ± 0.10 | 0.99 ± 0.03 | 0.98 ± 0.03 |
| Medication prior to exercise, number of inhalations/day | 0.74 ± 0.46 | 0.78 ± 0.46 | 0.63 ± 0.19 |
| Medication for symptom relief, number of inhalations/day | 0.07 ± 0.09 | 0.15 ± 0.24 | 0.14 ± 0.55 |

**Table 7: Intake of study drugs throughout the study. Data are presented as mean±SD.**

In summary, the EIB study **(Paper I)** demonstrated that a six-week treatment with inhalation of a combination of budesonide and formoterol, prior to training sessions and for symptom relief, was superior to on demand inhalation of the SABA terbutaline in reducing the airway response to exercise. This was assessed by an exercise test that was preceded by a 24-hour period free of any medication. Furthermore, it was found that the reduction in EIB after six weeks treatment regimen with inhalation of bud/form on demand was similar to regular, daily inhalation of budesonide. This finding clearly indicates that three to four weekly doses of the combination treatment are sufficient to reduce EIB, to a similar degree as regular treatment with low dose inhaled corticosteroids in these patients with rather mild asthma. It was also shown that the airway response to exercise after 24 hours free from all medication was unaltered, or even slightly increased, after six weeks in the group that was only treated with inhaled terbutaline on demand, which is in agreement with the results of previous studies with methacholine (Vathenen, Knox et al. 1988), exercise (Hancox, Subbarao et al. 2002) and repeated low dose allergen (Dahlen, Lantz et al. 2009) where SABAs or LABAs were used as monotherapy.

### 5.2.2 Mannitol challenge

The airway response to a bronchial challenge with mannitol was assessed at baseline (visit 3) prior to randomization and at the end of the treatment period (visit 6). The mannitol data were not included in the original paper and are thus unpublished. All subjects that performed the challenge had a positive exercise test at visit 2 i.e. had a confirmed EIB diagnosis. This permits a preliminary comparison of the sensitivity to mannitol and exercise challenge in this cohort of patients. Results were available from 60 out of 66 enrolled subjects and are seen in Figure 10, where the $FEV_1$ fall during the challenge was plotted against the inhaled cumulative dose of mannitol. Thirty subjects (50%) inhaled the maximal cumulative mannitol dose of 635 mg without reaching the threshold of 15% fall in $FEV_1$. The results are thus consistent with a lower sensitivity of the mannitol challenge in diagnosing EIB than previously published data

(Anderson, Brannan et al. 1997, Brannan, Koskela et al. 1998). The original studies however included subjects who were in the majority atopic with a more severe EIB compared to our group of asthmatics (e.g. $FEV_1$ fall $40 \pm 19\%$ SD in the study by Brannan et al). In another study where mannitol was compared to EVH in elite athletes (Holzer, Anderson et al. 2003), mannitol showed sensitivity of 83% to identify EIB but also in that group, the $FEV_1$ fall was greater than in our material ($25.4 \pm 15\%$ SD). In contrast, the sensitivity of mannitol to diagnose EIB was reduced to 59% when tested in a group of asthmatics with mild EIB that had a $FEV_1$ fall after exercise of $19 \pm 9.2\%$ (SD), which is similar to our results (Anderson, Charlton et al. 2009). Likewise, in another Scandinavian study with mild asthmatics mannitol was positive in 12 out of 22 subjects (55%) with a positive EVH challenge ($FEV_1$ fall > 10%) (Aronsson, Tufvesson et al. 2011).
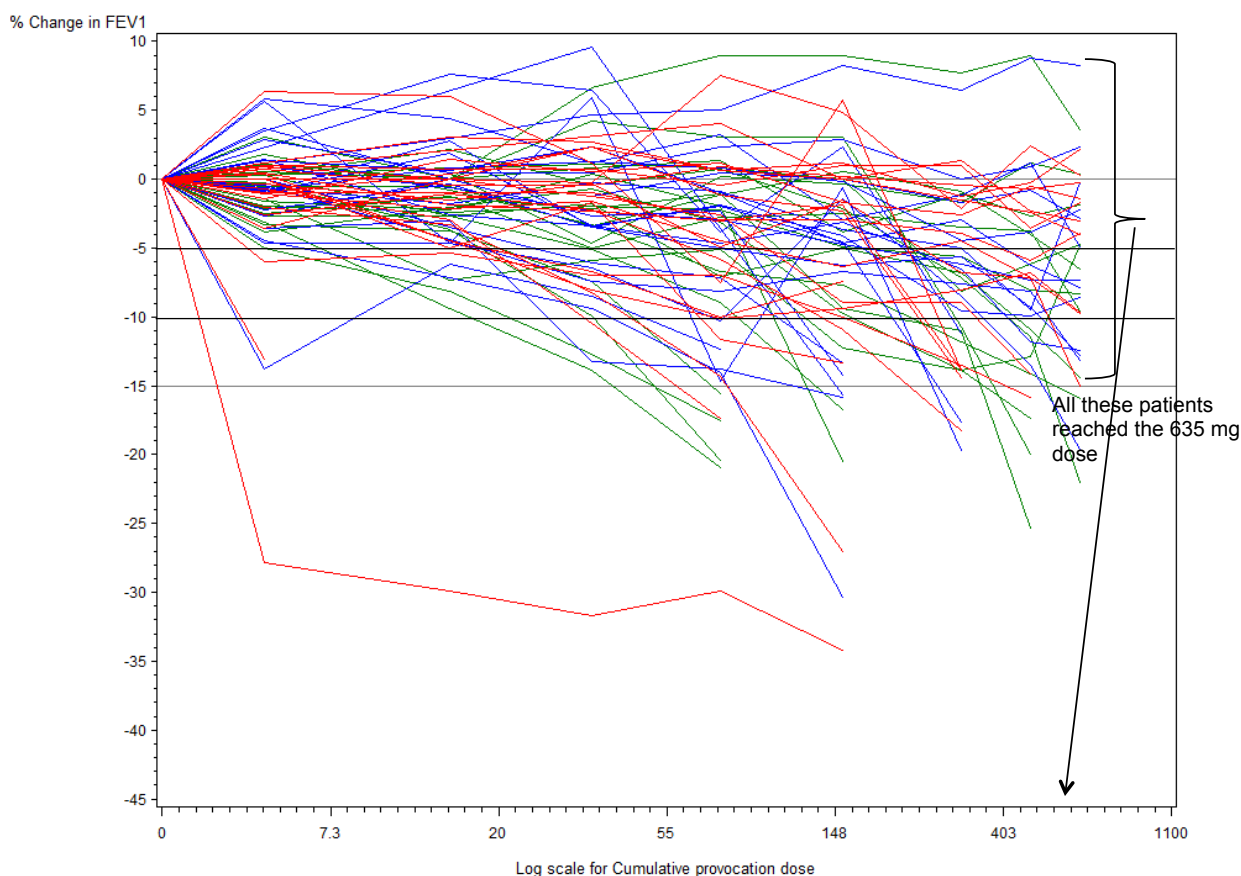


**Figure 10: $FEV_1$ fall versus mannitol dose at baseline (visit 3). Blue lines represent subjects that were randomized to receive terbutaline on demand, green lines budesonide once daily plus terbutaline on demand and red lines bud/form on demand**.

After 6 weeks of treatment (visit 6) the mannitol challenge was repeated and the $FEV_1$ fall for all subjects during the challenge is shown in Figure 11. Thirty-nine subjects inhaled the highest cumulated dose of mannitol compared to thirty at baseline; there was no significant difference in the mannitol airway response between baseline and end of study possibly due to the fact that at baseline already half of the subjects had reached the maximal inhaled dose. Interestingly of the remaining twenty-one subjects that were still mannitol positive at the end of the study, preliminary analysis indicates that fifteen belonged to the two groups that received ICS. Because

42

of the low responder rate to mannitol, there was no power to perform a meaningful comparison of the effects of the interventions on this outcome.
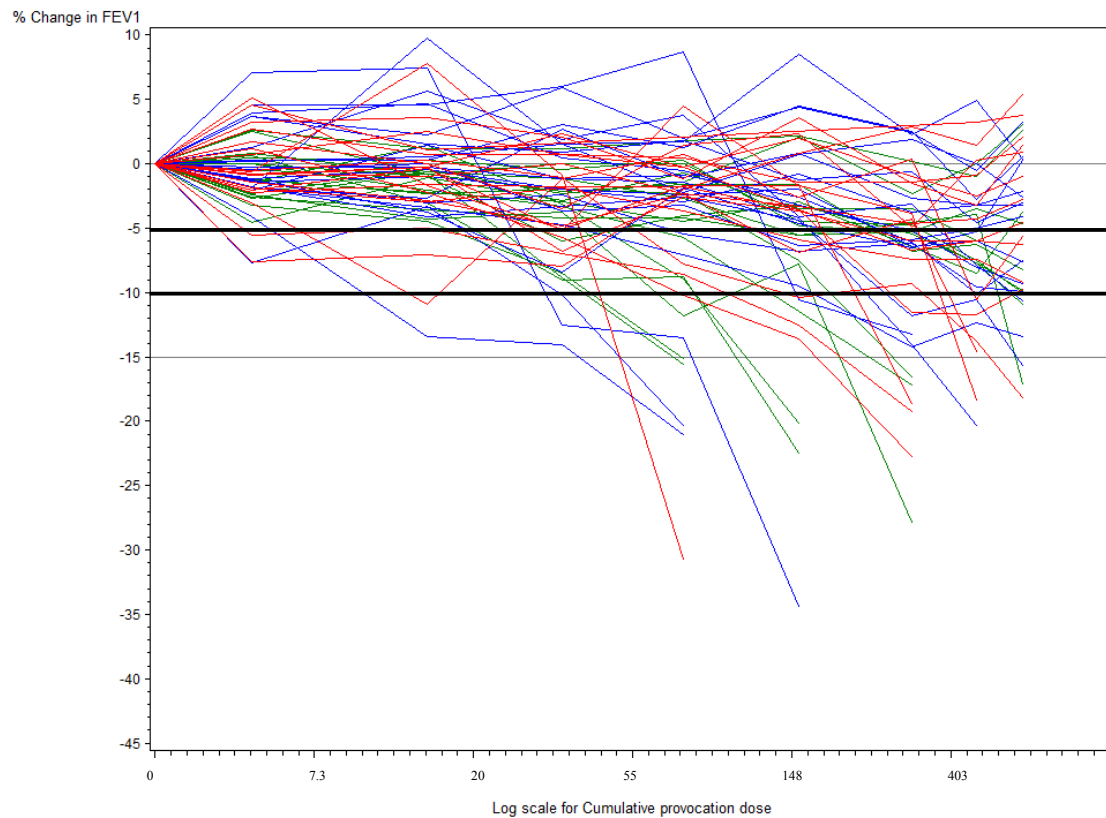


**Figure 11**: **FEV$_1$ fall versus mannitol dose at end of study (visit 6). Blue lines represent subjects that were randomized to receive terbutaline on demand, green lines budesonide once daily plus terbutaline on demand and red lines bud/form on demand.**

### 5.2.3  Allergen challenge and anti-IgE treatment

An allergen challenge was performed at baseline, after six and twelve weeks (end of treatment period) as well as eighteen weeks after the first dose. The change in allergen PC$_{15}$ was calculated and presented as fold change from baseline for each individual, data plotted against time are shown in Figure 12.
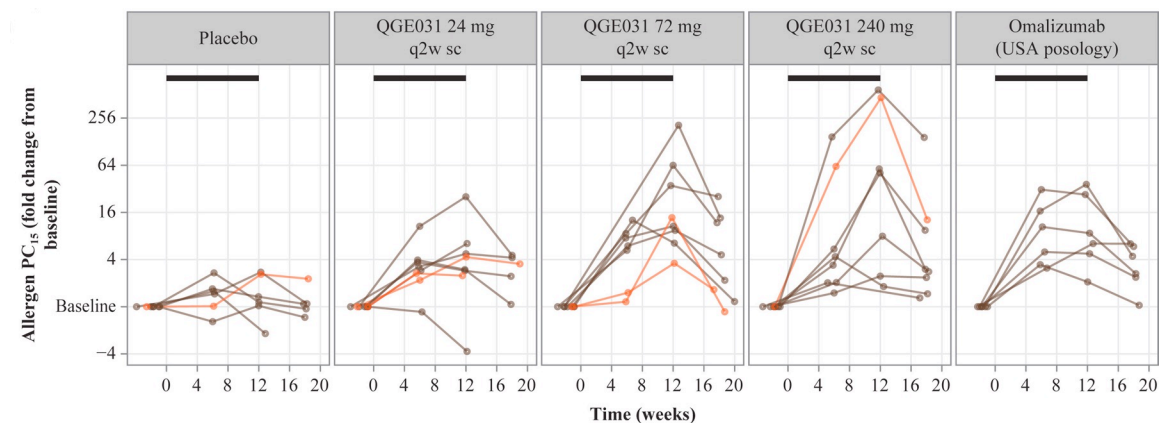


**Figure 12: Individual subject time-course data for placebo, the three doses of QGE031 and omalizumab. Red-coloured data are subjects with baseline IgE levels greater than 700 IU/ml.**

There was a great variety in the allergen $PC_{15}$ shift between subjects, especially for the subjects that were treated with 72 and 240 mg of QGE031, where the change from baseline ranged from less than 2 to more than 500-fold at week 12 (Figure 12). This change was on its way to returning to baseline by week 18. The mean change from baseline for $\log_2$-transformed allergen $PC_{15}$ was 4.96 (number of doubling dilutions) at week 12 for the group that received 240 mg of QGE031 with a range of 0.85 to 9.2 (Table 8).

| Change from baseline of log2 $PC_{15}$ at week 12 | QGE031 24 mg (n=8) | QGE031 72 mg (n=7) | QGE031 240 mg (n=8) | Omalizumab (n=6) | Placebo (n=6) |
|---|---|---|---|---|---|
| Mean (SD) | 1.78 (1.82) | 4.23 (1.92) | 4.96 (3.39) | 3.17 (1.56) | 0.39 (0.96) |
| CV% mean | 102.6 | 45.5 | 68.2 | 49.2 | 245.2 |
| Min-Max | (-1.90, 4.66) | (1.84, 7.70) | (0.85, 9.19) | (1.05, 5.19) | (-1.14, 1.47) |

**Table 8: Change from baseline of $\log_2$ $PC_{15}$ (number of doubling dilutions) at week 12. CV: Coefficient of variation; SD: Standard deviation**

The ratio of the geometric means for allergen $PC_{15}$ at week 12 compared to baseline was calculated for each of the three dose regimens of QGE031 and was compared to omalizumab. Treatment with 72 and 240 mg of QGE031 produced a mean change in the inhaled allergen concentration (allergen $PC_{15}$) that numerically was two to three-fold greater than that for omalizumab at week 12 but the difference was not statistically significant (p=0.14 and p=0.10 respectively). Treatment with 24 mg QGE031 was less effective than omalizumab (Figure 13).
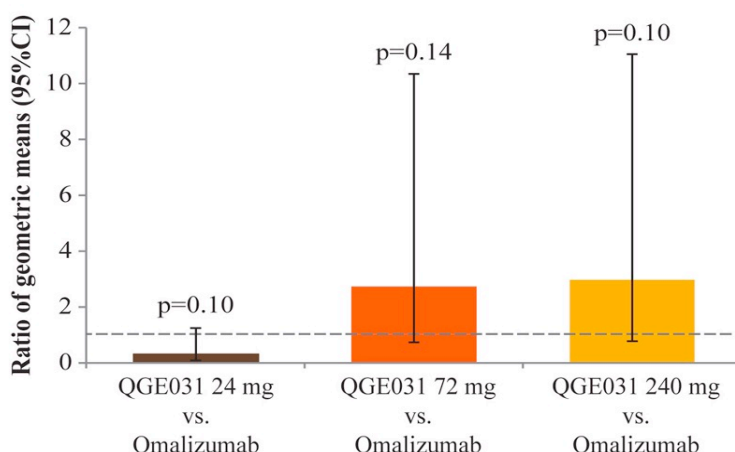


**Figure 13: Statistical analyses of the geometric mean ratios of allergen $PC_{15}$ for the three different QGE031 doses versus omalizumab at week 12.**

When compared to placebo at week 12 there was a change in allergen $PC_{15}$ of sixteen-fold for the groups receiving 240 mg of QGE031 (p< 0.001) and 72 mg of QGE031 (p<0.001) compared

to a five-fold improvement in the group that received omalizumab (p=0.02) (Figure 14). Treatment with 24 mg of QGE031 was no different than placebo (p=0.40).
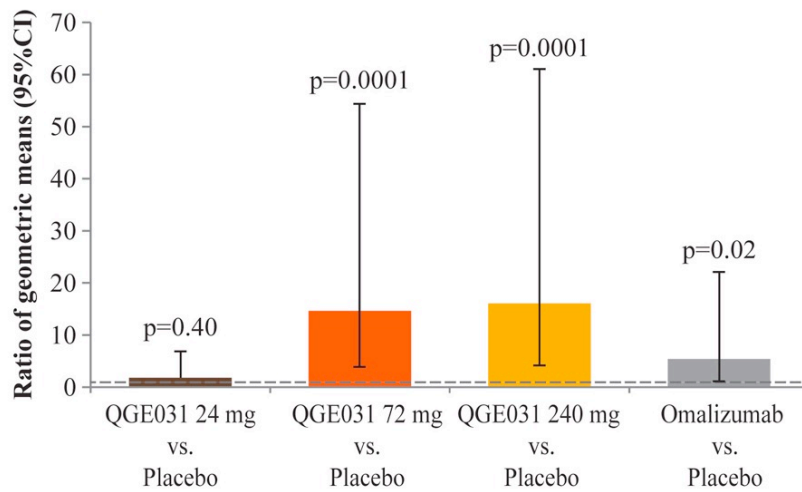


**Figure 14: Statistical analyses of the geometric mean ratios of allergen PC$_{15}$ for the three different QGE031 doses versus placebo at week 12.**

In summary, we have shown that treatment with the highest dose of QGE031 every two weeks inhibited the early airway response (EAR) to a standardized allergen bronchoprovocation challenge causing a shift in allergen PC$_{15}$ that was sixteen times greater than placebo, twelve weeks after the first dose. Omalizumab inhibited also the EAR with a change in allergen PC$_{15}$ that was five times greater than placebo and almost nine times compared with the baseline (3.2 doubling dilutions) at twelve weeks. These results for omalizumab are in agreement with previous allergen bronchoprovocation studies where the drug has shown similar efficacy in inhibiting both the EAR (Boulet, Chapman et al. 1997, Zielen, Lieb et al. 2013), as well as the late asthmatic reaction (Fahy, Fleming et al. 1997, van Rensen, Evertse et al. 2009). When comparing QGE031 with omalizumab, the shift in allergen PC$_{15}$ at twelve weeks was three times greater for 240 mg of QGE031 compared with that after omalizumab, although the difference was not statistically significant. The peak of the allergen PC$_{15}$ change for all QGE031 treatment arms occurred at twelve weeks after the first dose i.e. two weeks after the last dose, returning towards baseline levels by week 18, which is a pattern similar to the one observed with omalizumab. A great variety in the allergen PC$_{15}$ shift was observed in the subjects that were treated with QG031 indicating heterogeneity in the inhibition of the EAR. It is known that the airway response to inhaled allergen in asthmatics can be regulated through other pathways that are not Fc$\varepsilon$RI dependent (Neighbour, Boulet et al. 2014, Kaur, Gomez et al. 2015). Mast cells and basophils can be activated through other mediators such as the epithelial cell-derived cytokines thymic stromal lymphopoietin (TSLP) and interleukin-33 (IL-33) that can partly be involved in airway response to allergen inhalation (Salter, Oliveria et al. 2015, Saluja, Zoltowska et al. 2016). Thus anti-IgE treatment does not block all the mediators that are involved in the EAR after allergen inhalation, which might explain the great variety in the suppression of the response to allergen observed in the subjects treated with QGE031 in this study.

### 5.2.4 Leukotriene $E_4$ challenge and treatment with montelukast

A cumulative inhalation challenge with $LTE_4$ was performed at screening (visit 2) as well as at the last day of each treatment period (visits 4 and 6) with either the $CysLT_1$ receptor antagonist montelukast or matching placebo. At screening the mean maximal $FEV_1$ drop after $LTE_4$ inhalation was 28.7% (95% CI, -25.3% to -32%) compared to 26.3% (95% CI, -23% to -29.6%) after treatment with placebo and 0.45% (95% CI, -3% to 2.1%) after treatment with montelukast (Figure 15). Thus montelukast abolished the airway response to inhaled $LTE_4$ compared to placebo (p < 0.001).
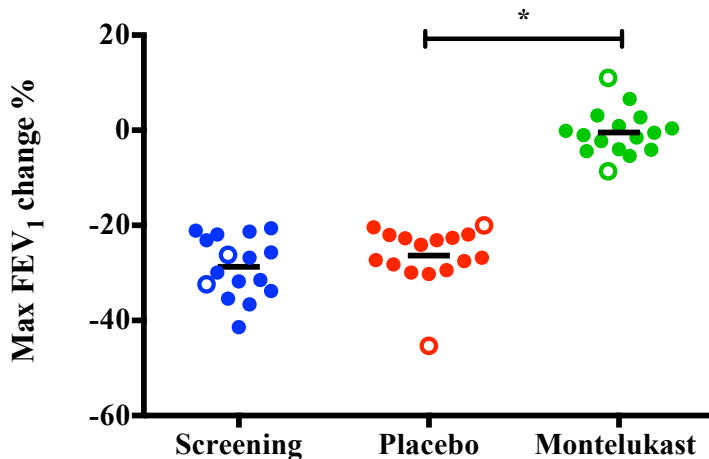


**Figure 15: Maximal $FEV_1$ fall  % at screening (blue plot), after treatment with placebo (red plot) and montelukast (green plot). Open circles represent values for AERD subjects. Horizontal bars indicate mean values; *p<0.001**

When treated with montelukast subjects inhaled on average a ten-fold higher cumulated dose of $LTE_4$ (geometric mean 380,277 pmol) compared to after treatment with placebo (geometric mean, 37,006 pmol; p< 0.001) (Figure 16). The airway response to inhaled $LTE_4$ for the two subjects with AERD was similar to the rest of the asthmatic subjects (open circles in Figures 15 and 16).
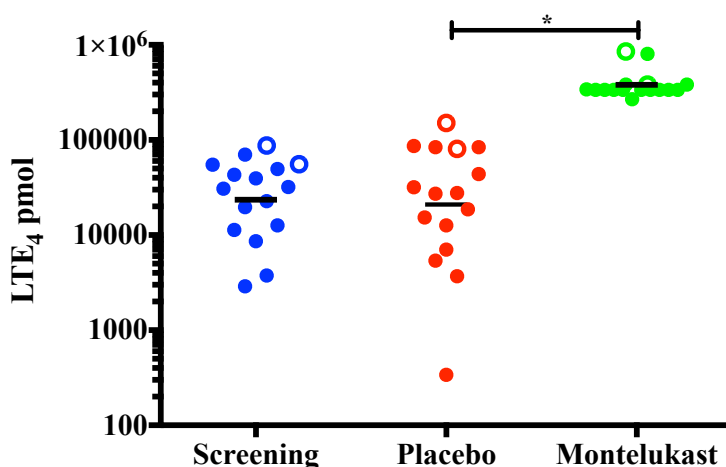


**Figure 16: $LTE_4$ $PD_{20}$ values at screening (blue plot) and after treatment with placebo (red plot), total dose of inhaled $LTE_4$ after treatment with montelukast (green plot). Open circles represent values for AERD subjects.  Horizontal bars indicate geometric mean values * p<0.001**

46

There was no significant difference in the LTE$_4$PD$_{20}$ when comparing results at screening (geometric mean 23,610 pmol) and after treatment with placebo (geometric mean 20,923 pmol) (Figure 16). Similarly the Bland-Altman analysis demonstrated that the challenge was highly repeatable (Figure 17), which is in agreement with previous studies with inhalation of leukotriene D$_4$ performed in our lab (Gyllfors, Kumlin et al. 2005, Gyllfors, Dahlen et al. 2006).
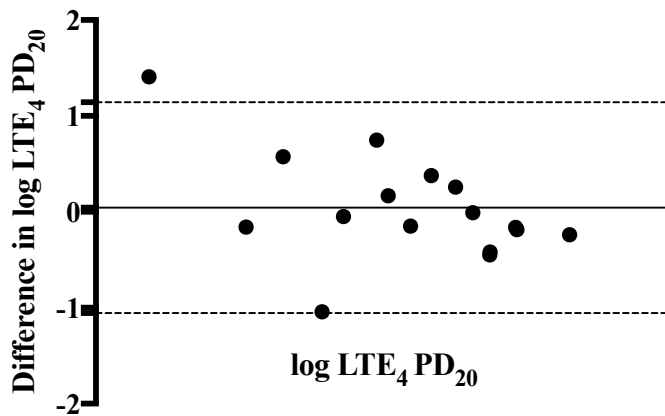


**Figure 17: Bland-Altman plot where the difference in log LTE$_4$ PD$_{20}$ between screening and after treatment with placebo were plotted against their mean.**

Interestingly, there was an inverse relation between the airway response to methacholine and LTE$_4$ where subjects with the highest responsiveness to methacholine had the lowest relative responsiveness to LTE$_4$ (Figure 18). This finding is in agreement with the relations demonstrated between inhaled methacholine and leukotriene C$_4$ and D$_4$ respectively in previous studies (Adelroth, Morris et al. 1986, Gyllfors, Kumlin et al. 2005).
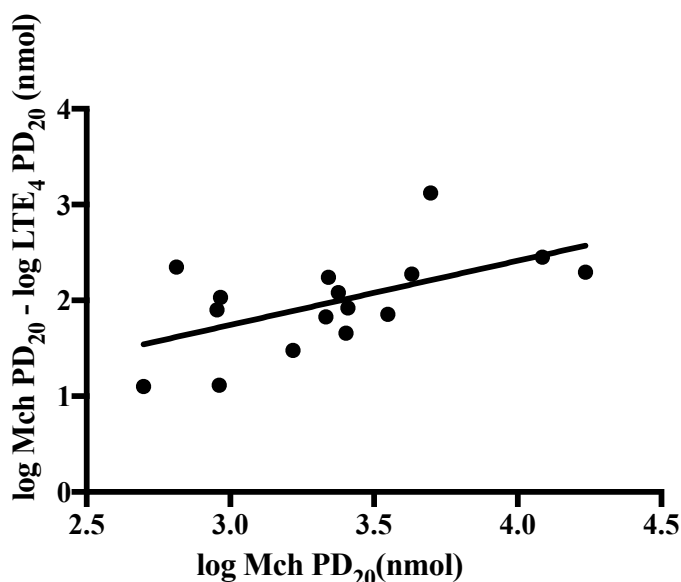


**Figure 18: Relation between airway responsiveness to methacholine (PD$_{20}$) and the relative potency of LTE$_4$ compared to methacholine (difference in logMchPD$_{20}$ and logLTE$_4$PD$_{20}$). r = 0.57 , p = 0.002 (Pearson)**

In order to extend the information about the airway response to inhaled $LTE_4$ and examine its influence in the small airways, IOS measurements were performed in eleven subjects including both AERD subjects. Measurements were performed prior to as well as at the end of each challenge after the last dose of $LTE_4$ was inhaled. Values for all major IOS parameters at baseline as well as after inhalation of $LTE_4$ at the end of each treatment period are presented in Table 9. There was a significant change in all IOS measurements after inhalation of $LTE_4$ when subjects were treated with placebo. After treatment with montelukast there was a slight increase in $R_{20}$ after inhalation of $LTE_4$, which is a parameter that reflects resistance mainly in the central airways. The total airway resistance $R_5$ increased with 58.9% (mean, 95% CI, 41.1% to 76.8%) from baseline after inhalation of $LTE_4$ with placebo compared to a 7.1% increase (mean, 95% CI, -3.2% to 17.2%) after montelukast, with the difference being highly significant $p < 0.001$. The increase in $R_5$ during placebo is similar to changes observed after methacholine challenge (Short, Anderson et al. 2015), EVH (Rundell, Evans et al. 2005, Price, Ansley et al. 2016) and slightly higher than the one after mannitol challenge (Horsman, Duke et al. 2009). Frequency dependent resistance ($R_5$-$R_{20}$), one of the most sensitive IOS markers in the bronchoprovocation setting (Naji, Keung et al. 2013) increased seven fold after $LTE_4$ inhalation during placebo treatment (Figure 19), which is a clear sign of peripheral airway obstruction and small airways impairment similar to that seen in patients with severe asthma and small airways disease (Williamson, Clearie et al. 2011, Anderson, Zajda et al. 2012). Changes in reactance were of the same magnitude, the area of reactance ($A_x$), which is considered to be also one of the most sensitive IOS indexes and the one that correlates strongest to $R_5$-$R_{20}$ (Goldman, Saadeh et al. 2005) increased ten-fold from baseline after $LTE_4$ inhalation (Figure 20). This conspicuous small-airway obstruction caused by $LTE_4$ was abolished when subjects were treated with montelukast (Figures 19 and 20).

| | Placebo | | Montelukast | |
|---|---|---|---|---|
| | Pre | Post | Pre | Post |
| $R_5$ (kPa/L/s) | 0.37 (0.23; 0.63) | 0.56 (0.43; 1.03)* | 0.31 (0.16; 0.56) | 0.33 (0.21; 0.65) |
| $R_{20}$ (kPa/L/s) | 0.35 (0.23; 0.59) | 0.44 (0.31; 0.65)* | 0.31 (0.15; 0.55) | 0.33 (0.22; 0.64)[¶] |
| $F_{res}$ (Hz) | 9.37 (7.8; 13.8) | 23.7 (11.8; 29.6)* | 9.26 (6.04; 12.8) | 8.94 (6.87; 15.8) |
| $X_5$ (kPa/L/s) | -0.09 (-0.15; -0.05) | -0.21 (-0.47; -0.11)* | -0.07 (-0.19; 0.1) | -0.09 (-0.25; -0.03) |
| $A_x$ (kPa/L) | 0.15 (0.09; 0.58) | 1.62 (0.33; 5.22)* | 0.14 (0.01; 0.48) | 0.16 (0.025; 0.8) |
| $R_5$-$R_{20}$ (kPa/L/s) | 0.02 (-0.03; 0.06) | 0.14 (0.04; 0.38)* | 0.01 (-0.01; 0.19) | 0.02 (-0.03; 0.08) |

**Table 9: All values represent median and range. $R_5$ resistance at 5Hz, $R_{20}$ resistance at 20Hz, $F_{res}$ resonant frequency, $X_5$ reactance at 5Hz, $A_x$ reactance area, $R_5$-$R_{20}$ frequency dependent resistance. * p<0.01, ¶p=0.04. Wilcoxon signed rank test, comparison between pre and post inhalation challenge with $LTE_4$.**
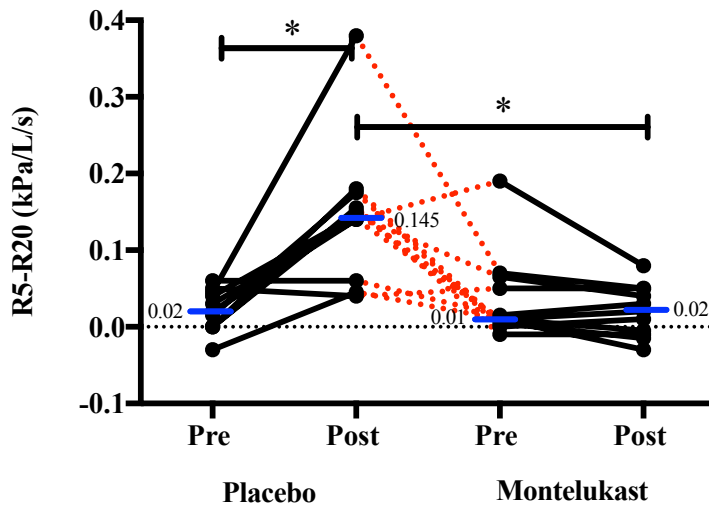
**Figure 19: Changes in frequency-dependent resistance ($R_5$-$R_{20}$) before and five minutes after LTE$_4$ inhalation challenge for both treatment periods. Horizontal bars indicate median values. * P<0.01, Wilcoxon signed rank test.**
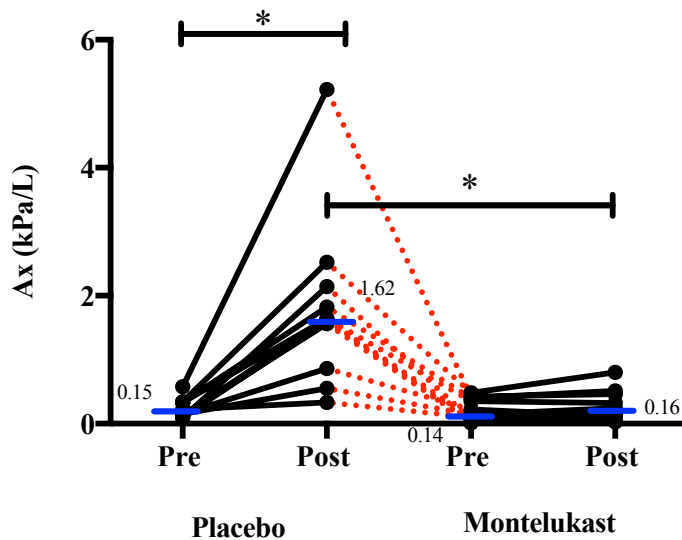


**Figure 20: Changes in reactance area ($A_x$) before and five minutes after LTE$_4$ inhalation challenge for both treatment periods. Horizontal bars indicate median values. * P<0.01, Wilcoxon signed rank test.**

In summary, the airway response to inhaled LTE$_4$ was completely blocked by treatment with montelukast. On the average, subjects tolerated a ten-fold higher dose of LTE$_4$ compared to placebo without a fall in FEV$_1$ indicating that the LTE$_4$ effects on smooth muscle in asthmatics are mediated solely through the CysLT$_1$ receptor. In addition, IOS results indicate that LTE$_4$ contributed to small-airways obstruction, which also was mediated through the CysLT$_1$ receptor. This small airways impairment induced by leukotrienes is probably due to both a constriction of the smooth muscle as well as mucosal edema because CysLT$_s$ are potent inducers of plasma exudation (Dahlen, Bjork et al. 1981) in the airways (Hua, Dahlen et al. 1985, Persson, Erjefalt et al. 1986) and provoke profound disturbances of pulmonary gas exchange in asthma (Echazarreta, Dahlen et al. 2001).

## 5.3  EFFECT OF INTERVENTIONS ON MARKERS OF AIRWAY INFLAMMATION

Measurement of $F_E$NO as well as measurement of inflammatory cells in induced sputum after inhalation of hypertonic saline have been widely used as surrogate markers for assessment of airway inflammation in subjects with asthma (Pizzichini, Pizzichini et al. 1996, Gibson 1998, Barnes, Dweik et al. 2010, Cowan, Cowan et al. 2010). In **Paper III** $F_E$NO was assessed on all study days in order to monitor airway inflammation and measurements were performed prior to baseline spirometry. Induced sputum was also collected in **Paper III** four hours after the end of the $LTE_4$ inhalation challenge performed on the last day of each treatment period with montelukast and placebo respectively.

### 5.3.1  Effects of montelukast on baseline $F_E$NO

There was no significant difference in $F_E$NO values measured at screening and during the two treatment periods, see Table 10. The variability in the data was low with most subjects displaying $F_E$NO values with in normal range on most study days with the exception of subject 5, thus indicating a low grade of inflammation in the airways. Previous studies have shown that montelukast may decrease $F_E$NO in asthmatics. However, in those studies montelukast was given as add on treatment in subjects already treated with ICS that had a higher degree of airway inflammation compared to our group (Ghiro, Zanconato et al. 2002, Montuschi, Mondino et al. 2007, Kononowa, Michel et al. 2013).

| Subject No | Baseline | Placebo start | Placebo end | Montelukast start | Montelukast end |
|---|---|---|---|---|---|
| 1 | 8 | 16 | 14 | 13 | 12 |
| 2 | 68 | 57 | 66 | 74 | 55 |
| 3 | 50 | 64 | 56 | 49 | 39 |
| 4 | 13 | 10 | 12 | 22 | 11 |
| 5 | 116 | 112 | 105 | 116 | 113 |
| 6 | 24 | 24 | 22 | 19 | 18 |
| 7 | 10 | 8 | 8 | 8 | 9 |
| 8 | 11 | 9 | 7 | 11 | 8 |
| 9 | 14 | 19 | 18 | 19 | 18 |
| 10 | 36 | 51 | 48 | 44 | 46 |
| 11 | 30 | 24 | 21 | 23 | 26 |
| 12 | 52 | 57 | 50 | 56 | 42 |
| 13 | 11 | 14 | 14 | 11 | 12 |
| 14 | 48 | 37 | 43 | 43 | 57 |
| AERD 1 | 14 | 8 | 11 | 9 | 8 |
| AERD 2 | 21 | 13 | 20 | 20 | 16 |
| Geom. Mean | 24.1 | 23.4 | 23.7 | 24.6 | 22.2 |

**Table 10: $F_E$NO values in ppb throughout the study.**

### 5.3.2  Effects of montelukast on sputum cells

Sputum examination was performed four hours after the end of the $LTE_4$ challenge on both treatment periods and data that met pre-defined quality criteria (< 20% squamous cells) were

obtained on both occasions in nine subjects (including one AERD subject). There was no significant difference in the post-challenge percentage of sputum eosinophils between placebo (mean 4.5%, 95 CI, 1.8% to 7.1%) and montelukast (mean 3.2%, 95 CI, 1.4% to 4.9%) p=0.11 (Figure 21).
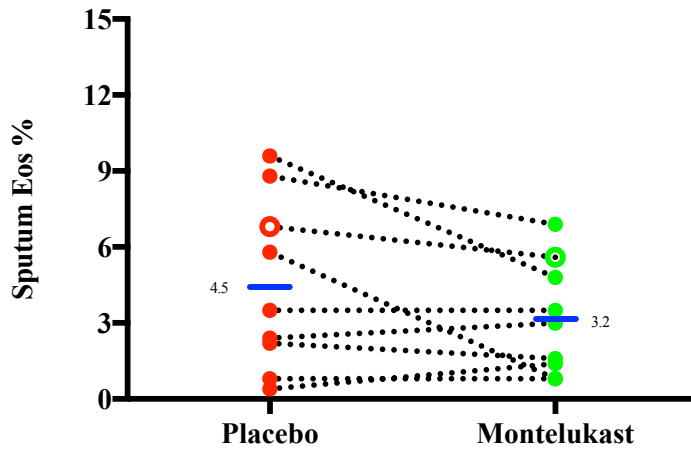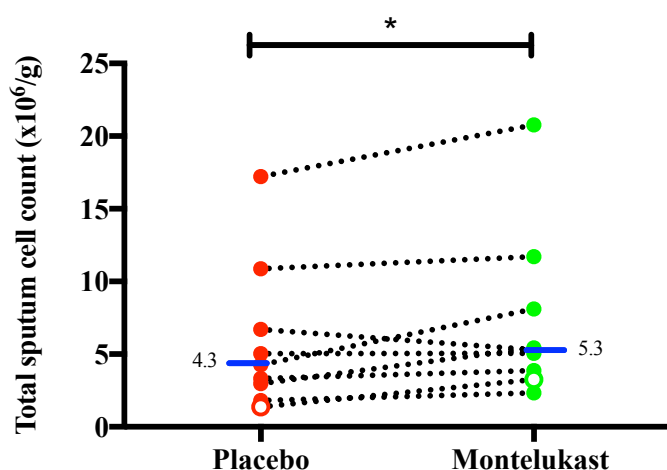


**Figure 21: Effects of montelukast on sputum eosinophil counts from 9 subjects with paired data. Open circle represents values in patient number 2 with AERD. Horizontal bars indicate mean values.**

There was no difference in the percentage of sputum neutrophils either (mean 28.8%, 95 CI, 18.5% to 39% for placebo compared to mean 30.4%, 95 CI, 12.9% to 47.9% for montelukast), p=0.82. The rest of the differential cell counts were also similar between treatments (Figure 22).
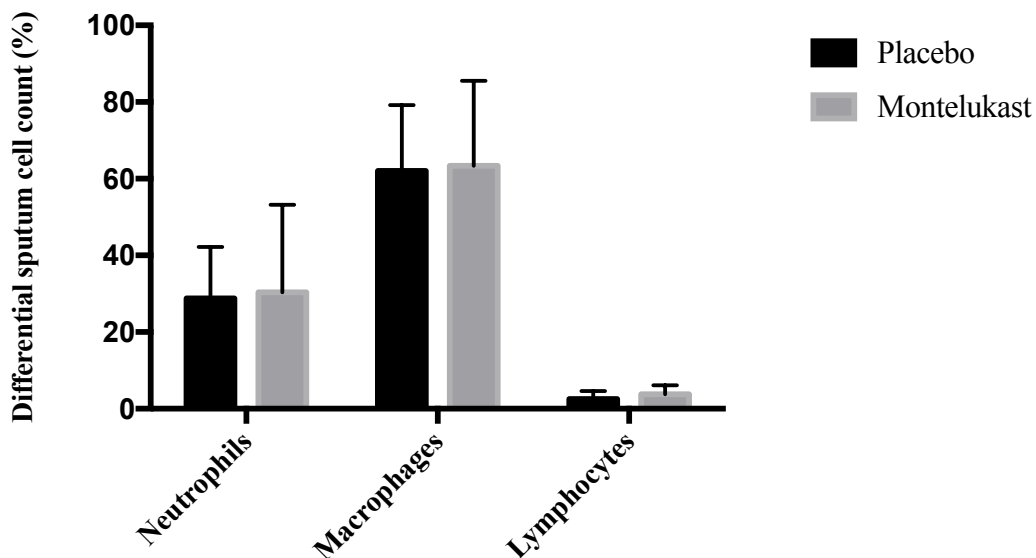


**Figure 22: Differential cell counts from sputum induction in the presence of placebo and montelukast respectively. Neutrophils p=0.82, macrophages p=0.87, lymphocytes p=0.094**

There was a small increase in the total number of cells when subjects were treated with montelukast compared to placebo; median 4.3, interquartile range, 2.4-8.8 x $10^6$/g sputum for placebo vs 5.3 median, interquartile range, 3.6-9.9 x $10^6$/g sputum for montelukast, p=0.039 (Figure 23).

**Figure 23: Effects of montelukast on total sputum cell counts from 9 subjects with paired data. The open circle represents values in patient number two with AERD. Horizontal bars indicate median values. P=0.039**

In summary, it was demonstrated that despite exposure to a ten times higher dose of $LTE_4$ during treatment with montelukast, the sputum eosinophil count was the same as after treatment with placebo. The results thus argue against an alternative receptor being involved in the pro-inflammatory effects of $LTE_4$ on airways. It is quite unusual in responses mediated by dual-receptors, that blockade of one receptor, does not allow the second receptor to be unmasked and express its response when higher agonist doses are administered. In this case, a ten times higher dose of $LTE_4$, i.e. a one-log order of magnitude in increase, did not produce more sputum eosinophils. If there had been an "E-type" alternative receptor driving eosinophilia, this receptor would have had remarkable opportunities to display functional effects once the montelukast treatment had abolished the $CysLT_1$ responses. The previous studies in the mouse ear oedema model, where the presence of three different CysLT receptors have been established in fact clearly demonstrate how sequential blockade or deletion of one receptor favours functional emergence of responses mediated by the remaining receptors (Kanaoka, Maekawa et al. 2013). In addition, the study suggests that the observed difference between inhaled $LTD_4$ and $LTE_4$ with respect to their ability to cause airway eosinophilia (Gauvreau, Parameswaran et al. 2001) is unlikely to be explained by a different receptor for $LTE_4$.

## 5.4 EFFECT OF INTERVENTIONS ON LIPID MEDIATOR RELEASE IN URINE

Urine samples were collected in **Paper III** before, during and after the end of the $LTE_4$ challenge at hourly intervals for up to four hours for measurement of the major lipid mediator metabolites of CysLTs, prostaglandins, tromboxanes and isoprostanes using UPLC-MS/MS and EIA (only for $11\beta$-$PGF_{2a}$). The aim was to explore both the pattern of the mediator excretion after the $LTE_4$ challenge, which has not been studied before as well as the effects of the intervention with a $CysLT_1$ antagonist. An overview of the pattern of mediator excretion after inhalation of $LTE_4$ after treatment with placebo and montelukast is presented in Figure 24. All data are presented as nanograms per nanomole of creatinine. The peak values varied in between

subjects and between different mediators, although in the majority of cases the peak values were measured at the first hour interval or directly at the end of the LTE$_4$ challenge.
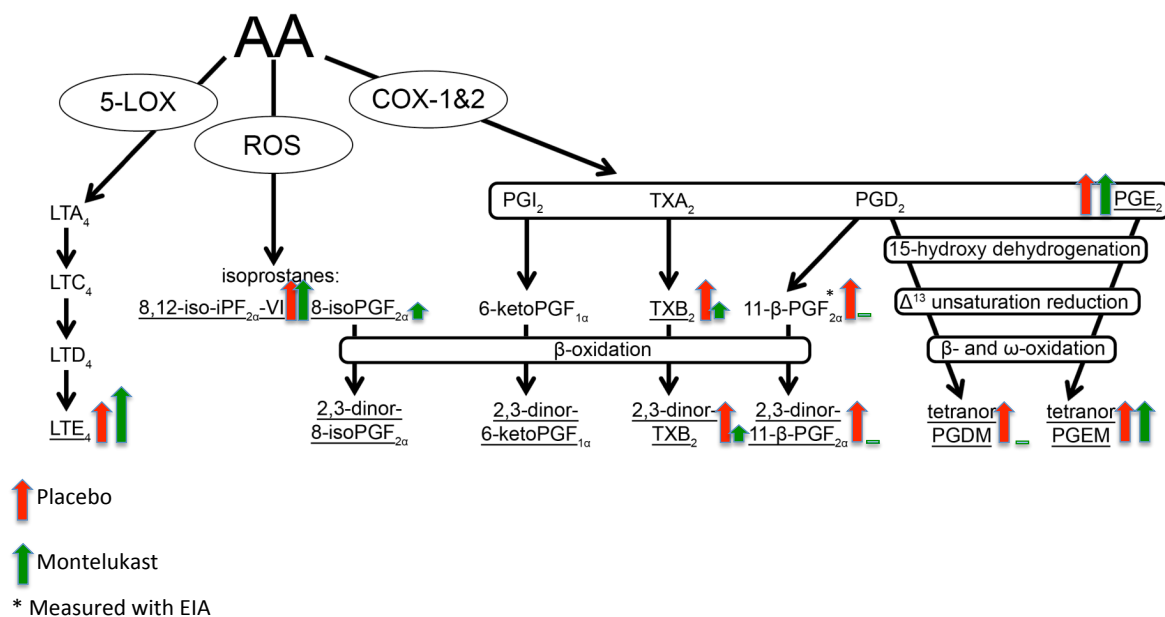


**Figure 24: Schematic overview of the lipid mediator excretion measured with UPLC-MS/MS after inhalation of LTE$_4$ at the end of each treatment period.**

## 5.4.1 LTE$_4$

As expected, the urinary excretion of LTE$_4$ increased significantly after inhalation of LTE$_4$ compared to baseline on both treatment periods (placebo: $15 \pm 10$ baseline vs $981 \pm 240$ after LTE$_4$ inhalation, p=0.0015; montelukast: $11 \pm 5$ baseline vs $3201 \pm 645$ after LTE$_4$ inhalation, p=0.0002). The peak excretion in between treatments was significantly higher for montelukast, p=0.0004 (Figure 25).
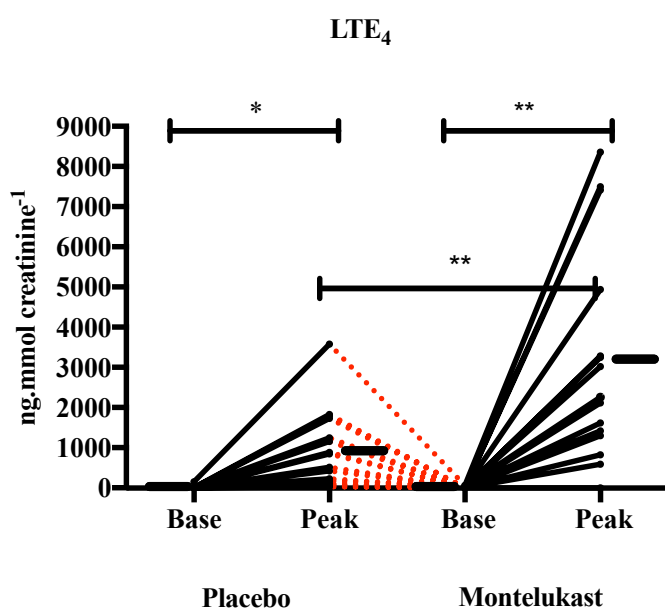


**Figure 25: Urinary excretion of LTE$_4$ before and after challenge with inhaled LTE$_4$ during the two treatment periods. Horizontal bars indicate mean values. \* P< 0.01 \*\* P< 0.001**

Comparing the released amount in urine during and following the challenge with the inhaled $LTE_4$ we found that there was a consistent 2% recovery of inhaled $LTE_4$ (2.3% for placebo and 1.9% for montelukast) across the range of inhaled concentrations (Figure 26).
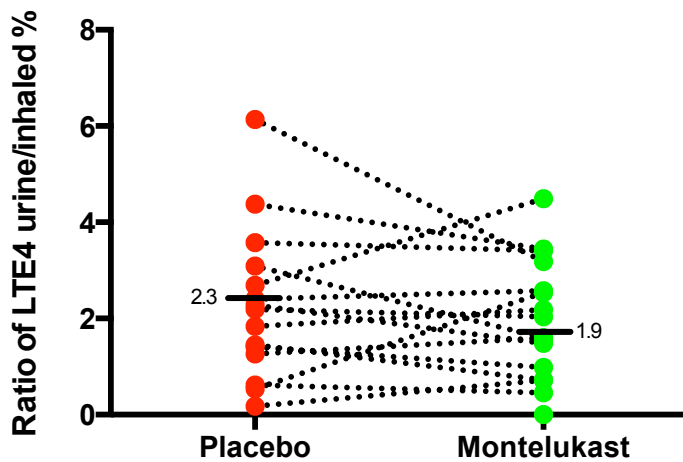


**Figure 26: Ratio of $LTE_4$ excreted in the urine to inhaled $LTE_4$ (%) for the two treatment periods.**

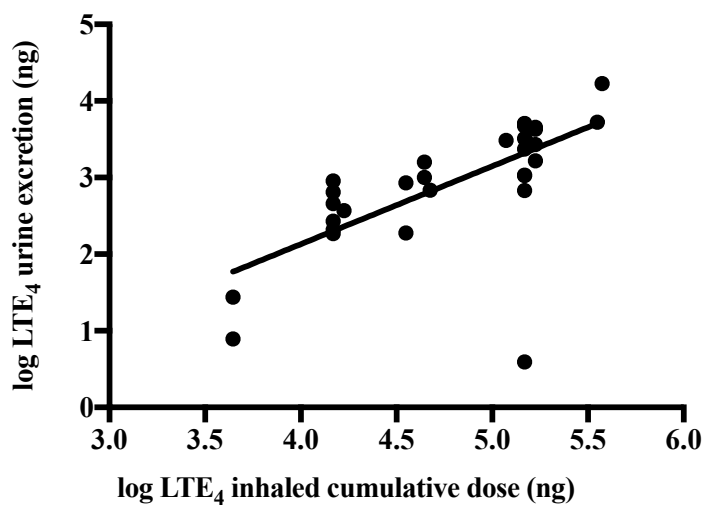We found a significant correlation between the inhaled dose and the amount of $LTE_4$ excreted into the urine (Figure 27).



**Figure 27: Relation between inhaled and excreted $LTE_4$ in the urine for both treatment periods r=0.68, P<0.0001 (Pearson)**

### 5.4.2  Prostaglandin $D_2$

Excretion of the most abundant metabolite, tetranor-PGDM, increased significantly from baseline after inhalation of $LTE_4$ during treatment with placebo (baseline $272 \pm 77$ vs $3743 \pm 1335$ peak, p=0.0189). This increase was abolished after treatment with montelukast (baseline $418 \pm 135$ vs $509 \pm 101$ peak, not significant) (Figure 28).
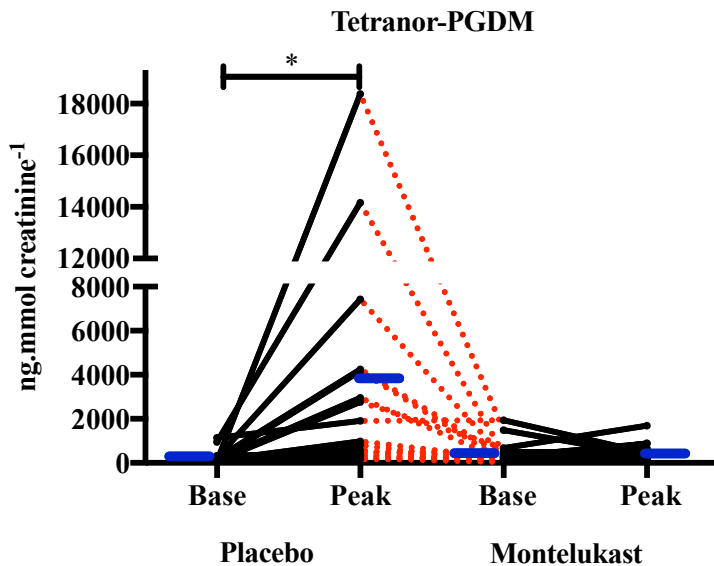
54

**Figure 28: Urinary excretion of the most abundant PGD$_2$ metabolite as measured with UPLC-MS/MS * P<0.05. Horizontal bars indicate mean values.**

Excretion of the early PGD$_2$ metabolite, 2,3-dinor-11β-PGF$_{2\alpha}$ demonstrated a similar pattern although the numeric increase during placebo did not reach statistical significance, p=0.072. This increase was again blocked completely by montelukast, (Figure 29).
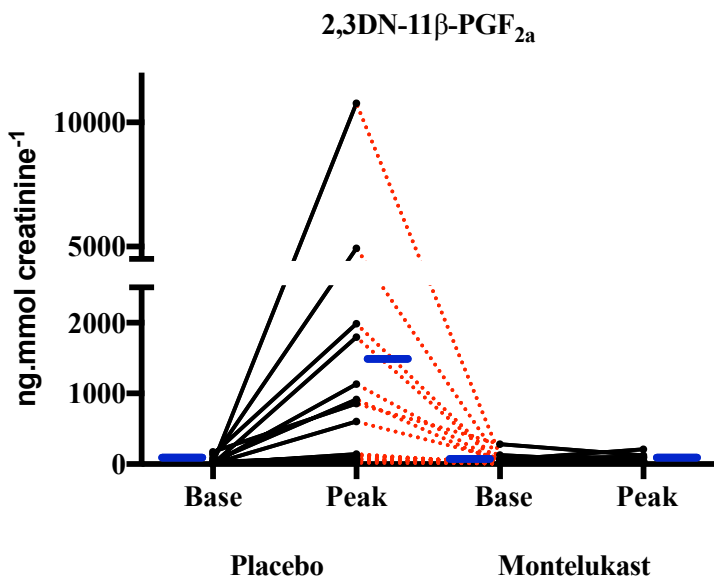


**Figure 29: Urinary excretion of the early PGD$_2$ metabolite as measured with UPLC-MS/MS. Horizontal bars indicates mean values.**

Next results for the urinary excretion of PGD$_2$ metabolites measured with UPLC-MS/MS were replicated by using a commercially available EIA kit for the very early PGD$_2$ metabolite 11β-PGF$_{2\alpha}$ (Figure 30). The excretion of 11β-PGF$_{2\alpha}$ increased significantly after inhalation of LTE$_4$ from baseline on placebo (baseline 58 ± 6 vs 383 ± 120 peak, p=0.0163) and this increase was again blocked by montelukast (baseline 69 ± 12 vs 80 ± 10 peak, not significant). Using UPLC-MS/MS we did not find detectable amounts of 11β-PGF$_{2\alpha}$,which is in concordance with

previous findings indicating that the 10% cross reactivity of the antibody against 11β-PGF$_{2\alpha}$ with 2,3-dinor-11β-PGF$_{2\alpha}$ explains the use of that particular EIA on urine (O'Sullivan, Mueller et al. 1999, Bood, Sundblad et al. 2015).
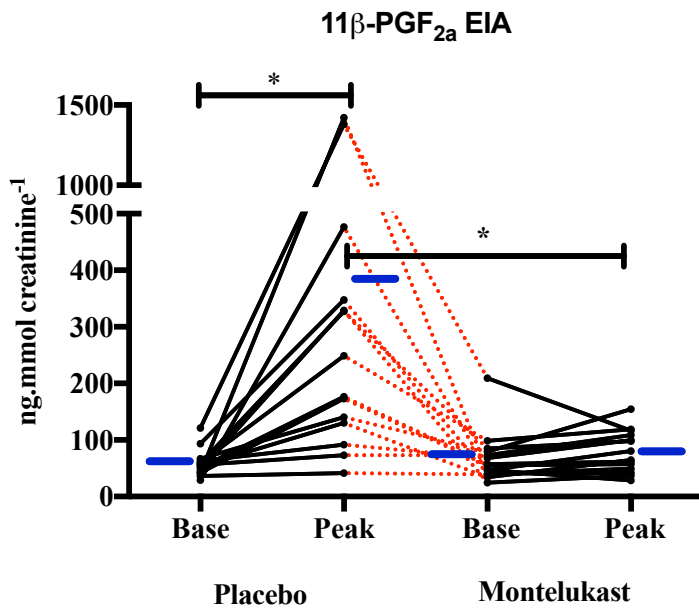


**Figure 30: Urinary excretion of the very early PGD$_2$ metabolite as measured using an EIA. Horizontal bars indicate mean values * P<0.05.**

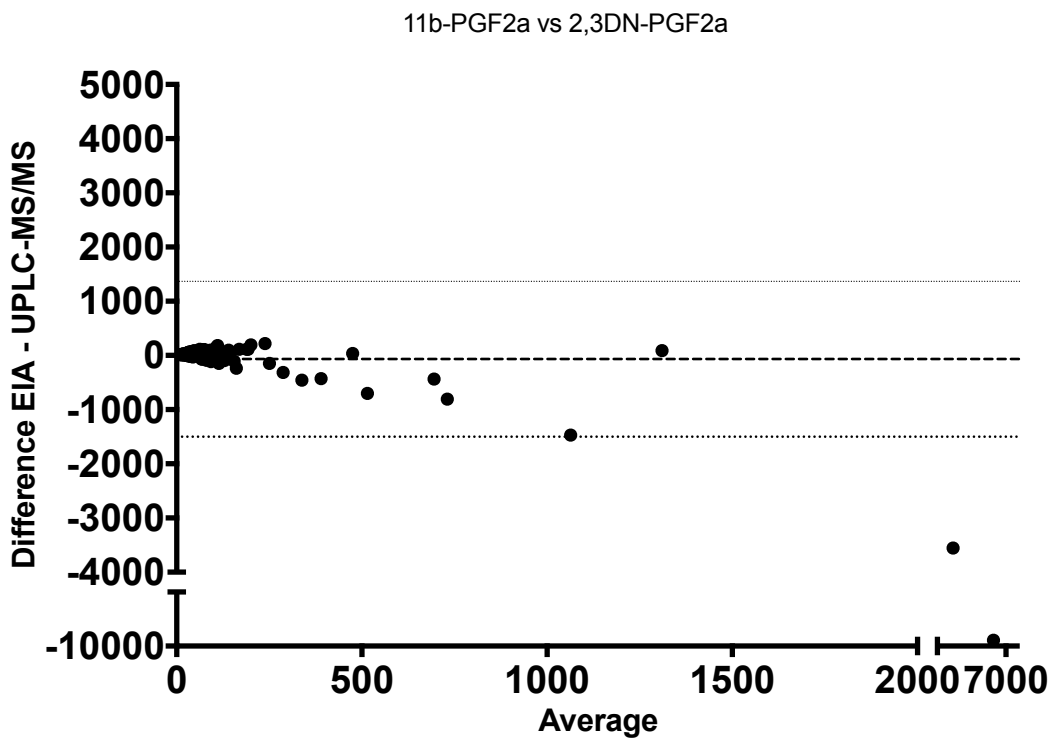There was a good agreement between the two measurements by using Bland-Altman analysis (Figure 31).



**Figure 31: Bland-Altman plot to compare measurements for the early PGD$_2$ metabolite 11β-PGF$_{2\alpha}$ between EIA and UPLC-MS/MS**

56

### 5.4.3 Thromboxane $B_2$ and its metabolites

The most abundant metabolite 2,3DN-$TXB_2$ increased significantly from baseline after inhalation of $LTE_4$ on both treatment periods (placebo; $201 \pm 74$ baseline vs $1853 \pm 460$ peak, p=0.0019. montelukast; $159 \pm 41$ baseline vs $561 \pm 116$ peak, p=0.0021). The peak excretion was however much greater with placebo compared to montelukast, p=0.0092 (Figure 32).
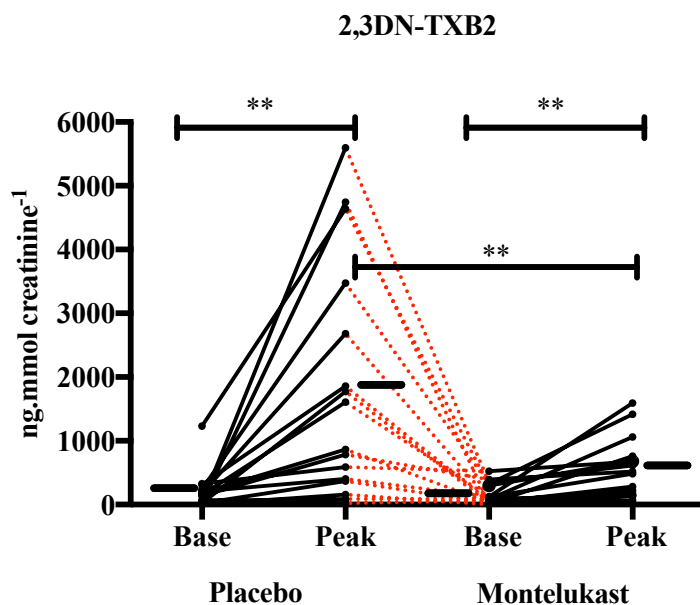


**Figure 32: Urinary excretion of the most abundant thromboxane $B_2$ metabolite measured with UPLC-MS/MS during the two treatments. Horizontal bars indicate mean values. **P<0.01**

Results for the primary $TXB_2$ showed a similar excretion pattern as levels increased following inhalation of $LTE_4$ on both treatments (placebo; $20 \pm 6$ baseline vs $136 \pm 35$ peak, p=0.0038, montelukast; $31 \pm 13$ vs $95 \pm 32$, p=0.0486 (Figure 33A). Excretion of the metabolite 11DH-$TXB_2$ increased after $LTE_4$ inhalation only during treatment with placebo $81 \pm 22$ baseline vs $220 \pm 54$ peak, p=0024 (Figure 33B).
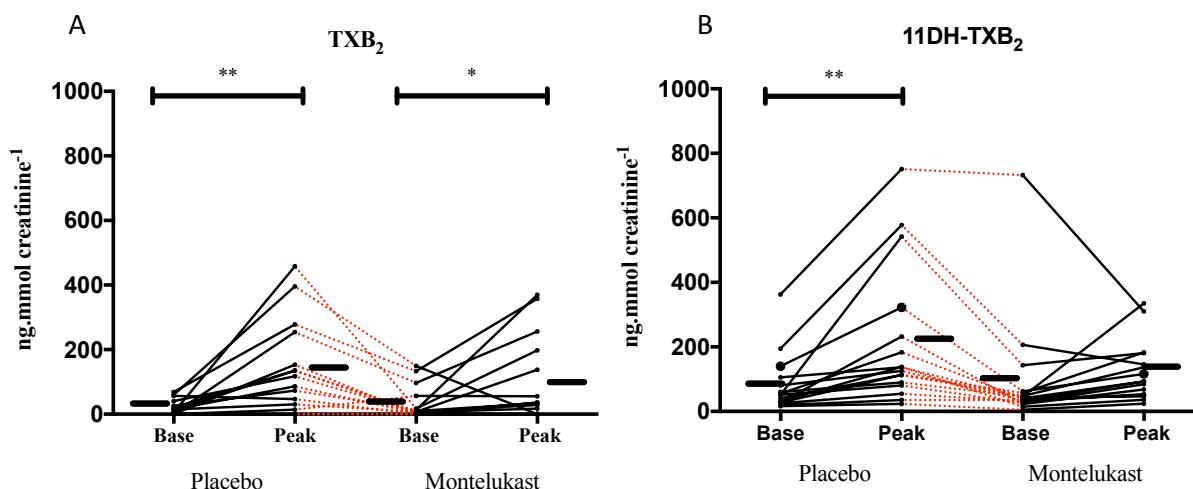


**Figure 33: Urinary excretion of (A) $TXB_2$ and (B) 11DH-$TXB_2$ for each treatment period. Horizontal bars indicate mean values. ** P<0.01 *P<0.05**

### 5.4.4 PGF$_{2\alpha}$

There was a significant increase in PGF$_{2\alpha}$ after inhalation of LTE$_4$ during placebo treatment 209 $\pm$ 74 baseline vs 711 $\pm$ 158 peak, p=0.029. During montelukast treatment there was a numerical increase that did not reach statistical significance 251 $\pm$ 74 baseline vs 411 $\pm$ 67 peak, p=0.06. Peak excretion was significantly higher with placebo compared to montelukast p=0.035, (Figure 34).
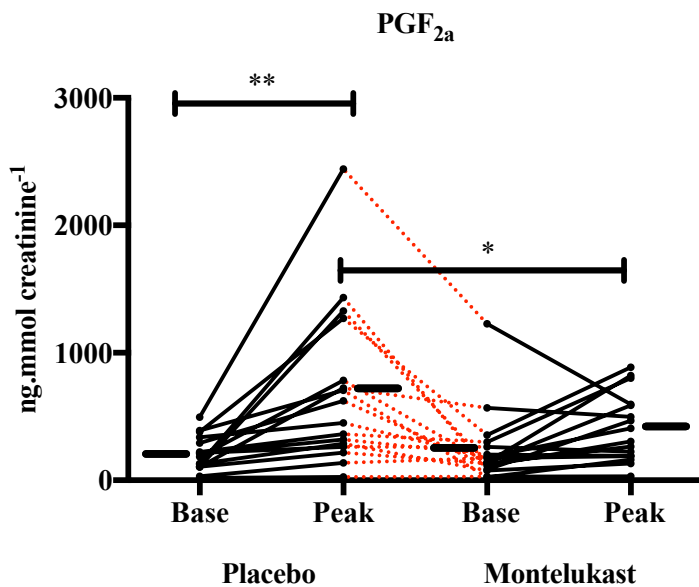


**Figure 34: Urinary excretion of PGF$_{2\alpha}$ during the two treatment periods. Horizontal bars indicate mean values. ** P<0.01 *P<0.05.**

### 5.4.5 Prostacyclin metabolite 2,3-dinor-6-keto-PGF$_{1\alpha}$

This metabolite did not increase significantly following LTE$_4$ on either treatment period, there was however a trend towards an increase after placebo treatment, p=0.055 (Figure 35).
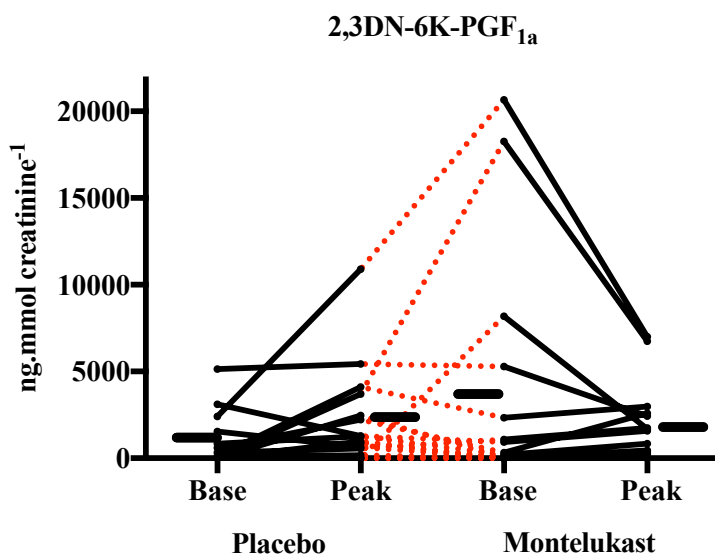


**Figure 35: Urinary excretion of 2,3-dinor-6-keto-PGF$_{1\alpha}$ during the two treatment periods. Horizontal bars indicate mean values.**

58

### 5.4.6 PGE$_2$ and its metabolites

Primary PGE$_2$ increased to the same extent following inhalation of LTE$_4$ on both treatment periods (placebo; 57 ± 21 baseline vs 143 ± 31 peak, p=0.0219 montelukast; 69 ± 39 baseline vs 147 ± 34 peak, p=0.0188) (Figure 36).
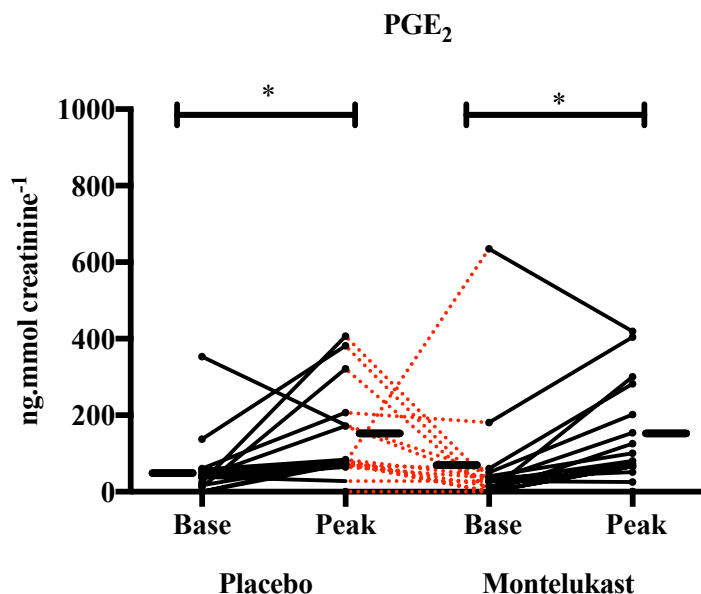


**Figure 36: Urinary excretion of PGE$_2$ during the two treatment periods. Horizontal bars indicate mean values. \*P<0.05.**

A similar excretion pattern was observed for the later and more abundant major metabolite of PGE$_2$, tetranor-PGEM (placebo; 363 ± 111 baseline vs 1555 ± 365 peak, p=0.0056 montelukast; 388 ± 90 baseline vs 1823 ± 407 peak, p=0.0037) (Figure 37).
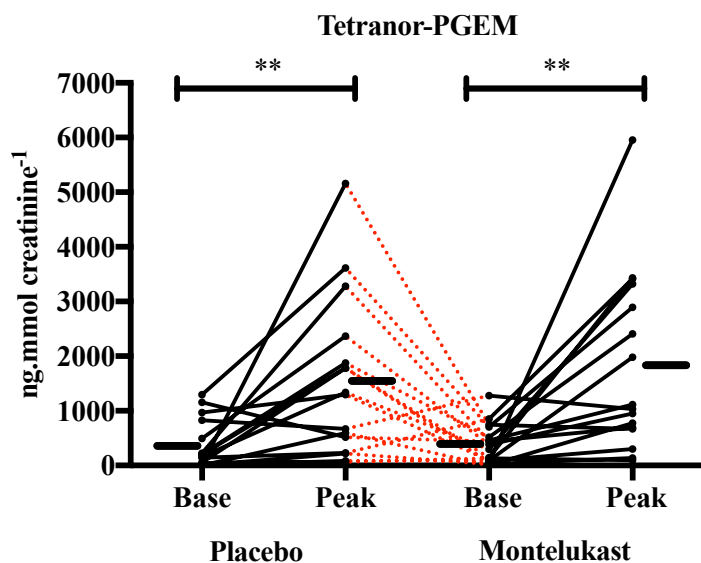


**Figure 37: Urinary excretion of the later PGE$_2$ metabolite during the two treatment periods. Horizontal bars indicate mean values. \*\* P<0.01.**

### 5.4.7 Isoprostanes

The most abundant metabolite, 8,12-iPF$_{2\alpha}$-VI increased following inhalation of LTE$_4$ on both treatment periods (placebo; $858 \pm 166$ baseline vs $2139 \pm 347$, p=0.0035 montelukast; $1081 \pm 220$ baseline vs $1756 \pm 273$, p=0.0274) (Figure 38).
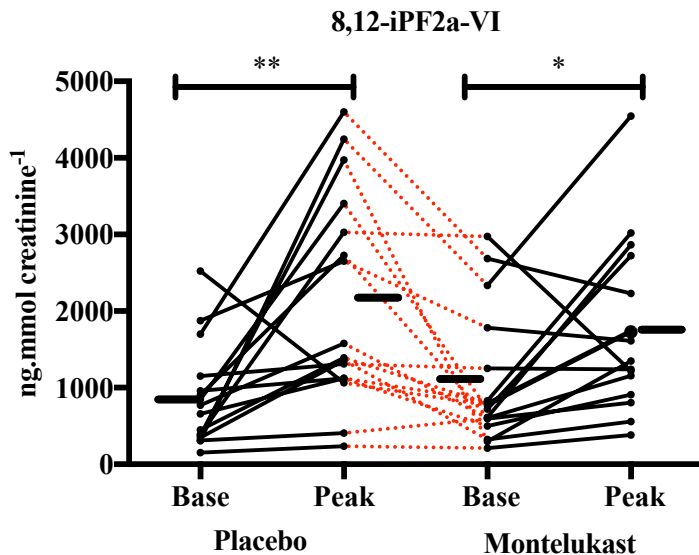
**8,12-iPF2a-VI**



**Figure 38: Urinary excretion of the most abundant isoprostane metabolite during the two treatment periods. Horizontal bars indicate mean values. ** P<0.01 *P<0.05.**

8-iso-PGF$_{2\alpha}$ increased after LTE$_4$ challenge only during montelukast treatment $66 \pm 20$ baseline vs $143 \pm 33$, p=0.0339 (Figure 39A). Excretion of 2,3-dinor-8-iso-PGF$_{2\alpha}$ did not increase significantly after LTE$_4$ inhalation on either treatment periods (Figure 39B).
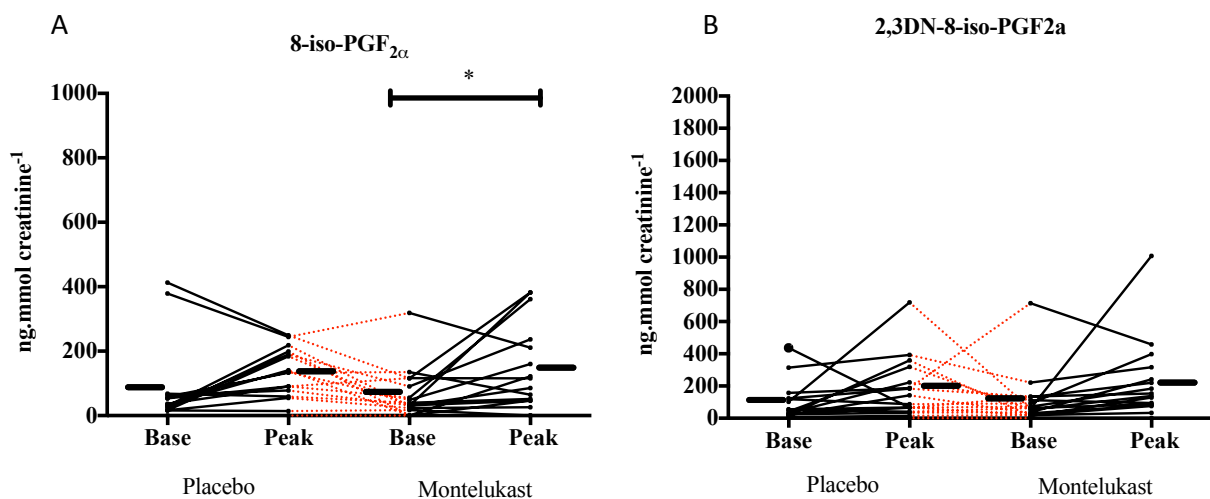


**Figure 39: Urinary excretion of two major isoprostane metabolites during the two treatment periods. Horizontal bars indicate mean values. *P<0.05.**

60

### 5.4.8 Validation of lipid mediator excretion in biobanked samples from previous study with inhalation of LTD$_4$

In order to validate the results, we went on and performed analysis of urine collected from a previous LTD$_4$ bronchoprovocation study performed in our lab (Gyllfors, Kumlin et al. 2005) using the same UPLC-MS/MS platform. We found significant increases in the following metabolites after LTD$_4$ inhalation: the PGD$_2$ metabolite 2,3-DN-11β-PGF$_{2\alpha}$; the TXB$_2$ metabolite 2,3-DN-TXB$_2$; the isoprostanes 8iso-PGF$_{2\alpha}$, 2,3-DN-8iso-PGF$_{2\alpha}$ and 8,12-iPF$_{2\alpha}$; and LTE$_4$. The results for 2,3-DN-11β-PGF$_{2\alpha}$ and LTE$_4$ are presented in Figure 40, while data for the rest are seen in Figure 41.
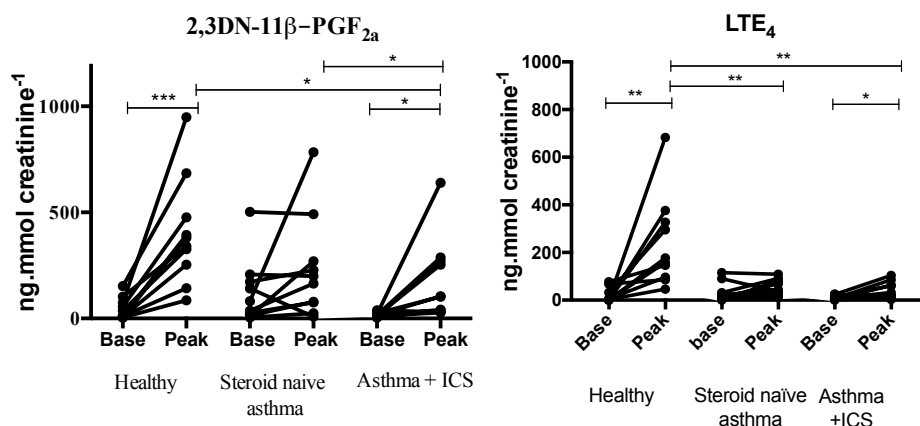


**Figure 40: Urinary excretion of the PGD$_2$ metabolite 2,3-DN-PGF$_{2\alpha}$ and LTE$_4$ after inhalation of LTD$_4$. *P<0.05, **P<0.01 and ***P<0.001. ICS: inhaled corticosteroids.**
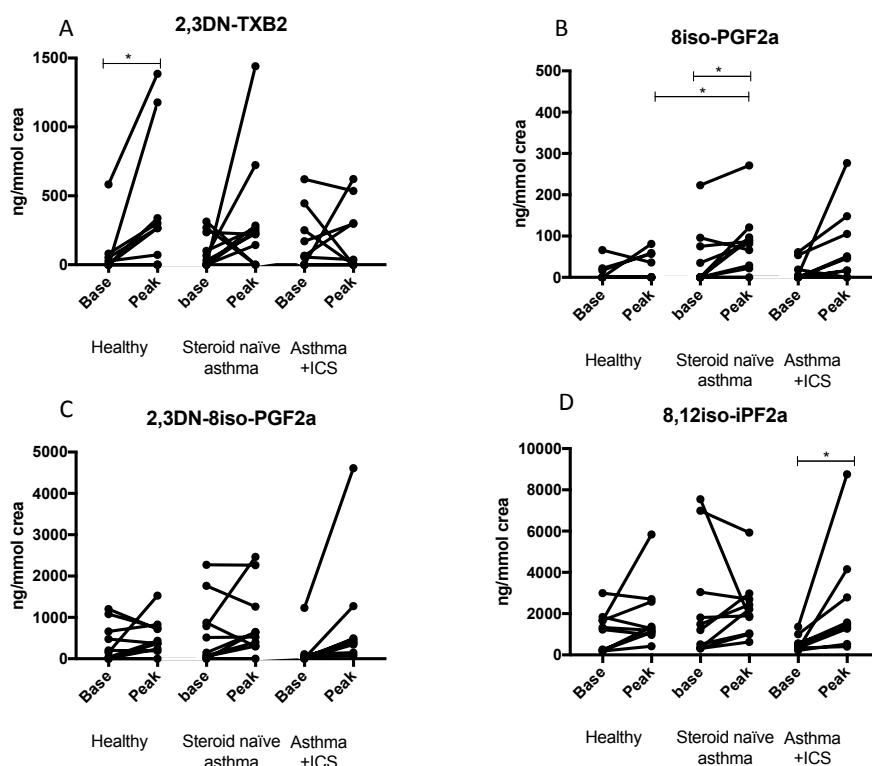


**Figure 41: Urinary excretion of 2,3-DN-TXB$_2$ (A) as well as isoprostanes (B)(C)(D) after inhalation of LTD$_4$. *P<0.05. ICS: inhaled corticosteroids.**

No significant increases after $LTD_4$ were observed for the following detected mediators: primary $TXB_2$, 11DH-$TXB_2$, $PGF_{2\alpha}$ and the prostacyclin 2,3-DN-6K-$PGF_{1\alpha}$ (Figure 42). Primary $PGE_2$, tetranor-PGEM and tetranor-PGDM could not be detected, presumably due to degradation during storage, which is in line with previous studies (Idborg, Pawelzik et al. 2014).
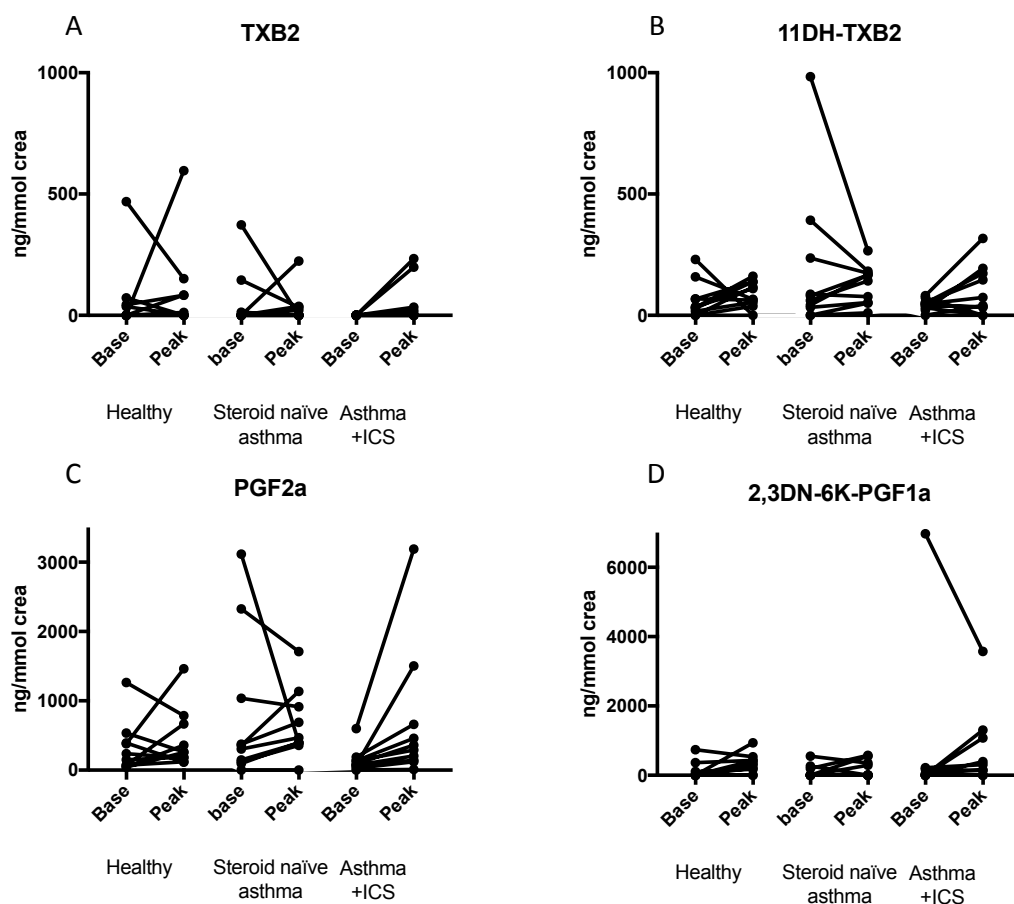


**Figure 42: Urinary excretion of primary $TXB_2$ (A), 11DH-$TXB_2$ (B), $PGF_{2\alpha}$ (C) and prostacyclin (D) after inhalation of $LTD_4$. ICS: inhaled corticosteroids.**

### 5.4.9  Summary of lipid mediator urinary excretion results

The study for the first time in human subjects *in vivo* demonstrated that inhalation of $LTE_4$ caused an increase in the urinary excretion of metabolites that are indicative of the release of $PGD_2$, $PGF_{2\alpha}$, $PGE_2$ as well as several isoprostanes, which are known markers of oxidative stress (Lawson, Rokach et al. 1999). The release of $PGD_2$ is considered to be a sign of mast cell activation because the mast cell is known to be the major source of $PGD_2$ in human subjects (Lewis, Soter et al. 1982, O'Sullivan, Dahlen et al. 1996, Bood, Sundblad et al. 2015). The increased excretion of $PGD_2$ was blocked by montelukast, thus confirming that the mast cell activation is $CysLT_1$ dependent. Studies in experimental models have shown that CysLTs can activate mast cells and cause secondary prostanoid release (Paruchuri, Tashimo et al. 2009, Liu, Garofalo et al. 2015). Next the effect of $LTE_4$ on $PGD_2$ release was replicated by measurements using the same UPLC-MS/MS platform on samples that had been biobanked from a previous $LTD_4$ bronchoprovocation study (Gyllfors, Kumlin et al. 2005). That study in addition to asthmatics, included healthy subjects. They tolerated a higher dose of $LTD_4$ and showed a more

pronounced $PGD_2$ mediator excretion in urine, indicating dose-dependent mast cell activation. The release of $TXA_2$ and to some extent $PGF_{2\alpha}$ showed a similar pattern to $PGD_2$ and was inhibited by montelukast. The mast cell could be the major source for these mediators too, although $CysLT_1$ mediated release from other sources is also possible. It still remains poorly characterized which lipid mediators are formed in human mast cells *in vivo*. In contrast, the release of $PGE_2$ and its major metabolite, tetranor-PGEM showed a different pattern from $PGD_2$, where the increased urinary excretion following $LTE_4$ inhalation was not abolished by montelukast. It could be that another receptor is involved in the $PGE_2$ release, possibly from the airway epithelium, which is known to be the major source of $PGE_2$ (Churchill, Chilton et al. 1989, Harrington, Lucas et al. 2008). However, because the peak of urinary excretion of $PGE_2$ was similar between the two treatment periods despite the fact that subjects inhaled a 10-fold greater dose of $LTE_4$ in the presence of montelukast, it is likely that the effect of $LTE_4$ on $PGE_2$ release at least partly is mediated through the $CysLT_1$ receptor.

## 5.5 EFFECT OF INTERVENTIONS ON SKIN PRICK TEST REACTIVITY

In **Paper II** a skin prick test titration over a range of dilutions was performed with the allergen selected for inhalation prior to the inhalation challenge. This was done in order to evaluate the allergen-induced skin test response and how it was affected by the interventions. The area under the curve (AUC) in the allergen dilution-response curves for skin wheal diameters was calculated by using the linear trapezoidal rule. QGE031 caused a dose- and time-dependent inhibition of the allergen-induced skin test response (Figure 43). The effect was greatest for the group that received the highest dose of QGE031 (240 mg). In contrast to the airway response to allergen, the suppression of the skin response in the 240 mg group was maximal at week 18 i.e. eight weeks after the last dose of QGE031 and was observed in all subjects (Figure 43).
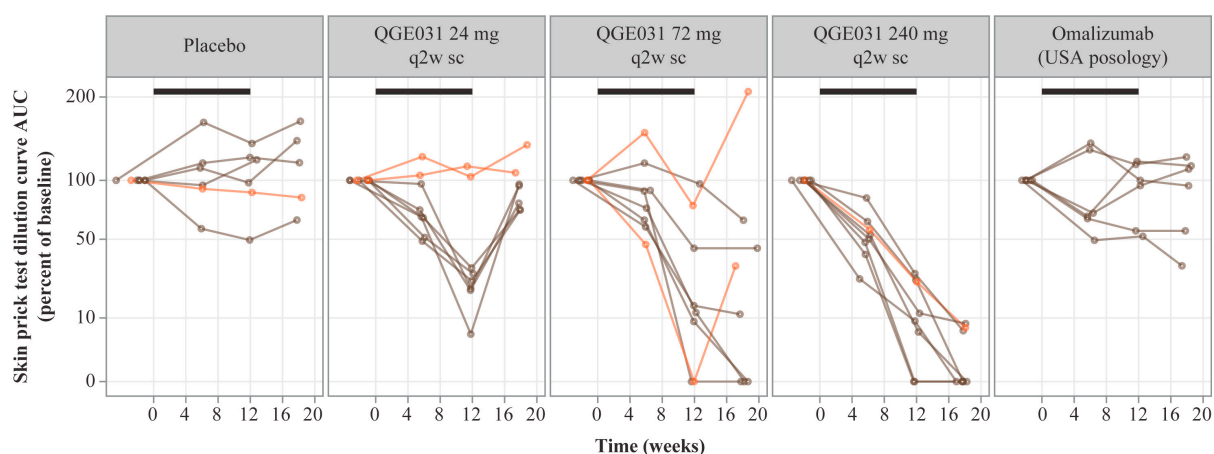


**Figure 43: Effect of the intervention on the AUC of the wheal response to allergen skin prick titration. Graphs represent individual subject time-course data from each treatment group. Data are represented as the percentage change in AUC from baseline. Red-coloured data represent subjects with IgE levels greater than 700 IU/ml.**

At week 12, there was a significant difference in the allergen-skin prick test response between all three QGE031 treatment groups and placebo (all $p<0.01$), whereas there was no significant

inhibition in the group that received omalizumab, p=0.47 (Figure 44B). All three doses of QGE031 demonstrated a greater inhibition of the skin prick test response compared to omalizumab, which was more pronounced for the group that was treated with 72 and 240 mg of QGE031 (p=0.002 and p<0.0001 respectively) (Figure 44A). Omalizumab elicited 22% suppression of the allergen-skin prick test response compared to 74% and 85% for the 72- and 240 mg QGE031 groups respectively at week 12.
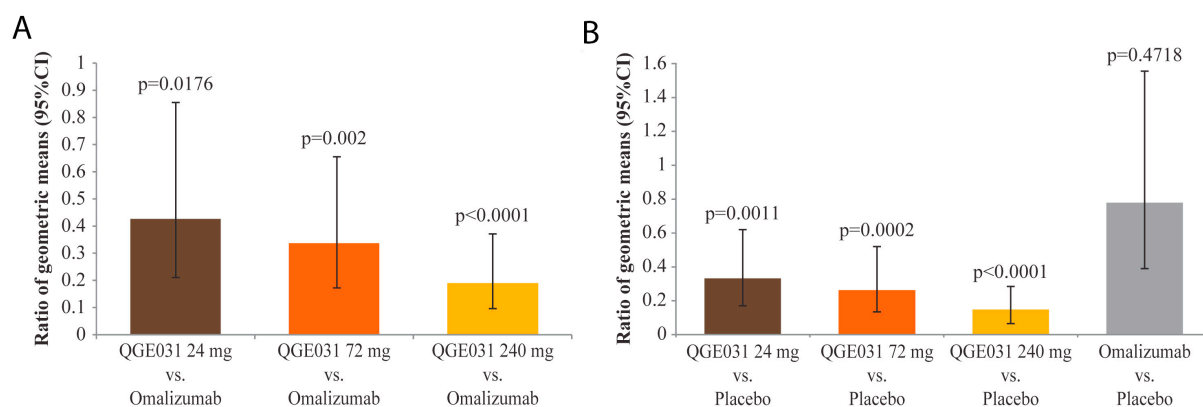


**Figure 44: Statistical analyses of the geometric mean ratios of AUC of wheal size across all dilutions at week 12 for the three QGE031 doses versus omalizumab (A) and placebo (B). Error bars represent 95% CIs. Lower ratios indicate lower sensitivity to allergen and therefore better suppression by the drug.**

In summary, it was demonstrated that QGE031 inhibited the allergen induced skin prick test responses. Similar to the airway response the inhibition was more pronounced in the two groups that received the two higher doses of QGE031 compared to the group that was treated with omalizumab. It is known that the allergen induced skin wheal reaction can be blocked by antihistamines (Gronneberg and Dahlen 1990), which indicates that QGE031 can effectively prevent release of histamine in the skin. For the 24- and 72-mg QGE031 groups the mean peak efficacy in the skin response was observed at 12 weeks; at 18 weeks it had returned to baseline for the 24 mg group while in the 72 mg group some subjects had no worsening or continued to improve with the exception of the two subjects with the highest IgE levels, see Figure 43. For the 240-mg QGE031 group maximal inhibition was observed at 18 weeks for all subjects unlike inhibition of the airway response, which was maximal at 12 weeks in this group. Thus our findings highlight the fundamental differences in the allergen response of airway mucosal surfaces compared to the skin.

## 5.6  EFFECT OF INTERVENTIONS ON MARKERS MEASURED IN BLOOD

In **Paper II** blood samples were collected during the study for measurements of serum total QGE031, serum total and free IgE, FcεRI on basophils and dendritic cells, CD23 on B cells and bound IgE on basophils. In **Paper III** blood samples were collected before challenge with $LTE_4$ and five minutes after the last dose was administered for measurement of circulating white blood cell counts.

64

### 5.6.1 Pharmacokinetics of QGE031

The average steady-state serum concentrations at week ten, before the last dose of QGE031 were 1.8 μg/ml (coefficient of variation CV 34%) for the 24-mg-QGE031 group, 5.6 μg/ml (CV, 33%) for the 72-mg-QGE031 group and 13.2 μg/ml (CV, 25%) for the 240-mg-QGE031 group. The results were similar at week twelve i.e. one dosing interval after the last dose: 2.3 μg/ml (CV, 38%), 6.7 μg/ml (CV, 36%) and 15.5 μg/ml (CV, 28%) respectively. Exposure increased with dose, with steady state accomplished at the end of the dosing period.

### 5.6.2 Total serum IgE levels

There was an increase in total IgE levels (sum of free and drug captured IgE levels) from baseline across all treatment groups except for placebo. The extent and duration of IgE binding was dependent on QGE031 dose (Figure 45A), which was also seen for omalizumab. There are several factors that influence levels of total circulating IgE such as cellular synthesis, binding affinity to FcεRI, occupancy of IgE receptors and elimination of free IgE and drug-IgE complexes. Measurements of free IgE were also performed although all QGE031 doses suppressed IgE levels to below the assay lower limit of quantification in most subjects. Because the results from the measurements of the downstream biomarkers basophil surface IgE and FcεRI showed a clear response, no further analysis of free IgE was performed.

### 5.6.3 Surface IgE and FcεRI expression

There were observed numeric dose-dependent reductions in basophil surface IgE levels in all treatment groups except for placebo. The responses were more pronounced in the groups receiving the two higher doses of QGE031 and less pronounced in the omalizumab and the 24 mg QGE031 group (Figure 45B). At week 12 the suppression of surface IgE from baseline reached 66%, 99%, 99% and 95% for 24, 72, 240 mg of QGE031 and omalizumab respectively. When compared to placebo surface IgE levels were reduced by 71%, 99%, 99% and 95% for 24, 72, 240 mg of QGE031 and omalizumab respectively and by 86% and 87% with 72 and 240 mg of QGE031 when compared to omalizumab.

At week 12 the reduction of basophil expression of FcεRI from baseline reached 27%, 82%, 85% and 77% for 24, 72, 240 mg of QGE031 and omalizumab respectively. When compared to placebo the reduction was 22%, 81%, 84% and 75% with 24, 72, 240 mg of QGE031 and omalizumab respectively and by 21% and 34% with 72 and 240 mg of QGE031 when compared to omalizumab. There was no reduction achieved for the two subjects with the highest levels of IgE in the group of 24 mg QGE031. Suppression of FcεRI after stopping treatment was longest in the group receiving the highest dose of QGE031 240 mg (Figure 45C).

In total there was a clear reduction in the basophil FcεRI and surface IgE levels after treatment with QGE031, which has also been demonstrated in previous phase 1 studies (Arm, Bottoli et al. 2014). This effect of QGE031 on expression of basophil FcεRI, surface IgE and even total IgE levels was rapid and in some cases a maximal change was observed in the first blood sample

taken two weeks after treatment start. This was different from the response both in the airways and the skin that lagged behind.
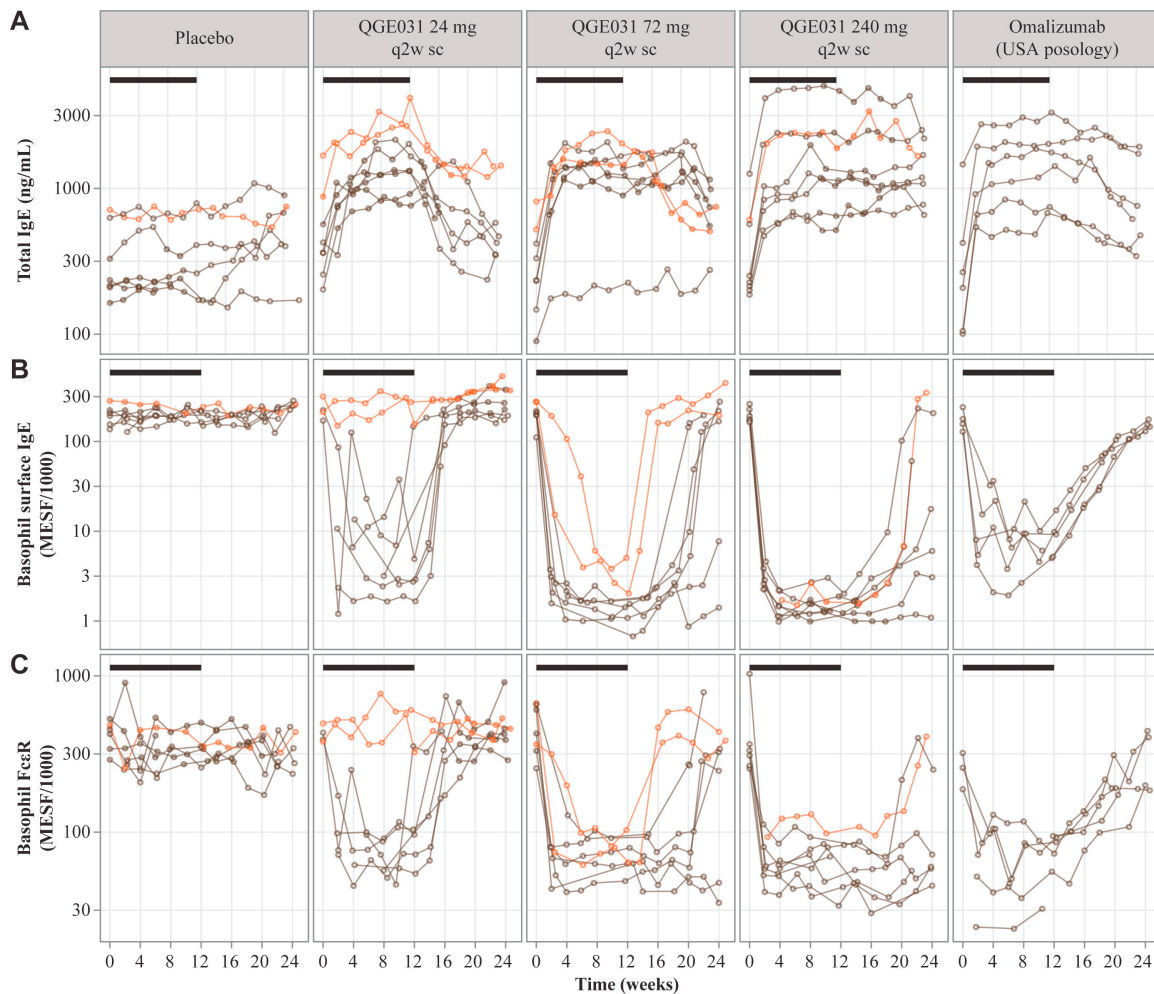


**Figure 45: Effect of QGE031 and omalizumab in IgE and IgE receptor expression. Individual subject time course of circulating total IgE (A), basophil surface IgE levels (B) and basophil expression of FcεRI (C) for placebo, the three dose levels of QGE031 and omalizumab. Red-coloured data represent subjects with baseline IgE levels of greater than 700 IU/mL. Units for the basophil FACS assay were molecules of equivalent soluble fluorophore (MESF) divided by 1000.**

### 5.6.4  White blood cell counts

Inhalation of $LTE_4$ elicited a significant increase in the white blood cell count during placebo treatment (baseline $6.4 \pm 1.5$ x 10(9)/L vs. $7.01 \pm 2.06$ x 10(9)/L after, p=0.0257), which was not significant after treatment with montelukast (baseline $6.7 \pm 1.6$ vs. $7.2 \pm 1.9$ after, p=0.13) (Figure 46A). Neutrophils increased slightly after the challenge on both treatment periods although this increase was not significant (placebo; baseline $3.5 \pm 1.2$ vs. $3.6 \pm 1.6$ after, p=0.52 and montelukast; baseline $3.6 \pm 1.2$ vs. $3.8 \pm 1.3$ after, p=0.49), (Figure 46B). There was predominant increase in lymphocytes after inhalation of $LTE_4$ on both periods (placebo; baseline $2.0 \pm 0.5$ vs. $2.5 \pm 0.5$ after, p<0.001 and montelukast; baseline $2.1 \pm 0.6$ vs. $2.5 \pm 0.6$ after, p=0.0235) (Figure 46D), while eosinophils slightly decreased (placebo; baseline $0.27 \pm 0.15$ vs. $0.22 \pm 0.14$ after, p=0.0013 and montelukast; baseline $0.23 \pm 0.17$ vs. $0.19 \pm 0.17$ after,

p=0.0552) (Figure 46C). There was also a non-significant decrease in monocytes after LTE$_4$ challenge (placebo; baseline $0.55 \pm 0.16$ vs. $0.54 \pm 0.2$ after, p=0.9 and montelukast; baseline $0.58 \pm 0.15$ vs. $0.54 \pm 0.12$, p=0.11) (Figure 46E), while basophils were undetectable on both treatment periods.
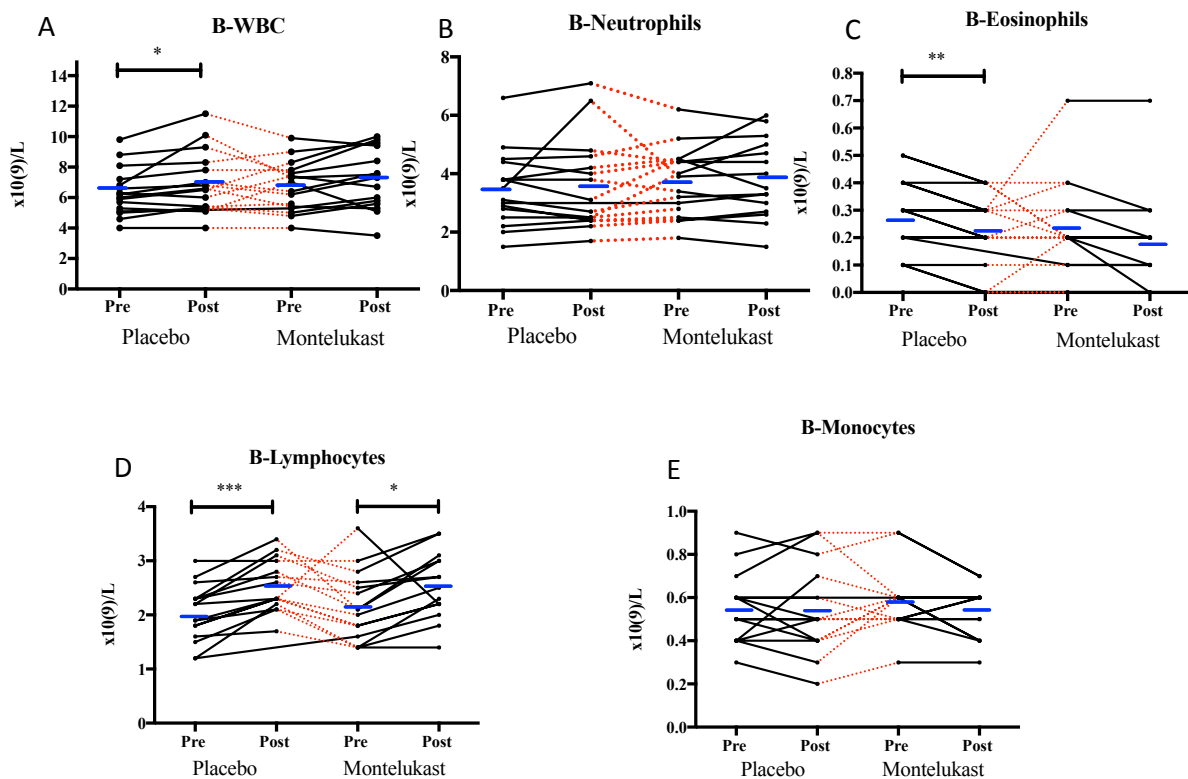


**Figure 46: Effect of inhaled LTE$_4$ on circulating total white blood cells, neutrophils, eosinophils, lymphocytes and monocytes during the two treatment periods. Horizontal bars indicate mean values.**

In summary, we have shown for the first time that LTE$_4$ inhalation caused a rapid increase in numbers of circulating white blood cells in human subjects. This was predominantly due to an increase in the numbers of circulating lymphocytes and a decrease in eosinophil counts; both of these effects were unaffected by montelukast, which might suggest involvement of other receptors. Future studies are needed in order to characterize this new finding in greater detail.

## 5.7 EFFECT OF INTERVENTIONS ON MEASURES OF ASTHMA CONTROL

In **Paper I,** participants recorded asthma symptoms as well as their physical activity in an electronic diary on a daily basis, and completed the ACQ5 questionnaire on study days. Results from ACQ5 and reported asthma symptoms are presented in Table 11. Already at baseline i.e. prior to treatment, all subjects had a relatively low ACQ5 score supporting that they, as intended, had mild asthma. After 6 weeks of treatment ACQ5 scores decreased to less than 1.0 in all groups, which indicates that their asthma was under control. Subjects recorded symptoms on approximately half of the days exercise was performed with low symptom scores in all three groups, while there were almost no symptoms on exercise free days (Table 11). Neither symptom nor ACQ5 scores differed between the three treatment groups at any visit.

| | | Regular placebo once daily + budesonide/formoterol as needed<br><br>N=23 | Regular placebo once daily + terbutaline as needed<br><br>N=22 | Regular budesonide once daily + terbutaline as needed<br><br>N=21 |
|---|---|---|---|---|
| ACQ5 | Baseline | 1.2 ± 0.7 | 1.3 ± 0.7 | 1.0 ± 0.6 |
| | After 6 weeks | 0.75 ± 0.58 | 0.93 ± 0.74 | 0.66 ± 0.61 |
| Symptoms associated with exercise | Asthma symptoms (%)* | 49.8 ± 26.1 | 50.9 ± 36.9 | 51.1 ± 30.5 |
| | Cough (0-4) | 0.31 ± 0.24 | 0.26 ± 0.31 | 0.22 ± 0.25 |
| | Wheeze (0-4) | 0.29 ± 0.35 | 0.31 ± 0.56 | 0.20 ± 0.26 |
| | Shortness of breath (0-4) | 0.42 ± 0.48 | 0.63 ± 0.77 | 0.37 ± 0.47 |
| | Mucus (0-4) | 0.33 ± 0.28 | 0.52 ± 0.66 | 0.59 ± 0.78 |
| | Breathlessness (0-4) | 0.69 ± 0.62 | 0.95 ± 1.03 | 0.82 ± 0.75 |
| Symptoms not associated with exercise | Asthma symptoms (%)* | 0.69 ± 0.62 | 0.95 ± 1.03 | 0.82 ± 0.75 |
| | Cough (0-4) | 0.13 ± 0.30 | 0.22 ± 0.39 | 0.08 ± 0.16 |
| | Wheeze (0-4) | 0.06 ± 0.14 | 0.07 ± 0.22 | 0.02 ± 0.06 |
| | Shortness of breath (0-4) | 0.06 ± 0.15 | 0.12 ± 0.24 | 0.03 ± 0.05 |
| | Mucus (0-4) | 0.08 ± 0.23 | 0.14 ± 0.27 | 0.11 ± 0.29 |
| | Breathlessness (0-4) | 0.07 ± 0.15 | 0.14 ± 0.18 | 0.06 ± 0.16 |

**Table 11: ACQ5 and symptom scores at baseline and after six weeks of treatment during days with and without exercise. *Asthma symptoms associated with exercise are calculated on days when exercise was performed. *Asthma symptoms not associated with exercise indicate days with any symptom not related to exercise during the whole study. Symptoms are measured on a scale from 0 to 4, where 0 indicates no symptoms and 4 maximal symptoms. Data are presented as mean ± SD.**

In summary, results from subject-reported measures indicate that all subjects including the ones receiving only terbutaline on demand perceived that they had a good asthma control and an effective treatment of EIB with low symptom scores on exercise days similar to the two other groups that were treated with ICS.

## 5.8 PHARMACOKINETICS MODEL AND SIMULATION

A mathematical pharmacokinetics/pharmacodynamics model descriptive of drug pharmacokinetics for QGE031 and omalizumab was built based on the data from the current study; model curves fitted the observed subject data well (Figure S1 in the supplementary material to **Paper II**). The model included both biomarker and clinical responses, with agreement between simulated and observed data, normal distributions of random variation between subjects and accurate predictions in quantile-quantile plots, see figure S2 in the papers supplementary material. Together with the precise pharmacokinetics, biomarker and allergen challenge pharmacodynamic parameter estimates (Tables E3 and E4 in the supplementary material of **Paper II**), were such that the model was considered valid for providing predictions

through simulation. Simulations were therefore performed for approximately 1000 virtual subjects and predicted the biomarker and allergen test response time courses from pre-treatment to six months for doses from zero to 600 mg of QGE031 administered every four weeks and omalizumab dosed as per the US dosing table (Xolair 2010). Simulation results showed that maximal suppression of the skin allergic response would occur after 12 weeks of treatment, while the airway response to allergen was predicted a bit earlier, after about 8 weeks of treatment (Figure S3 in the supplementary material of **Paper II**). The biomarker and allergen responses from the 168th day were taken to plot QGE031 dose responses. These indicated that for subjects with baseline IgE levels of less than the median value of 250 IU/mL, 36 mg of QGE031 every four weeks should provide equivalent inhibition of allergen-induced skin and airway response to omalizumab (Figure 47). Maximal QGE031- induced responses that exceeded those of omalizumab would occur with higher doses; subjects with lower baseline IgE values would reach maximal effects with 120 mg of QGE031 every four weeks, while those with higher values would require higher doses, such as 240 mg every four weeks.
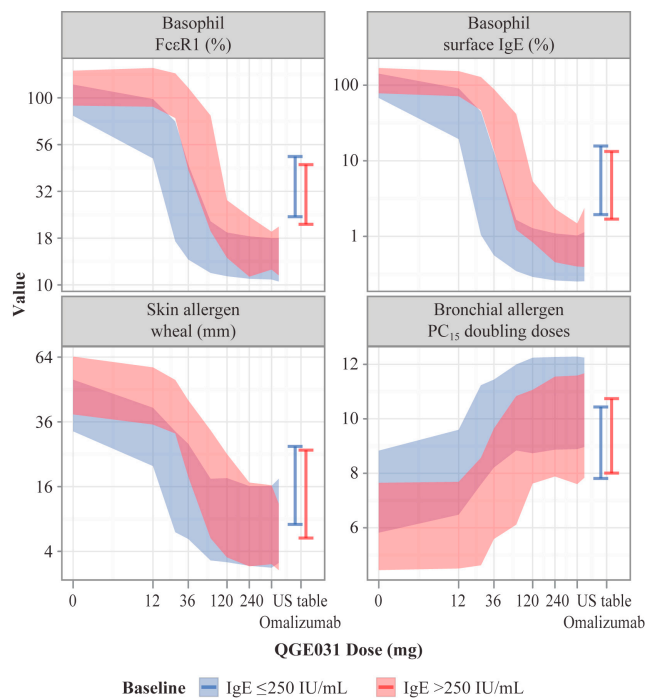


**Figure 47: Simulation of steady state (week 24) dose responses to QGE031 for a population split by the median baseline IgE level. Simulated doses on the x-axis are those for QGE031 administered every 4 weeks. Omalizumab error bars refer to simulated measurements administered according to the US dosing table.**

## 5.9  SAFETY

All interventions were well tolerated and there were no severe adverse events or withdrawals due to adverse events in the active treatment groups/periods. For subjects receiving QGE031 nasopharyngitis and asthma worsening were the most common reported adverse events, which were not dose dependent. Most suspected drug-related adverse events were related to injection-site reactions that were of mild nature. No cases of urticarial or anaphylaxis were reported.

# 6 CONCLUSIONS

The main conclusions of this thesis are:

- Treatment with a combination of bud/form on-demand was non inferior to regular treatment with budesonide in subjects with mild asthma and EIB.
- Treatment with three to four doses per week with bud/form combination on-demand was sufficient to provide good asthma control, indicating that this could be an alternative to regular budesonide treatment in mild asthmatics.
- Both treatment with a combination of bud/form on-demand and regular budesonide were superior to terbutaline monotherapy on-demand on inhibiting EIB.
- Monotherapy with terbutaline slightly increased the bronchial response to exercise propably due to tachyphylaxis regarding the $\beta_2$ protective effect, indicating that terbutaline monotherapy should be avoided in subjects with mild asthma.
- The mannitol bronchial challenge was not as sensitive as the exercice challenge in diagnosing EIB in subjects with mild asthma.
- Treatment with QGE031 caused a shift in the allergen $PC_{15}$ that was three times greater for the highest dose of QGE031 (240 mg) compared to omalizumab and sixteen times greater compared to placebo.
- There was a remarkable variability on the shift of allergen $PC_{15}$ among subjects treated with QGE031 (2-fold to 500-fold change).
- The maximal response regarding allergen $PC_{15}$ was seen at 12 weeks (2 weeks after the last dose) in subjects treated with QGE031, with effects declining by week 18.
- Allergen skin prick test responses were inhibited by treatment with QGE031 with greater inhibition for the highest dose, whereas omalizumab made no difference from treatment with placebo.
- There was no variability regarding the skin response among subjects treated with QGE031, and those receiving the highest dose (240 mg) continued to improve at week 18, six weeks after the last dose.
- The effects of QGE031 on total serum IgE levels, surface IgE and expression of FcεRI were dose dependent with greater effects for subjects receiving the two higher doses.
- Subjects with high baseline IgE levels had poor responses to lower doses of QGE031 whereas dependancy on baseline IgE was not seen for those treated with the highest dose of 240 mg.
- QGE031 was well tolerated in all treatment groups.
- Montelukast completely inhibited the bronchoconstriction induced by inhaled $LTE_4$ indicating that the effect of $LTE_4$ on smooth muscle in asthmatics is mediated solely through the $CysLT_1$ receptor.
- Inhalation of $LTE_4$ elicitated significant small airway obstruction that was also inhibited by montelukast.
- Sputum eosinophils post $LTE_4$ inhalation remained the same when subjects were treated with montelukast compared to placebo, despite inhaling a ten-fold higher cumulative

dose of $LTE_4$. This argues against the presence of another receptor for the proinflammatory effects of $LTE_4$ on airway cells.

- Inhalation of $LTE_4$ led to increased urinary excretion of $PGD_2$ metabolites, which is a sign of mast cell activation; this activation was also mediated through $CysLT_1$.
- The findings indicate that CysLTs may need to be reclassified as dual bronchonconstrictors, with direct effects on smooth muscle and indirect effects by mast cell activation and release of $PGD_2$ which is a known bronchoconstrictor.
- $TXA_2$ and $PGF_{2\alpha}$ release was aslo increased after $LTE_4$ inhalation and was likewise sensitive to inhibition by montelukast.
- $PGE_2$ release was however not blocked by montelukast, possibly implying the presence of another receptor probably on the airway epithelium.
- $PGE_2$ release was however not increased during treatment with montelukast despite subjects inhaling a much higher dose of $LTE_4$ during this period, which suggests that the $CysLT_1$ nonetheless is partially involved.

# 7 GENERAL DISCUSSION

## 7.1 HOW SHOULD WE TREAT MILD INTERMITTENT ASTHMA AND EIB

Mild asthma is estimated to affect about 50% to 75% of all asthmatic subjects and can lead to severe exacerbations that represent approximately 30% to 40% of asthma exacerbations that require emergency consultation (Dusser, Montani et al. 2007). One component of mild intermittent asthma is EIB. Subjects may have symptoms that are related to exercise despite otherwise well controlled asthma or exercise is the only asthma trigger.

### 7.1.1 Monotherapy with β₂ agonists on demand

According to guidelines (Bateman, Hurd et al. 2008, Parsons, Hallstrand et al. 2013, British Thoracic and Scottish Intercollegiate Guidelines 2014), the first treatment of choice to alleviate EIB is administration of a SABA 5 to 20 minutes before exercise, which is effective for 2-4 hours (Tan and Spector 2002, Carlsen, Anderson et al. 2008). This is somewhat controversial because it is known that airway inflammation is present already at this stage of the disease (Vignola, Chanez et al. 1998, Hallstrand, Moody et al. 2005a, Hallstrand, Moody et al. 2005b). The initial recommended treatment is therefore focusing on symptom relief rather than the underlying mechanism. The results of my exercise bronchoprovocation study in **Paper I** clearly demonstrate that after six weeks of intermittent treatment with terbutaline there was no long-term bronchoprotective effect against exercise, in fact EIB slightly worsened compared to baseline. The focus of my study was the effect of treatment on the bronchoprotective effect against EIB and not the direct bronchodilator effect. The latter was maintained as indicated by the rapid recovery of $FEV_1$ back to baseline after terbutaline inhalation (30 minutes after the end of the challenge) in all treatment groups. This included the group receiving terbutaline monotherapy (Figure 9 in section 5.2.1). The low symptom scores on exercise days and the decrease in ACQ5 value after six weeks of treatment compared to baseline for all groups including the terbutaline group also indirectly indicate this.

The loss of bronchoprotection (tachyphylaxis) against exercise observed in this study has also been reported previously after regular use of both SABAs and LABAs with cross-tolerance to other β₂ agonists. The degree of tachyphylaxis appears to be slightly greater regarding protection against indirect stimuli, such as exercise and allergen challenge compared to stimuli that act directly on the smooth muscles, such as methacholine challenge (Lipworth 1997). In previous bronchoprovocation studies using methacholine, regular treatment with salmeterol has led to tachyphylaxis within the first hours after treatment start in both steroid naïve subjects (Cheung, Timmers et al. 1992, Bhagat, Kalra et al. 1995, Drotar, Davis et al. 1998) as well as in subjects using ICS (Booth, Bish et al. 1996, Kalra, Swystun et al. 1996, Lipworth and Aziz 1999). Interestingly, it has been also reported that regular use of β₂ agonists may lead to loss of the bronchodilator effect of rescue SABA used after bronchoconstriction with either methacholine (Hancox, Aldridge et al. 1999, Wraight, Hancox et al. 2003, Haney and Hancox 2005, Haney and Hancox 2007) or exercise (Hancox, Subbarao et al. 2002, Storms, Chervinsky et al. 2004).

This increases the risk that asthma patients with an on-going worsening have less bronchodilator responsiveness when they need it the most.

Similar to methacholine, tachyphylaxis with regard to the protective effect of $\beta_2$ agonists has been reported following indirect challenges with allergen (Cockcroft, McParland et al. 1993, Giannini, Carletti et al. 1996, Dahlen, Lantz et al. 2009), AMP (O'Connor, Aikman et al. 1992, Aziz, Tan et al. 1998) and of course exercise (Ramage, Lipworth et al. 1994, Inman and O'Byrne 1996, Simons, Gerstner et al. 1997, Nelson, Strauss et al. 1998, Villaran, O'Neill et al. 1999, Edelman, Turpin et al. 2000).

Taken together, it has been shown that the onset of tachyphylaxis is rapid, can occur within twelve hours after the first dose, can increase with continuous $\beta_2$ agonist use before reaching a plateau with recovery within 72 hours after the last dose, and can affect also subjects receiving ICS (Weiler, Brannan et al. 2016). Moreover, some of these studies have reported that daily use of $\beta_2$ agonists can actually increase both EIB (Inman and O'Byrne 1996, Hancox, Subbarao et al. 2002) and the bronchial responsiveness to methacholine (Vathenen, Knox et al. 1988) and cause partial bronchodilator subsensitivity (Newnham, Dhillon et al. 1993, Grove and Lipworth 1995, Newnham, Grove et al. 1995, Hancox, Cowan et al. 2000).

The underlying mechanism or mechanisms of tachyphylaxis are still not clear but the most possible explanation is that chronic exposure of the $\beta_2$ receptors to $\beta_2$ agonists can cause uncoupling and internalization of the receptors in the cells where they are degraded (Johnson 2006). This $\beta_2$ receptor loss elicits a downregulation of the receptors and the responsiveness to $\beta_2$ agonists, which can be reversed by resynthesis of the receptors that has been shown to occur clinically within 72 hours after the last $\beta_2$ agonist dose (Haney and Hancox 2005, Haney and Hancox 2006). However, this downregulation of the $\beta_2$ receptors causes only a partial reduction in the bronchodilator response to $\beta_2$ agonists due to the large reserve of these receptors in the airway smooth muscle cells. This is not the case for other inflammatory cells such as mast cells and lymphocytes, where $\beta_2$ agonists can cause a rapid downregulation *in vitro*, which reflects the low density of $\beta_2$ receptors in these cells (Chong, Morice et al. 1995, McGraw and Liggett 1997, Barnes 1999, Scola, Chong et al. 2004). If it is taken into consideration that $\beta_2$ agonists also may block the release of bronchoconstrictive mediators from mast cells such as CysLTs and histamine (Chong and Peachell 1999), it appears consistent that $\beta_2$ receptor downregulation leads to loss of the bronchoprotective effect of $\beta_2$ agonists against EIB, where mast cells have a key role. Moreover, downregulation of the $\beta_2$ receptor may result in augmentation of pathways mediated through the CysLTs, histamine and thromboxane receptors that can potentially enhance EIB (Anderson 2006, McGraw, Elwing et al. 2007). It is evident that monotherapy with $\beta_2$ agonists leads to tachyphylaxis and loss of the drugs bronchoprotective effect and thus eventually a greater risk for asthma related complications, which is why it is avoided in more severe asthma (Spitzer, Suissa et al. 1992, Nelson, Weiss et al. 2006, Salpeter, Buckley et al. 2006). The results of my bronchoprovocation study clearly demonstrate that $\beta_2$ agonist monotherapy should be avoided also in subjects with mild asthma and EIB, and alternative treatment given.

### 7.1.2 Regular treatment with ICS

According to previous versions of the GINA guidelines published at the time our study was conceived, subjects with EIB should inhale a SABA prior to exercise (step 1) and if reliever medication was needed more than two times a week, regular treatment with ICS should be considered (step 2). One of the main issues is that these subjects often inhale a SABA before exercise as a routine, meaning that the exact need for treatment is not known. The guidelines have however, been updated through the years. In the latest versions of the GINA guidelines, due to the emerging evidence regarding the insufficient safety of SABA monotherapy and risk of exacerbations already in this stage of mild asthma, low dose ICS is in fact already considered an alternative at step 1 (GINA report 2018). Continuous treatment with ICS is considered the best controller option for EIB due to its direct anti-inflammatory effect and can reduce both the frequency and severity of EIB but not necessarily completely abolish it (Koh, Tee et al. 2007, Weiler, Brannan et al. 2016).

This change of view is very much in agreement with the results in **Paper I** where regular treatment with low dose ICS for six weeks was the best treatment option for EIB but did not completely eliminate it. After three weeks of treatment there was a small, although not significant reduction in EIB. This finding also confirms previous studies showing that regular ICS treatment can provide some bronchoprotection against EIB after one week, although a greater protection is observed after three to four weeks of treatment depending upon the dosage (Hofstra, Neijens et al. 2000, Petersen, Agertoft et al. 2004, Subbarao, Duong et al. 2006, Stelmach, Grzelewski et al. 2008). It seems that the protective effect begins to plateau after one week (Duong, Subbarao et al. 2008) with further improvement upon the next weeks with continuous treatment until the final plateau is reached, which may relate to the degree of underlying inflammation (Koh, Tee et al. 2007). In fact, there is substantial evidence indicating that even a single high dose of ICS can have some bronchoprotective effect against EIB as early as four hours after the dose in children (Thio, Slingerland et al. 2001, Driessen, Nieland et al. 2011) and against EVH induced bronchoconstriction in adults (Kippelen, Larsson et al. 2010). In the latter study, it was also demonstrated that ICS treatment reduced the urinary excretion of bronchoconstrictive eicosanoid mast cell mediators, indicating that this effect is due to mast cell inhibition in the airways. Similarly, a single dose of ICS significantly inhibited the bronchoconstrictive effect of inhaled AMP, which is also mediated through mast cell degranulation, as early as two hours after the first dose (Ketchell, Jensen et al. 2002). A significant reduction in sputum eosinophils and protection against hypertonic saline induced bronchoconstriction has also been reported as early as six hours after the dose (Gibson, Saltos et al. 2001). In line with previous data, the chosen interval of six weeks of treatment in **Paper I** was sufficient to demonstrate efficacy against EIB.

The effects of regular ICS treatment on EIB are dose dependent. Because the study in **Paper I** included a mixed group of children over 12 years of age and adults, a low daily dose of budesonide 400 µg was chosen. Low doses of ICS provide significant protection against EIB in children (Freezer, Croasdell et al. 1995, Pedersen and Hansen 1995, Jonasson, Carlsen et al. 2000, Visser, Wind et al. 2015) and a high dose of budesonide (1600 µg daily) protected against

EIB in adults with more severe asthma and with a greater pretreatment $FEV_1$ fall post exercise compared to our group of mild asthmatics (Vathenen, Knox et al. 1991). In the study by Subbarao et al, treatment with low dose of ciclesonide protected against EIB in adults, although a greater protection was observed with higher doses (Subbarao, Duong et al. 2006). Thus, it is possible that a higher dose of budesonide in the group that received regular treatment in **Paper I** would have had even greater protection against EIB.

However, even if regular treatment with ICS has great efficacy in protecting against EIB, this option has its own pitfalls. The main one is that regular ICS treatment will not completely abolish EIB, meaning that the need for the use of a reliever medication such as a SABA before exercise remains. Considering that these subjects have otherwise well controlled asthma as indicated by the low ACQ5 values in **Paper I**, there is a risk of poor adherence to treatment. Studies in this segment of patients show that they take less than 50% and usually about 30% of the prescribed ICS, and that most therefore nevertheless rely on as needed SABA (Jonasson, Carlsen et al. 2000, Williams, Pladevall et al. 2004). In addition, with this approach, while initially with the use of SABA as needed patients learned to have autonomy and their own perception of medication need and disease control, this now has to be unlearned with the regular use of ICS irrespectively of symptoms. This can be confusing for many asthmatics and lead to the paradox that in case of asthma worsening, most of them tend to rely on their reliever medication instead of using their ICS inhaler (O'Byrne, Jenkins et al. 2017). It is known that early intervention with ICS in mild asthma can prevent more severe exacerbations (Pauwels, Pedersen et al. 2003). In order to cope with these issues an alternative treatment option with a combination of bud/form was tested in **Paper I** and the results are going to be further discussed in the next section.

### 7.1.3 Treatment with a combination of ICS/formoterol on demand

In **Paper I** it was demonstrated for the first time that treatment with a combination of bud/form three to four times per week provided the same magnitude of bronchoprotection against exercise compared to regular treatment with low dose budesonide, implying that this could be an alternative treatment in patients with mild asthma; in addition combination treatment was clearly superior to monotherapy with terbutaline taken on demand. These results have implications for asthma guidelines. In the GINA 2018 report, combination treatment is recommended in step 3, while low dose ICS can be considered as an alternative option already at step 1 (GINA report 2018). However, the results of the study in **Paper I** argues that the combination of bud/form might be preferred to SABA monotherapy in step 1, and also should be considered as an alternative to regular ICS treatment at step 2.

The scientific rationale for using a combination therapy with ICS/LABA in asthma (and its benefits) has been promoted for several years (Barnes 2002). Formoterol should be the LABA of choice in an as-needed treatment option due to its rapid onset of action, which makes it an effective reliever medication in asthma, as well as its long bronchodilator effect with systemic side effects similar to a SABA (Tattersfield, Lofdahl et al. 2001, Pauwels, Sears et al. 2003, Kaae, Agertoft et al. 2004, Cheung, van Klink et al. 2006). In one study when used three times

per week, little or no tolerance developed (Davis, Reid et al. 2003). Formoterol has also an inhibitory effect on plasma leakage in the airways, which should not be considered negligible (Erjefalt and Persson 1991, Tokuyama, Lotvall et al. 1991, Baluk and McDonald 1994), as well as a mast cell stabilising effect, which is an important mechanism in reducing EIB (Ketchell, Jensen et al. 2002). There is also data suggesting synergetic effects of ICS and LABA when taken together; corticosteroids can increase $\beta_2$ receptor expression and reverse the $\beta_2$ receptor uncoupling in experimental animal models and LABAs can enhance the translocation of the glucocorticoid receptors (GR) with also an increase in glucocorticoid receptor element (GRE) binding of the GR (Barnes 2007).

The interactions between ICS and LABA as well as the favourable profile of formoterol for as-needed use have contributed to the development of the combination of bud/form in a single inhaler for both maintenance and reliever treatment in asthma i.e. SMART treatment regimen (Kew, Karner et al. 2013). SMART has shown better efficacy in preventing exacerbations and treating asthma symptoms compared to a higher dose of budesonide with SABA for relief (Scicchitano, Aalbers et al. 2004, Rabe, Pizzichini et al. 2006, Jenkins, Eriksson et al. 2017), as well as compared to a fixed combination of salmeterol/fluticasone with SABA for relief (Vogelmeier, D'Urzo et al. 2005, Bousquet, Boulet et al. 2007, Kuna, Peters et al. 2007). SMART is also more effective in reducing exacerbations compared to the same fixed combination of budesonide/formoterol with SABA or LABA for relief (O'Byrne, Bisgaard et al. 2005, Bisgaard, Le Roux et al. 2006, Rabe, Atienza et al. 2006, Edwards, von Maltzahn et al. 2010). In addition, SMART appears to be more cost-effective than treatment with a higher maintenance dose of ICS/LABA with a rapid-acting $\beta_2$ agonist for relief (Lundborg, Wille et al. 2006, Price, Wiren et al. 2007).

The efficacy of treatment with ICS on symptom-driven as needed basis alone, or when combined with $\beta_2$ agonists versus traditional regular ICS treatment, has been debated over the years. It has been proposed that SABA monotherapy should be replaced with a combination treatment taken on demand and that more clinical trials are needed to test this approach (Papi, Caramori et al. 2009). This is one of the main reasons the study presented in **Paper I** of this thesis was developed. The protocol including exercise challenges was chosen because it is also known that the severity of EIB is considered a reflection of asthma control, or lack of control. Persistent EIB despite treatment often indicates a need for reassessment of therapy (Hofstra, Neijens et al. 2000). One previous study using beclomethasone combined with SABA in a single inhaler taken on an as needed basis showed similar efficacy with regular beclomethasone treatment (Papi, Canonica et al. 2007), whereas in another study from the same group, the combination of bud/form taken as-needed was inferior to regular treatment with the same combination (Papi, Marku et al. 2015). Because the main question remained unanswered, two large phase 3 clinical trials of the efficacy and safety of bud/form as needed were studied in slightly less than 8000 subjects with mild asthma (SYGMA programme) (O'Byrne, FitzGerald et al. 2017). Both trials were double blind including adults and adolescents 12 years of age or older with mild asthma that were followed for one year.

76

In the SYGMA 1 trial (O'Byrne, FitzGerald et al. 2018) a total of 3849 patients were randomised to three treatment groups: terbutaline as needed, budesonide 400 μg daily plus terbutaline as needed, or a combination of bud/form as needed. The interventions studied in the SYGMA 1 trial were the same as used in **Paper I**. The primary endpoint in this trial was to compare the efficacy of as-needed bud/form to as-needed terbutaline with regard to electronically recorded weeks with well-controlled asthma. Treatment with bud/form as needed was superior to SABA monotherapy with terbutaline regarding the primary endpoint (mean percentage of weeks per patient 34.4% bud/formo vs 31.1% terbutaline, p=0.046). Bud/form as needed also resulted in a 64% lower rate of severe and 60% lower rate of moderate-to-severe exacerbations compared to terbutaline used as needed, whereas there was no difference compared to the regular budesonide group regarding exacerbations. It should be mentioned that 19.7% of the subjects that underwent randomisation in this trial had reported a serious exacerbation the previous year. Regarding the mean percentage of weeks with well-controlled asthma per patient bud/form as needed was inferior to regular treatment with budesonide (34.4% bud/form vs 44.4% regular budesonide). However, the median daily dose of inhaled budesonide in the bud/form as-needed group was 17% of the dose in the regular budesonide group.

In the SYGMA 2 trial (Bateman, Reddel et al. 2018), 4215 patients with mild asthma that were eligible for regular ICS treatment (step 2 according to GINA guidelines) were randomised to either bud/form as-needed or regular treatment with budesonide 400 μg daily plus terbutaline. This was a non-inferiority study with the primary endpoint comparing the two treatment regimens regarding the annualized rate of severe exacerbations. The as-needed treatment with bud/form was non-inferior to regular treatment with budesonide (0.11 bud/form vs. 0.12 regular budesonide). The time to the first exacerbation was also similar between the two treatments, while there was a slightly greater improvement in ACQ5 in the regular budesonide group, which nonetheless was not considered clinically significant.

Thus the results of both these two large studies in patients with mild asthma have confirmed the results from **Paper I** presented in this thesis i.e. that bud/form taken as-needed is superior to SABA monotherapy also taken as needed and non-inferior to treatment with low dose ICS. It seems now that there is enough evidence for a revision of the current asthma guidelines and a first simplified proposal of the new guidelines is presented in this thesis (Figure 48).
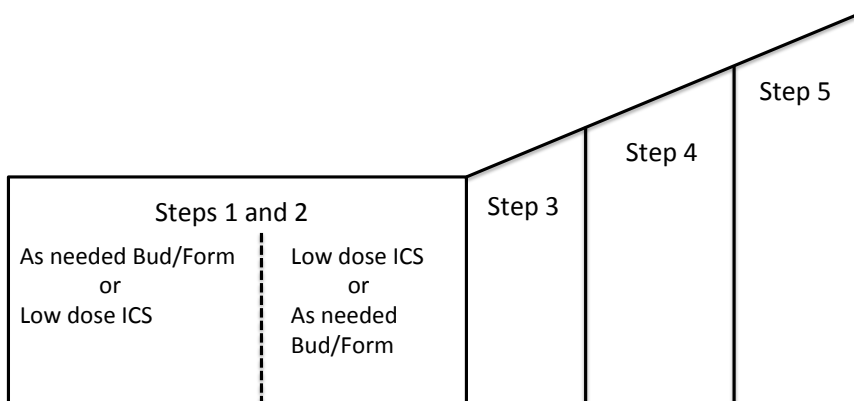


**Figure 48: Recommended revision of the GINA guidelines stepwise approach for treating asthma.**

According to the proposed revision of the guidelines in step 1, treatment with SABA monotherapy for symptom relief is replaced by the combination treatment of bud/form taken also for symptom relief, while at step 2 intermittent use of bud/form is considered an alternative controller medication to regular treatment with low dose ICS.

## 7.2   ANTI IGE THERAPY IN THE ALLERGEN PROVOCATION SETTING

Anti IgE therapy with omalizumab, the only approved humanized anti-IgE monoclonal antibody (mAb), has been used for more than 15 years for treatment of allergic asthma with efficacy shown in several randomised control trials (RCTs). A recent Cochrane review including 25 RCTs in subjects with moderate-to-severe allergic asthma concluded that omalizumab reduced asthma exacerbations by 25%, reduced hospitalizations due to asthma, and allowed tapering of the daily used ICS dose compared to placebo (Normansell, Walker et al. 2014). Omalizumab has shown similar efficacy in reducing asthma exacerbations also in real-life studies (Niven, Saralaya et al. 2016, Casale, Luskin et al. 2019), while in one study there was also a small improvement in lung function (Humbert, Beasley et al. 2005). Omalizumab dosing and administration frequency (every two or four weeks) depends on body weight and pre-treatment serum total IgE levels (approved range for adults 30 to 700 IU/mL in the United States and 30 to 1500 IU/ml in the European Union). There are nonetheless reports from previous studies supporting the benefit of omalizumab treatment even for subjects outside the recommended range of bodyweight/total IgE (Kwong and Jones 2006, Zielen, Lieb et al. 2013, Kornmann, Watz et al. 2014, Hew, Gillman et al. 2016).

In **Paper II,** treatment with different doses of a second generation of anti-IgE antibody (QGE031/ligelizumab) was compared to omalizumab regarding the inhibition of EAR after allergen challenge. The results showed that the highest dose of QGE031 caused a shift in the allergen $PC_{15}$ that was numerically three times greater than omalizumab, although this difference did not reach statistical significance. There was variability in the degree of inhibition of the EAR among subjects treated with QGE031, which was not observed in the skin prick test responses. All doses of QGE031 caused a significantly greater inhibition of the skin prick test responses to allergen compared to omalizumab, which was dose dependent with best efficacy for the highest dose. As expected, serum total IgE levels increased in all groups, including omalizumab. There was a reduction in basophil surface IgE levels in all subjects treated with the two highest doses of QGE031, whereas this reduction was less pronounced for the lowest QGE031 dose and for omalizumab. Basophil expression of FcεRI was also reduced in all groups, including omalizumab, although no suppression was observed for the two subjects that had the highest IgE levels and were treated with the lowest dose of QGE031. In general, it was observed that subjects with high IgE levels (> 700 IU/mL) had poorer responses to the two lower doses of QGE031, especially regarding responses from the skin, as well as effects on basophil IgE and FcεRI. This difference was abolished in the group that received the highest dose of QGE031 with respect to all the efficacy measurements. In addition, the response duration for all outcomes was longest for subjects receiving the highest dose of QGE031, and was longer than for omalizumab. However, there was a variation of the time the maximal response to QGE031 was

achieved in the different compartments; the effect on total IgE, surface IgE and FcεRI expression was achieved after two weeks, while the airway response was maximal at 12 weeks, two weeks after the last dose and returned to baseline at week 18, which was also seen for omalizumab. Moreover, the skin response peaked also at 12 weeks for the two lowest doses of QGE031, whereas the group that received the highest dose continued to improve at week 18, i.e. six weeks after the last dose. No cases of anaphylaxis were reported with QGE031 treatment possibly due to the fact that similar to omalizumab it does not bind to cell-bound IgE, which results in avoidance of FcεRI cross-linking that would potentially increase the anaphylaxis risk.

The results of this allergen bronchoprovocation study demonstrate the complexity of allergen-induced responses with differences regarding the various tissues and the efficacy of anti-IgE therapy. Anti-IgE therapy with omalizumab has been shown to inhibit the EAR and LAR after allergen inhalation (Boulet, Chapman et al. 1997, Fahy, Fleming et al. 1997) as well as allergen induced eosinophilic inflammation in the airways (van Rensen, Evertse et al. 2009). Similarly, outside the bronchoprovocation setting, anti-IgE therapy significantly reduced the eosinophil count in both the bronchial mucosa as well as in induced sputum compared to placebo in subjects with mild to moderate persistent asthma (Djukanovic, Wilson et al. 2004). The mechanism of action of anti-IgE therapy is that the drug binds to free IgE thus forming IgE/drug complexes that reduce free IgE levels and prevent interaction of IgE to FcεRI receptors and low-affinity IgE receptors on the surface of mast cells and basophils (Casale, Bernstein et al. 1997, Busse, Corren et al. 2001, Kuhl and Hanania 2012). This prevents mast cell degranulation and the release of bronchoconstrictive and pro-inflammatory mediators in the airways. Moreover, IgE regulates its own receptor, thus the reduction in serum free IgE causes downregulation of FcεRI expression on mast cells, basophils and DCs (MacGlashan, Bochner et al. 1997, Beck, Marcotte et al. 2004, Lin, Boesel et al. 2004, Pelaia, Gallelli et al. 2011). After three months of anti-IgE therapy the density of FcεRI receptors were reduced from 220,000 to 83,000 per basophil (MacGlashan, Bochner et al. 1997). Interestingly, this effect was also observed in non-atopic asthma (Garcia, Magnan et al. 2013). This reduction in FcεRI expression can contribute to a further dampening of the effector cell response to allergen (Oliver, Tarleton et al. 2010). Irrespectively of the mode of action it is shown at the population level, that the reduction in free IgE correlates with a reduction in asthma symptoms suggesting that greater IgE suppression leads to better clinical benefits (Lowe, Tannenbaum et al. 2009, Slavin, Ferioli et al. 2009, Zhu, Zheng et al. 2013). After initiation of anti-IgE therapy total IgE increases due to measurement of both free IgE and drug/IgE complexes, hence measurement of total IgE cannot be used for the assessment of the response to therapy. After the initial increase, total IgE levels will be reduced in parallel to the reduction in IgE production suggesting the regulation of IgE production through a feedback loop determined by the free IgE levels; a pharmacokinetic-pharmacodynamics model showed that treated subjects would be expected to reach a new equilibrium after approximately five years of anti-IgE therapy (Lowe and Renard 2011). The same model predicted that when anti-IgE therapy is withdrawn, IgE production slowly returns to baseline levels. This is also supported by the results of the XPORT trial where both free IgE levels and FcεRI expression on basophils increased after discontinuation of anti-IgE therapy

(Ledford, Busse et al. 2017), which was also associated with more exacerbations and worse asthma control compared to the group that continued on anti-IgE therapy.

**Paper II** shows that the allergen bronchoprovocation model can be used as a research tool to better understand the complexity of the airway response to allergen as well as to investigate the blocking effects of new interventions, such as QGE031 treatment. The excellent repeatability and reproducibility of the allergen bronchoprovocation model has been validated in previous studies, applying incremental allergen doses (Inman, Watson et al. 1995, Gauvreau, Watson et al. 1999, Gauvreau, Watson et al. 1999). These studies have shown high within-subject repeatability of both the EAR and LAR, irrespectively the way data was analysed (maximal % decrease in $FEV_1$ from baseline or area under the time-response curve) as well as high repeatability when other outcome measure were explored such as the degree of sputum eosinophilia. They have also shown excellent reproducibility with sample sizes of less than 10 subjects providing sufficient power to detect a 50 % inhibition using a crossover design. When using parallel-group studies it has been shown that 15 subjects per treatment group can also detect a 50 % inhibition of LAR (Gauvreau, Boulet et al. 2011). A recovery period of at least two weeks in-between challenges is recommended for both cross-over studies as well as parallel-group studies with multiple challenges (Diamant, Gauvreau et al. 2013). In **Paper II** the use of biologics as interventions that are known to have a long half-life, made the parallel-group design the only practical option. The primary endpoint in this study was the change in the EAR and no measurements of other markers of airways inflammation such as $F_ENO$ or sputum eosinophils were performed.

The findings in **Paper II** elucidate some fundamental differences in the allergen responses on the airways compared to the skin. Skin prick test responses were measured ten minutes after exposure to allergen and were caused by histamine release and were effectively suppressed in all subjects treated with the highest dose of QGE031. On the other hand on the airways the maximal decrease in $FEV_1$ is detected between 10 and 30 minutes after inhalation and is due to release of histamine, CysLTs and $PGD_2$; there was variability in the airway response to QGE031 that was not observed in the skin response. It is known that although cross-linking of IgE on mast cells and basophils is the initial "trigger" for the airway response to allergen, activation of other cells that express FcεRI such as DCs and B cells, can enhance and prime type 2 inflammation in the airway microenvironment (Holgate, Smith et al. 2009, Massanari, Holgate et al. 2010). Alternate pathways, such as activation through protease-activated receptor 2 can cause IgE-independent mast cell activation by alarmins such as TSLP and IL-33 (Boitano, Flynn et al. 2011). Hence, anti-IgE therapy does not target all the pathways involved to the development of the allergen response in the airways, which might explain the heterogeneity in the suppression of the EAR by QGE031. In addition, it was shown that subjects with higher IgE levels might require higher doses of QGE031. Although previous studies have shown some correlations between levels of free IgE and clinical benefits of anti-IgE therapy, they were based on statistical correlations across large populations of patients, thus for the individual subject, it is unlikely that suppression of free IgE or FcεRI expression alone will be able to predict responses to treatment (Chanez, Contin-Bordes et al. 2010). Moreover, there are also other factors that are involved that are of

importance regarding the response to anti-IgE treatment, including cellular IgE receptor expression (FcεRI and FcεRII/CD23), specific/total IgE ratios and cellular sensitivity (MacGlashan 2009), before even considering local tissue IgE concentrations and permeation of the drug to the tissue site. Previous retrospective analyses have suggested that asthmatic subjects with high blood eosinophil counts, high levels of $F_ENO$ and serum periostin would benefit most of anti-IgE therapy (Hanania, Wenzel et al. 2013); nonetheless this could be due to a higher rate of exacerbations in subjects with high type 2 biomarker, thus allowing more space for improvement with anti-IgE therapy. A recent prospective real-world study demonstrated that both type-2 high inflammatory profile (blood eosinophil count $\geq 300$ cells/μL and FeNO $\geq 25$ ppb) and type-2 low had a similar benefit of anti-IgE therapy (Casale, Luskin et al. 2019).

In addition, anti-IgE therapy has been investigated in other conditions than allergic asthma. A proof-of-concept study in subjects with severe non-atopic asthma demonstrated that anti-IgE therapy significantly reduced FcεRI expression on basophils and DCs compared to placebo and was associated with an increase of lung function compared with baseline (Garcia, Magnan et al. 2013). There was no significant reduction in the asthma exacerbation rate, although it should be noted that the study was only 16 weeks long. These findings indicate that anti-IgE therapy might be effective in the treatment of non-allergic asthma. Moreover, anti-IgE therapy has been investigated in aspirin-exacerbated respiratory disease (AERD) in an open label study in 21 adults with AERD that were also sensitised to one or more common environmental allergens. Anti-IgE therapy significantly improved asthma exacerbations and asthma related symptom scores, although it must be noted that there was no placebo comparator arm. The most important finding was that anti-IgE therapy induced a rapid reduction in urinary excretion of $LTE_4$ and the $PGD_2$ metabolite 9α,11β-prostaglandin $F_2$ compared to the period prior to treatment, thus indicating inhibition of mast cell activation (Hayashi, Mitsui et al. 2016). If one takes into consideration the results from **Paper III** regarding the excretion of a variety of eicosanoid lipid mediators in the urine, it would be of great interest in future studies regarding the efficacy of anti-IgE therapy to include as a primary or secondary endpoint the ability of the interventional drug to suppress these responses. It would be extremely useful to be able to predict the responders to anti-IgE therapy by a simple urine test. Lastly, anti-IgE therapy has shown efficacy in the treatment of chronic spontaneous urticaria (CSU) (Kaplan, Ledford et al. 2013, Maurer, Rosen et al. 2013). After the results regarding the effects of QGE031 in suppressing allergen-induced skin prick responses in **Paper II**, the drug is now tested for treatment of CSU. One study regarding the dose of QGE031 in CSU has been completed (NCT02477332) and a continuation study regarding the long-term safety of 240 mg QGE031 (the highest dose used in **Paper II**) given every four weeks for 52 weeks is estimated to be completed in June 2019 (NCT02649218).

## 7.3  NEW INSIGHTS INTO THE ROLE OF LTE$_4$ IN ASTHMA

The study presented in **Paper III** shows how a bronchoprovocation study with drug interventions and measurement of molecular markers can elucidate important pathophysiological and pharmacological mechanisms in asthma. The results of this study have several

pathobiological implications. This the first study where the most widely clinically used $CysLT_1$ antagonist, montelukast, was used to assess efficacy against bronchoconstriction induced by $LTE_4$. While previous studies have investigated thoroughly the bronchoconstrictive effect of $LTD_4$, there have been only a handful of studies that have looked how the terminal CysLT $LTE_4$ affects human airways *in vivo*. Treatment with montelukast completely abolished the $LTE_4$ induced bronchoconstriction in all subjects while on average subjects inhaled a 10-fold higher dose of $LTE_4$ without any fall in lung function. Hence, the results support that the *in vivo* effect of $LTE_4$ on airway smooth muscle in human subjects is mediated solely by the $CysLT_1$ receptor. These findings are in agreement with those of previous studies using other leukotriene receptor antagonists against inhaled $LTE_4$ (Christie, Spur et al. 1991, Laitinen, Lindqvist et al. 2005). This is also in agreement with *in vitro* results from experiments performed in isolated human bronchi and other models where also $LTE_4$ has a mode of action solely through the $CysLT_1$ receptor (Buckner, Krell et al. 1986, Mechiche, Naline et al. 2003, Back, Dahlen et al. 2011, Foster, Fuerst et al. 2016). As it was expected, the study confirmed that $LTE_4$ is a more potent bronchoconstrictor than methacholine demonstrating 75 times greater potency on a molar basis. Using an identical bronchoprovocation protocol it was found that inhaled $LTD_4$ is approximately 1000 times more potent than methacholine (Gyllfors, Kumlin et al. 2005). These results confirms the potency differences observed in previous studies where inhaled $LTE_4$ was compared with methacholine or histamine on the one hand, and $LTC_4$ or $LTD_4$ on the other hand (Arm, O'Hickey et al. 1990). Another finding of the study was that subjects with the highest hyperresponsiveness for methacholine had the lowest relative airway responsiveness to $LTE_4$, which has also been found for relations between methacholine and inhaled $LTC_4$ or $LTD_4$ in previous studies (Adelroth, Morris et al. 1986, Gyllfors, Kumlin et al. 2005).

Because $FEV_1$ reflects mainly changes to the larger airways, IOS measurements were applied in **Paper III** in order to examine the effect of $LTE_4$ in the small airways and how that is modified by montelukast. The small airways are usually defined as having a luminal diameter less than 2 mm and are a major site of airway inflammation and obstruction in asthma (Kraft, Djukanovic et al. 1996, Hamid, Song et al. 1997, Verbanck, Schuermans et al. 2010). Small airways disease has gained recognition due to the rapid evolvement of the methods to measure it during the past few years. There are reports showing that it is present at about 50-60 % of all subjects with asthma (Usmani, Singh et al. 2016). IOS was used in **Paper III** for the first time in the bronchoprovocation setting with $LTE_4$. At baseline prior to challenge with $LTE_4$ during both treatment periods, $R_5$-$R_{20}$ was found to be within the upper limit of normal (ULN), which was considered at 0.030 kPa/L/s as previously reported (Williamson, Clearie et al. 2011, Alfieri, Aiello et al. 2014). Inhalation of $LTE_4$ after placebo treatment led to a seven-fold increase of $R_5$-$R_{20}$ from baseline, and a mean 59 % increase in $R_5$, which evidences small airways impairment. IOS has recently more widely been used in the bronchial challenge setting, especially in studies with children where it has been demonstrated that the methacholine dose that provokes a 45% increase in $R_5$ ($PD_{45}$ $R_5$) had the optimal correlation with $PD_{20}FEV_1$ (Schulze, Smith et al. 2012). In adults, it is recommended that $PC_{40}$ $R_5$ can be used to approximately extrapolate to $PC_{20}$ $FEV_1$ after methacholine challenge (Galant, Komarow et al. 2017). The mean fall in $FEV_1$ after

inhalation of $LTE_4$ and after treatment with placebo was 26.3% and it was calculated that a 20 % decrease in $FEV_1$ translated to a 44.8 % increase in $R_5$, which is in agreement with previous findings. These results are similar to the findings of a previous study using IOS after inhalation of $LTD_4$ in asthmatic subjects (Guan, Zheng et al. 2013). Moreover, treatment with montelukast completely abolished this small airways impairment, with essentially no significant changes in IOS parameters after $LTE_4$ inhalation. There are previous studies that have tested IOS as a marker of therapeutic response to inhaled therapies (ICS/LABA) in adults with asthma (Galant, Komarow et al. 2017). In one open label study in the pediatric setting, treatment with 10 mg of montelukast for four weeks showed modest improvement in most IOS parameters (Nieto, Pamies et al. 2006). In addition, montelukast has been shown to improve regional air trapping on CT-scan due to small airways obstruction in adults with asthma (Zeidler, Kleerup et al. 2006).

In **Paper III** a higher dose of montelukast (40 mg) than the ordinary daily clinical dose for adults of 10 mg, was chosen deliberately. In absolute terms, 40 mg is not a very high dose, but a medium range dose given the knowledge about the pharmacology of montelukast. Montelukast is a very selective pharmacologic antagonist of the $CysLT_1$ receptor (Jones, Labelle et al. 1995). In the dose ranges achieved by oral administration of montelukast up to 250 mg, a range of other known G-protein coupled receptors have been found to be un-affected by the drug. This has been substantiated in a number of pharmacologic assays over the years (Back, Dahlen et al. 2011). Of central importance to the hypothesis tested in our study, montelukast has no antagonistic effect whatsoever on $CysLT_2$ receptors or $CysLT_3$/GPR99 receptors *in vitro* (Kanaoka, Maekawa et al. 2013). Moreover, the early clinical development of montelukast included 50, 100 and 250 mg dosing in adults. In bronchoprovocation studies against $LTD_4$ or in clinical treatment, there were dose-dependent progressively increasing effects from 2 up to 250 mg. For example, the median fold shift in $LTD_4$ responsiveness in the study of De Lepeleire et al at the time-points corresponding to peak plasma concentration (around 4 hours) was 85, 113, 161 and 181 fold for 5, 20, 100 and 250 mg, respectively (De Lepeleire, Reiss et al. 1997). Furthermore, in the first published clinical treatment trials (Reiss, Altman et al. 1996, Altman, Munk et al. 1998) data were reported for groups treated with several different doses from 10 mg up to 200 mg taken once up to three times daily. Interestingly, the $FEV_1$ improvements in those early trials were ranging between 10-to17 % for the 100 and 200 mg doses, whereas the results reported for the 10 mg once daily dose in later trials have been lower, for example 7.4% in the study by Malmström et al (Malmstrom, Rodriguez-Gomez et al. 1999). The study populations were similar with baseline $FEV_1$ predicted around 65% and a mix of steroid-treated and steroid-naïve subjects. The clinical dose-selection was however much guided by the findings in an exercise-provocation dose-ranging study (Bronsky, Kemp et al. 1997). ). It did not find striking statistical significance between 10 mg and higher doses, although the numerical values for some outcomes suggested better protection by 50 or 100 mg. The final clinical dose selection was also based on commercial factors. The main goal of this study was to understand if there was a significant component of the bronchoconstriction induced by inhalation of $LTE_4$, which was resistant to blockade by the prototype $CysLT_1$ receptor antagonist montelukast. Given the clear published data from the study with short-term administration of montelukast in the $LTD_4$

bronchoprovocation setting (De Lepeleire, Reiss et al. 1997) it was not wished to test the hypothesis of the distinct $LTE_4$ receptor using a too low dose of montelukast. Partial inhibition of $LTE_4$ might then lead to the inconclusive result that either there is another receptor or that the receptor antagonism was not sufficient. Again, given the previous data and the remaining very selective effect of much higher doses of montelukast, the 40 mg dose was chosen to optimise pharmacologic antagonism without moving up into the real high dose interval. In De Lepeleire et al this dose at trough gave more than a 56-fold shift in the responsiveness to $LTD_4$. As a corollary of the findings, perhaps it is time to revisit the dosing of montelukast?

The results furthermore showed that despite exposure to one order of magnitude higher dose of $LTE_4$ after treatment with montelukast, the sputum eosinophil count was the same as during the placebo period. The first thing that needs to be discussed is if the window of four hours post $LTE_4$ inhalation was enough in order to assess the components of this response. There seems to be some uncertainty about the kinetics of the influence of the primary challenge with $LTE_4$ and secondary factors released from mast cells on the cellular responses. There were unfortunately only a few previous studies to use as guide on the design of this particular part of the study protocol when the investigation was planned. In the original report of $LTE_4$-induced airway eosinophilia by Laitinen et al (Laitinen, Laitinen et al. 1993b), the finding was documented at 4 hours after inhalation of $LTE_4$. Deykin and colleagues (Deykin, Belostotsky et al. 2000) reported an increase in sputum eosinophils at 4 hours following $LTE_4$ inhalation in subjects with mild intermittent asthma, thus being a similar study population as in our investigation. Furthermore, Laitinen et al replicated their finding of increased bronchial tissue eosinophils at 4 hours post $LTE_4$ challenge (Laitinen, Lindqvist et al. 2005). It appears therefore that eosinophil accumulation may be detected in human airways at four hours post challenge with $LTE_4$ irrespective of where assessed, indicating that the interval chosen in **Paper III** was sufficient. Hence, the absence of an increase in sputum eosinophils when subjects were exposed to one-log order of magnitude greater amounts of $LTE_4$ in the presence of montelukast argues against the presence of another CysLT receptor promoting eosinophil accumulation in the sputum. If a dual receptor scenario had been present, blockade of $CysLT_1$ with montelukast would have uncovered effects at another active receptor. Of course, it might be speculated that a putative second receptor is inhibitory. There is however, no experimental data supporting negative effects of $LTE_4$ or other CysLTs on human eosinophil migration, therefore such a theoretical interpretation appears unlikely.

One of the discoveries of the study in **Paper III** is that inhaled $LTE_4$ induced increased urinary excretion of metabolites indicative of pulmonary release of several COX products as well as several isoprostanes. It is the first time this is demonstrated in human subjects *in vivo* and confirms previous results from original work on CysLTs mechanisms in animal airways showing also that CysLTs can cause profound release of COX products that contribute to the overall biological responses (Piper and Samhoun 1982, Dahlen, Hedqvist et al. 1983). In addition, these results may provide one potential mechanistic explanation for the previous finding that in asthmatic subjects prior inhalation of $LTE_4$ enhanced histamine-induced bronchoconstriction that was inhibited by indomethacin, indicating that a cyclooxygenase

product mediated this particular hyperresponsiveness (Christie, Hawksworth et al. 1992). However, it was not discussed which prostanoid that might have been involved and there were no data on prostanoid release in that study. It must be also noted that the cyclooxygenase inhibition with indomethacin causes a global inhibition of the release of all PGs, including $PGE_2$, which is bronchoprotective by inhibition of mast cell mediator release (Safholm, Manson et al. 2015).

It was accordingly shown in **Paper III** that $LTE_4$ induced bronchoconstriction was associated with sharp increases in urinary excretion of metabolites of the mast cell derived mediator $PGD_2$, as well as $TXA_2$ and other COX pathway mediators such as $PGF_{2a}$ and $PGE_2$. Moreover, this increased excretion was abolished by montelukast, with noteworthy exception of $PGE_2$, thus confirming most of the secondary lipid mediator release to be $CysLT_1$ dependent. This is the first study that directly demonstrates that $LTE_4$ causes $CysLT_1$ dependent activation of mast cells and confirms that $LTE_4$ is able to elicit formation of several COX products, of which many are likely derived from mast cells (Figure 49). Increased excretion of $PGD_2$ is considered a sign of mast cell activation because this cell is the major source of $PGD_2$ in human subjects (Lewis, Soter et al. 1982, Bood, Sundblad et al. 2015). $PGD_2$ is known to be a potent bronchoconstrictor and can contract human airways through a mechanism that is blocked by thromboxane prostanoid (TP) receptor antagonism (Safholm, Manson et al. 2015). The results suggest that some of the bronchoconstrictive effects of $LTE_4$ are secondary to prostanoid release from mast cells, thus CysLTs may need to be reclassified as dual bronchoconstrictors (Figure 49). Another implication of this finding is that because mast cells can also produce CysLTs, this $CysLT_1$ dependent mast cell activation might represent a positive feedback loop sustaining the response to the initial stimulus. In addition, eosinophils, which are proficient producers of CysLTs, could prime mast cells by this particular mechanism. Moreover, $PGD_2$ acting through the chemoattractant receptor-homologous receptor ($CRTH_2$) can induce chemotaxis for Th2 lymphocytes and eosinophils as well as activate ILC2 cells that also have a key role in asthmatic inflammation (Salimi, Stoger et al. 2017). In fact results from a recent *in vitro* study showed that cytokine-induced endogenous production of $PGD_2$ is essential for human ILC2 cell activation (Maric, Ravindran et al. 2018). These $PGD_2$ driven pro-inflammatory effects can be counteracted at least partially by $PGE_2$, which was also increased in the urine after $LTE_4$ inhalation, although its excretion was not blocked by montelukast. This indicated that the release of $PGE_2$ is not $CysLT_1$ dependent, although $CysLT_1$ could nonetheless be involved partially because a ten-fold greater dose of $LTE_4$ in the presence of montelukast did not further increase urinary excretion of $PGE_2$ compared to placebo. Thus, it is possible that another receptor ($CysLT_x$) might be involved in $PGE_2$ release, most likely in the airway epithelium, which is a major source of $PGE_2$ (Churchill, Chilton et al. 1989, Harrington, Lucas et al. 2008). Irrespectively of the involved receptor, it was shown that inhalation of $LTE_4$ triggered secondary release of $PGE_2$, which has anti-inflammatory effects on both mast cells (Raud, Dahlen et al. 1988, Safholm, Manson et al. 2015) as well as ILC2 cells (Zaslona, Okunishi et al. 2014, Maric, Ravindran et al. 2018). This finding of increased $PGE_2$ excretion might be a protective negative feedback response intended to aid resolution (Figure 49). These results suggest that a potential

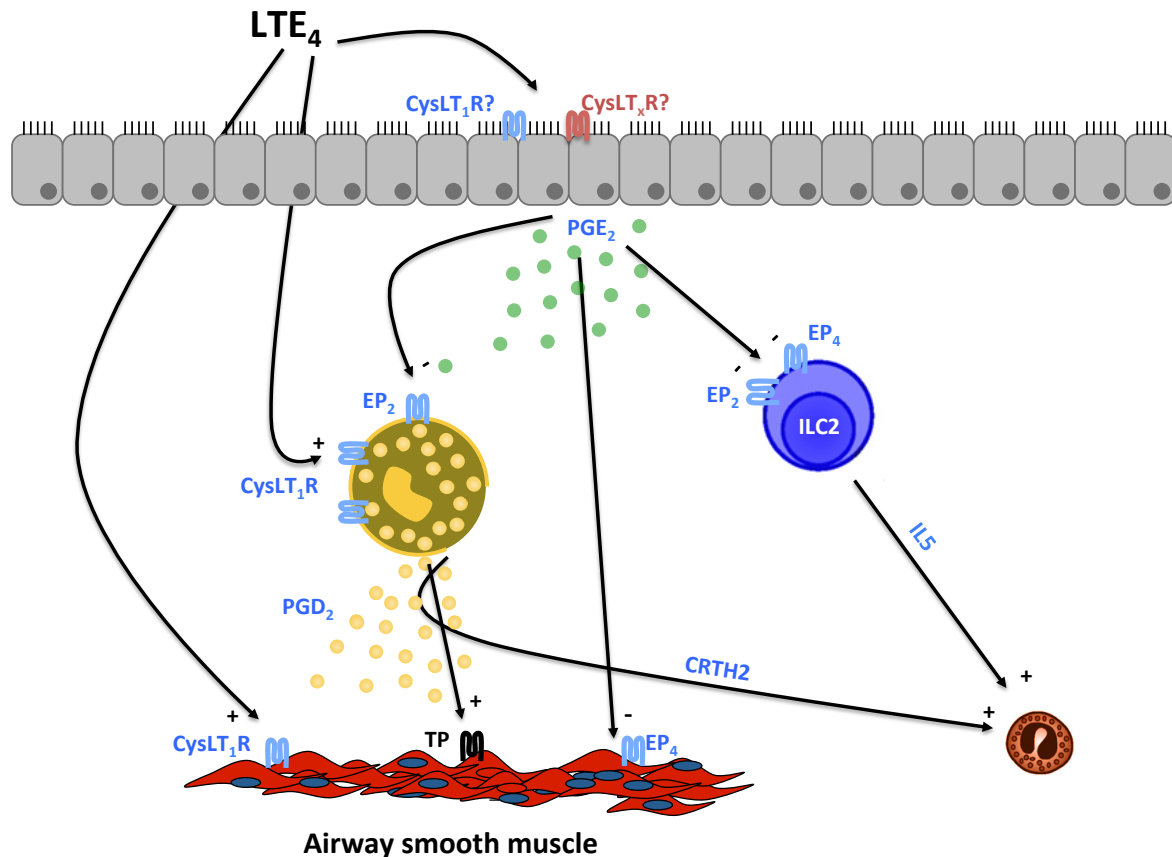imbalance in the production of endogenous prostanoids may have an important role in asthma pathogenesesis.



**Figure 49: Summary of the effects of LTE$_4$ in asthma according to findings in Paper III.**

## 7.4   SUMMARY

The aim of this thesis was to use bronchial provocations and interventions in order to investigate mechanisms in asthma and airway inflammation. Looking back at the results of the three different projects one could say that major discoveries have been made. The results of the first study have subsequently been confirmed in two large cohort studies. Together this will probably change current recommendations for treatment of mild asthma. The results of the second study elucidated the complexity of the IgE pathway, differences between skin and airways, and new unmet needs regarding effective anti-IgE therapy. The third study gave clear-cut answers regarding the role of the receptors for CysLTs that had not been answered in years and opened new doors for future research in the field. Of course all the questions are not answered yet, there is still much to do. Bronchoprovocation studies still after more than 70 years of use remain an effective research model in asthma that can ultimately integrate our understanding of the different pathophysiological mechanisms in asthma.

# 8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Astma är en folksjukdom som drabbar cirka 10-12% av den svenska befolkningen, Den orsakas av kronisk inflammation i luftrören som är överretbara och symptomen kännetecknas av varierande grad av andnöd och pipande andning. När astman utlöses drar musklerna i luftrören ihop sig och det blir trängre för luften att komma ner i lungorna. Dessutom det blir en svullnad i slemhinnorna som orsakar ökad slembildning och hosta. Ett astmaanfall kan utlösas av olika faktorer, såsom ansträngning, exponering för allergener, virusinfektioner eller intag av acetylsalicylsyra (ASA) och inflammationsdämpande läkemedel (NSAID). Astma är en variabel sjukdom som innebär att patienter med framförallt lindrig astma ofta mår bra i sin astma när dem inte exponeras för trigger faktorer. Astma är också en heterogen sjukdom där det ingår olika subgrupper som kallas för fenotyper som skiljer sig avsevärt både avseende dem underliggande mekanismer samtidigt till en viss del vad det gäller behandlingen.

Dessa karakteristika dvs. sjukdomens variabilitet och att exponering för visa stimuli kan framkalla ett anfall, kan vi använda vid astmaforskning genom att utföra en s.k. provokationsstudie. Vid en sådan studie provoceras dvs. exponeras, försökspersoner med oftast stabil, lindrig för olika ämnen som kan framkalla ett standardiserat och kontrollerat astmaanfall på forksningsenheten. Lungfunktionen mäts både innan, under och efter provokationen och man samlar prover från utandad luft, blod och urin för att mäta vilka molekyler som frisätts efter att luftvägarna har exponerats för ämnet. Provokationen upprepas efter en behandlingsperiod med en aktiv medicin som dämpar astmaanfallet och skyddar luftvägarna och efter behandling med placebo eller ett annat aktivt behandlingsalternativ. På det sättet kan man studera både underliggande mekanismer vid astma och samtidigt testa om en ny medicin är effektiv som astmabehandling. Nästan alla mediciner som är godkända idag för astmabehandling har studerats med provokationsstudier. I den här avhandlingen ingår 3 provokationsstudier där jag har studerat olika astmamekanismer och även testat nya behandlingsalternativ.

Första delarbetet var en provokationsstudie med fysisk ansträngning hos försökspersoner med lindrig astma, där syftet var att jämföra den skyddande effekten av att bara använda en kombination av inandat kortison och luftrörsvidgande medicin (budesonide/formoterol) vid behov med en kortverkande luftrörsvidgande (terbutalin) vid behov eller kontinuerlig behandling med inandat kortison (budesonid) och terbutalin vid behov. Den primära effektvariabeln var sänkning av lungfunktionen efter ett 6 minuters standariserat ansträngningsprovokation på rullande matta som utfördes fore samt efter 3 och 6 veckors behandling. Vi fann att kombinationsbehandling med budesonid-formoterol som profylaktisk behandling innan ansträngning, var mer effektivt än standardbehandling terbutalin. Terbutalin hade ingen skyddande effekt mot ansträngningsutllöst luftvägsobstruktion sannolikt pga toleransutveckling efter användningen. Vi fann också att behandling med budesonid-formoterol som profylaktisk behandling innan ansträngning, var lika effektiv som kontinuerlig behandling med budesonid och terbutalin vid behov. Resultaten kan ha implikationer vid framtida riktlinjer för astmabehandling.

Andra delarbetet var en allergenprovokationsstudie hos försökspersoner med lindrig astma. Syftet var att studera effekten av olika doser av ett nytt läkemedel (QGE031-ligelizumab) mot IgE, dvs den typ av immunoglobulin som spelar roll vid allergi, för att se om detta läkemedel kunde skydda mot inandat allergen och hämma den astmatiska reaktionen. I studien jämfördes QGE031 med omalizumab, som är hittils det enda registrerade läkemedlet mot IgE och med placebo. Den primära effektvariabeln var ändringen i allergen koncentrationen som gav en 15-procentig minskning av lungfunktionen (allergenPC$_{15}$) efter högsta dosen av QGE031 och i jämförelse med omalizumab. Sekundära variabler var effekten av de två mindre doserna av QGE031 jämfört med omalizumab och placebo på luftvägssvaret samt effekten av QGE031 vad gäller att hämma hud prick test reaktivitet på allergen jämfört med omalizumab och placebo. Vi fann att högsta dosen av QGE031 ändrade allergenPC$_{15}$ tre gånger mer än omalizumab och 16 gånger mer än placebo, dock skillnaden med omalizumab blev inte signifikant. Däremot QGE031 var mer effektivt än omalizumab att hämma den allergen inducerat prick test reaktivitet på hud i alla doser. QGE031 tolererades bra av alla försökspersoner.

Tredje delarbetet var en provokationsstudie med inandat leukotrien E$_4$ (LTE$_4$) hos försökspersoner med lindrig astma; studien var en rent experimentell undersokning av hur den kroppsegna mediatorn LTE$_4$ framkallar obstruktion av luftrören hos patienter med astma. Det finns sedan länge ett godkänt astmaläkemedel mot leukotriener, montelukast, som blockerar en receptor, nämligen CysLTR$_1$ som är viktigt för leukotrienernas verkan i luftägarna. Det finns nu experimentella data som tyder att LTE$_4$ kan utöva sin effekt via andra receptorer än CysLT$_1$. Det innebär då att montelukast endast delvis skyddar mot LTE$_4$´s effekt på luftvägarna. Den primära effektvariabeln var ändringen i den dos av LTE$_4$ som ger en 20-procentig sänkning i FEV$_1$ efter en standardiserat inhalations ptovokation med LTE$_4$ efter behandling med montelukast och placebo. Sekundära variabler var skillnaden i graden av inflammationen i upphostningsprov mätt som antal eosinofila celler i inducerat sputum vilket samlades 4 timmar efter provokationen och mätning av utsöndringen i urinen av kroppsegna lipidmediatorer med hjälp av mass spektrometri. Studien hade crossover design. Vi fann att montelukast skyddade mot LTE$_4$ inducerad luftvägsobstruktion och att försökspersonerna tolererade 10 gånger högre dos av LTE$_4$ när de fick montelukast jämfört med placebo. Det var ingen skillnad i sputum eosinofili mellan montelukast och placebo. LTE$_4$ inhalation ledde till utsöndring i urinen av prostaglandin D$_2$ metaboliter som blockerades av montelukast behandling. Prostaglanding D$_2$ frisätts huvusakligen från mastceller efter deras aktivering. Prostglandin D$_2$ framkallar också luftvägsobstruktion Detta är således den första studie som visar att leukotriener kan aktivera mast celler *in vivo* vilka i sin tur frisätter prostaglandiner som kan bidra till luftvägsreaktivitet.

Sammanfattnings visar avhandlingens resultat att provokationsstudier kan framgångsrikt användas för att studera viktiga mekanismer vid astma och luftvägsinflammation, samt för att testa effekten av nya behandlingsalternativ.

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110

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114

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