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Clinical studies of asthma phenotypes focusing on the role of the leukotrienes

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Till Simon:

Att ta in mycket ny kunskap under kort tid
handlar mycket om att utstå kaos, en
kunskap som inte varit applicerbar vid
tillkomsten av denna bok

ABSTRACT

Inflammation in the airways in connection to asthma is a complex phenomenon and the mechanisms underlying the associated clinical symptoms involve the interaction of many different kinds of cells and mediators, giving rise to different phenotypes. The aim of the present thesis was to investigate the molecular and cellular mechanisms that results in two of these phenotypes, i.e., aspirin-intolerant asthma and allergic asthma. The main focus was on leukotrienes and other eicosanoids, metabolites of arachidonic acid, and the major experimental approach employed was bronchial challenge.

Thirty-three subjects known to be suffering from aspirin-intolerant asthma were challenged with celecoxib a selective inhibitor of COX-2. Both escalating doses from 5-100 mg (administered in a blinded, placebo-controlled study) and an open label challenge with 200 + 200 mg celecoxib were tolerated well by these individuals. This finding indicates that the intolerance reaction leading to bronchoconstriction in patients with aspirin-intolerant asthma is due to inhibition of COX-1 and, furthermore, provides a scientific basis for administration of selective inhibitors of COX-2 to alleviate prostaglandin-mediated pain and inflammation in these patients.

With the ultimate objective of finding a marker that can be used to identify patients with leukotriene-associated asthma, the capacity to produce leukotrienes and responsiveness to inhaled leukotrienes was determined in 20 subjects with intermittent-to-mild asthma and 10 healthy control individuals. Neither group exhibited a correlation between the formation of LTB_4 by their whole blood in response to *ex vivo* stimulation or urinary levels of LTE_4 and airway responsiveness to LTD_4. In further attempts to predict which asthmatic patients will respond well to antileukotriene treatment, investigations on the capacity for leukotriene synthesis and responsiveness to these agents and expression of their specific receptor in the lungs are presently being performed.

When 8 individuals with allergic asthma were challenged repeatedly with low doses of allergen, the level of nitric oxide in the air they exhaled and their responsiveness to histamine rose significantly. At the same time, these subjects did not report any symptoms of asthma, required rescue by bronchodilator medication or display any change in the calibre of their airways. Accordingly monitoring of exhaled nitric oxide on a daily basis might allow for early detection of exacerbation in subjects with allergic asthma.

Thirteen patients with allergic asthma were subjected to bronchial challenges with methacholine and LTD_4 prior to and after administration of 500 µg fluticasone twice daily for two weeks, and their levels of exhaled nitric oxide and urinary LTE_4 was determined. Inhalation of glucocorticoid attenuated the responsiveness to methacholine and reduced the level of exhaled nitric oxide, but neither the responsiveness to LTD_4 nor urinary excretion of LTE_4 was affected. Thus, neither the release nor the actions of leukotrienes appear to be sensitive to inhaled glucocorticoids, strengthening the rationale for using a combination of glucocorticosteroids and antileukotrienes to treat allergic asthma.

In summary, we have shown the following here: 1) There is now a rationale basis for using selective inhibitors of COX-2 to alleviate prostaglandin mediated-pain and inflammation in individuals with aspirin-intolerant asthma. 2) The bronchial responsiveness of subjects with asthma cannot be predicted on the basis of the ability of their whole blood to produce LTB_4 in response to stimulation *ex vivo* or their urinary levels of LTE_4. 3) Regular monitoring of exhaled nitric oxide might allow early detection of exacerbation in subjects with allergic asthma. 4) There is a mechanistic rationale for combination treatment of allergic asthma with glucocorticosteroids and antileukotrienes.

Keywords: asthma, aspirin intolerance, leukotrienes, leukotriene D_4 responsiveness, methacholine responsiveness, exhaled nitric oxide

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals.

I **Gyllfors P***, Bochenek G*, Overholt J, Drupka D, Kumlin M, Sheller J, Nizankowska E, Isakson PC, Mejza F, Lefkowith JB, Dahlen SE, Szczeklik A, Murray JJ, Dahlen B.
Biochemical and clinical evidence that aspirin-intolerant asthmatic subjects tolerate the cyclooxygenase 2-selective analgetic drug celecoxib.
J Allergy Clin Immunol. 2003 May;111(5):1116-21.

II **Gyllfors P,** Kumlin M, Dahlen SE, Gaber F, Ehrs PO, Dahlen B.
Relation between bronchial responsiveness to inhaled leukotriene D_4 and markers of leukotriene biosynthesis.
Thorax. 2005 Nov;60(11):902-8. Epub 2005 Jul 29.

III Ihre E, **Gyllfors P**, Gustafsson LE, Kumlin M, Dahlen B.
Early rise in exhaled nitric oxide and mast cell activation in repeated low-dose allergen challenge.
Eur Respir J. 2006 Jun;27(6):1152-9. Epub 2006 Mar 1.

IV **Gyllfors P,** Dahlen SE, Kumlin M, Larsson K, Dahlen B.
Bronchial responsiveness to leukotriene D_4 is resistant to inhaled fluticasone propionate.
J Allergy Clin Immunol. 2006 Jul;118(1):78-83. Epub 2006 May 30.

* Authors have contributed equally to the study.

CONTENTS

LIST OF ABBREVIATIONS

AIA	aspirin-intolerant asthma
AMP	adenosine 5'-monophosphate
ASA	acetylsalicylic acid
ATA	aspirin-tolerant asthma
BAL	bronchoalveolar lavage
BHR	bronchial hyperresponsiveness
BSA	bovine serum albumin
COX	cyclooxygenase
CysLT	cysteinyl leukotriene
EAR	early allergic reaction
EIA	enzyme immunoassay
FE_{NO}	fraction of exhaled nitric oxide
FEV_1	forced expiratory volume in one second
FLAP	5-lipoxygenase-activating protein
FP	fluticasone propionate
FVC	forced vital capacity
IgE	immunoglobulin E
IL-1	interleukin 1
ICS	inhaled corticosteroids
LABA	long-acting β_2 agonist
LAR	late allergic reaction
LT	leukotriene
MCh	methacholine
NO	nitric oxide
NSAID	non-steroid antiinflammatory drug
PC_{20}	provocative concentration causing a 20% fall in FEV_1
PD_{20}	provocative dose causing a 20% fall in FEV_1
PG	prostaglandin
RV	rhinovirus
RT	room temperature
SEM	standard error of the mean
SD	standard deviation
TX	thromboxane
5-LO	5-lipoxygenase

BACKGROUND

1.1 ASTHMA - A GLOBAL HEALTH PROBLEM

Asthma is a major, chronic disorder of the airways that affects people of all ages and both genders, and has become a serious public health problem in many countries throughout the world. International comparisons reveal that asthma is most prevalent in Western Europe, Australia, New Zealand and North America [1, 2], where this prevalence increased dramatically from the middle to the end of the 20th century. In Sweden today, approximately 8% of the population suffers from asthma. Although certain studies indicate that the situation in Europe has now stabilized [3, 4] others document a nearly linear increase in the prevalence from 1960 till 2003 [5, 6]. Nevertheless, asthma is a considerable burden, not only in terms of healthcare costs, but also lost productivity, reduced participation in social activities and lowered quality of life [7, 8].

1.2 DEFINITION OF ASTHMA

The Global Strategy for Asthma Management and Prevention supported by GINA (Global Initiative for Asthma) describes asthma as a chronic inflammatory disorder of the airways in which many different types of cells and cellular elements are involved. This chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness tightness in the chest, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable obstruction of the airways that is often reversible either spontaneously or with proper treatment [8]. However, the general consensus now emerging is that asthma is unlikely to be a single disease entity [9].

1.3 ASTHMATIC PHENOTYPES

The airway inflammation associated with asthma is complex and the mechanisms underlying the clinical symptoms involve as mentioned above, interactions between many different types of cells and mediators. The initiation and maintenance of inflammation by different mechanisms in different patients probably explains the

general clinical impression that asthma is a heterogeneous disorder. “Phenotype” is defined as “the visible characteristics of an organism resulting from the interaction between its genetic makeup and the environment” [10]. Although clinicians have recognised different asthmatic phenotypes for many years, until recently no attempt of a detailed classification of these phenotypes have been undertaken [11] and biomarkers for the different phenotypes have still not been identified. These phenotypes are still generally categorised under the broad term “asthma” merely because they all fulfil the simple criteria for clinical diagnosis of this disease. The present thesis deals with two distinct clinical asthmatic phenotypes i.e., allergic asthma and aspirin-intolerant asthma.

1.3.1 Allergic asthma

Allergic or atopic asthma affects around 50% of all adult asthmatics [12, 13]. These individuals develop bronchoconstriction and airway inflammation upon exposure to specific air-borne allergens [14]. In Sweden the most common of these allergens originate from the plants birch, timothy and mugworth, as well as cats and dogs [15]. The house-dust mite, which globally is a major source of asthma-provoking allergens, is presently only a minor clinical problem in Sweden due to our Nordic climate.

Allergic asthma can develop at any age, even during the very first years of life. When symptoms of pulmonary obstruction develop in infants, these are usually transient and the individuals affected do not run an enhanced risk of developing asthma or allergies later on in life [16]. However, when eczema, food allergy and/or sensitization are present together with a family history of allergic disease, the risk of developing true allergic asthma is higher [17].

When individuals with atopic asthma are exposed to allergens to which they are sensitive, their immune system reacts by cross-linking specific IgE antibodies on the surface of mast cells, which gives rise to an Early Allergic Reaction (EAR) and, sometimes, to a Late Allergic Reaction (LAR) as well [18]. The EAR occurs when a sufficiently large number of IgE molecules on the surface of mast cells or basophils have bound molecules of allergen [19], which leads to a massive efflux of histamine from the granules of mast cells, as well as *de novo* biosynthesis of leukotrienes and prostaglandins by these cells. Both histamine and leukotrienes cause smooth muscle

cells to contract and thereby constrict the bronchi. Furthermore, this degranulation leads to recruitment of inflammatory cells and, in about half of the patients, to a LAR involving prolonged bronchial obstruction approximately 4-8 hours following exposure as well [20]. The diagnosis of allergic asthma is based on a typical history of asthmatic symptoms evoked by exposure to airborne allergens together with sensitization to that particular allergen, as demonstrated by a skin prick test or blood analysis.

1.3.2 Aspirin-intolerant asthma

Another distinct asthmatic phenotype is Aspirin-Intolerant Asthma (AIA), also referred to as ASA Intolerant Asthma, since the active ingredient of aspirin is acetyl salicylic acid. Aspirin® was introduced into the market by Bayer in 1898 and shortly thereafter implicated as the cause of serious respiratory attacks in subjects with asthma [21, 22]. In 1922, the association between asthma, aspirin intolerance and rhinitis with nasal polyposis was first recognised by Widal et al. [23] and in the late 1960's Samter and Beer [24] described this peculiar syndrome with its clinical triad of asthma, nasal polyposis and aspirin intolerance, in greater detail.

This particular kind of asthma is present in approximately 5-10% of the adult asthmatic population [25-27], affecting women and men in the ratio 2:1 [28]. It is not found in children, being characterized by late onset, usually between the age of 25 and 55, often following a viral infection [29]. In general the symptom of rhinitis develops first, followed by asthma and sensitivity to ASA on an average of 2 and 4 years later, respectively [30], but any one of the three aspects can be the first to appear. The chronic rhinitis with which these individuals are afflicted often involves nasal polyposis with nasal congestion, loss of smell and taste and reduced quality of life. For a more detailed natural history of AIA see references [24] and [30].

Individuals suffering from AIA exhibit a higher number of eosinophils in their bronchial mucosa biopsies than do patients with Aspirin-Tolerant Asthma (ATA) [31], as well as greater production of cysteinyl-leukotrienes under stable conditions, measured as urinary LTE_4 [32] and a significant elevation in this production when exposed to aspirin [33]. In fact, the anaphylactic-like reactions which individuals with AIA develop when they ingest aspirin or any other chemically unrelated traditional Non-Steroidal Anti-inflammatory Drug (NSAID) by mistake can be life-threatening.

This reaction frequently occurs in connection with the initial exposure to a new NSAID, suggesting that immunological cross-reactivity is unlikely to be involved.

Accurate diagnosis of AIA can be achieved by oral, pulmonary or nasal provocation with increasing doses of aspirin [34-36]. The inhalation challenge first described by Bianco and coworkers in 1977 [37] was later shown to be as reliable as an oral challenges in this respect [38]. In combination with a typical history such provocation tests represent the golden standard in diagnosing AIA. At present, no reliable *in vitro* test is available and the syndrome of AIA probably remains undiagnosed in many individuals, largely because of this lack of a rapid, safe and reliable diagnostic test.

Once ASA sensitivity develops, it is usually present for the rest of the patient's life. This creates a major clinical problem, because the persistent sensitivity to all NSAIDs leads to difficulties in treating inflammation and pain, as well as in finding alternative means to protect against cardiovasculature diseases. Regular usage of ASA can, however be employed to desensitize certain sensitive subjects [39, 40]. The underlying inflammation associated with AIA is often more severe than in patients with ATA and usually satisfactorily, administration of antileukotrienes is often effective [41]. Patients with AIA almost always require inhalation of high doses of corticosteroid and frequently take corticosteroids orally for maintenance as well [42].

1.4 FROM ASPIRIN TO SELECTIVE INHIBITORS OF CYCLOOXYGENASE-2

Some medical historians believe that even Hippocrates prescribed Non-Steroid Anti-inflammatory Drugs, claiming that he knew that an extract of the bark from the willow tree (*Salix alba*) could reduce fever. In the 19th century production of salicylic acid was achived and the chemist Felix Hoffman succeeded in synthesizing acetylsalicylic acid (ASA) in the 1870's. Curiously, this drug was first thought to be less toxic towards the stomach. As mentioned previously Bayer first launched Aspirin®, as a drug in 1898 and even now, 118 years later aspirin is frequently used by patients to alleviate pain and inflammation and, since the 1970's also to prevent cardiovascular pathology.

In the 1950's oral administration of glucocorticoids was first used to treat rheumatoid arthritis. The side-effects associated with this treatment stimulated the

search for more effective drugs with fewer and less severe undesired effects. In 1963 for example, a phase III trial was carried out with one of the first new NSAIDs, Indometacin [43].

In 1971 an important milestone was achieved when John Vane and his associates demonstrated that aspirin and other NSAIDs act by inhibiting the production of prostaglandins [44]. Later, it became clear that this inhibition reflects interference with the activity of the enzyme cyclooxygenase (COX). The work at Karolinska Institutet by Sune Bergström and Bengt Samuelsson on the metabolism of prostaglandins provided the background knowledge necessary for John Vane's discovery.

However, the new synthetic NSAIDs also cause side-effects. The most frequent and undesirable side effect of today's commonly used NSAIDs is an increase in the risk for ventricular and duodenal ulcers by 5- and 4-fold, respectively [45]. For example, a Finnish study [46] in 1995 concluded that 47 (3%) of 1666 patients with rheumatoid arthritis had died as a result of their medication. 64% of these deaths were related to treatment with NSAID and almost all (i.e., 93%) of these deaths were due to side-effects on the gastrointestinal tract. This disturbing situation motivated further research designed to find NSAIDs with even fewer side-effects.

After the effect of NSAIDs on COX had been discovered, it soon became clear that the degree of inhibition, at least *in vitro* did not correlate well with the anti-inflammatory, analgesic and antipyretic effects of these drugs. In particular, salicylic acid itself exert little or no effect at all on COX [47]. These observations indicated early on that there might be different forms of COX, an hypothesis which received substantial support from the demonstration that exposure of fibroblasts to the proinflammatory cytokine IL-1 stimulated their production of prostaglandins [48].

In 1994 the three-dimensional structure of the first COX enzyme, now referred to as COX-1 was reported [49]. This enzyme is responsible for important housekeeping functions in connection with the regulation of physiological processes, is expressed constitutively by all cells in the body, and is not inhibited by glucocorticoids. In 1996 the 3-D structure of COX-2 was solved independently by two research groups [50, 51]. This enzyme is inducible in connection with inflammation and believed to play an important role during injury, in addition to which its production can be inhibited by

glucocorticoids. When it became clear that there are at least 2 different COX enzymes, one of which is up-regulated in connection with inflammatory processes, an obvious question concerned the possible role of selective inhibition of COX in gastrointestinal and other forms of toxicity exerted by NSAIDs. Soon thereafter the first two selective inhibitors of COX-2, celecoxib and rofecoxib, were introduced onto the market.

1.5 THE CYCLOOXYGENASE THEORY AND UNDERLYING MECHANISMS

The cyclooxygenase (COX) theory – concerning the intolerance reactions associated with AIA is founded on the common ability of ASA/NSAIDs to inhibit the COX enzymes (Figure 1). The major evidence that support this theory [52] can be summarized as follows: 1) Analgesics that exert anticyclooxygenase activity invariably precipitate bronchoconstriction in aspirin-sensitive patients. 2) Analgesics that do not affect cyclooxygenase do not cause such bronchoconstriction [53] 3) There is a positive correlation between the potency of analgesics in inhibiting cyclooxygenase *in vitro* and their potency in inducing asthmatic attacks in sensitive patients [54]. And finally 4) if a subject with AIA is desensitized by repeated treatment with ASA, cross-desensitization to other NSAIDs that inhibit COX also occurs [55].

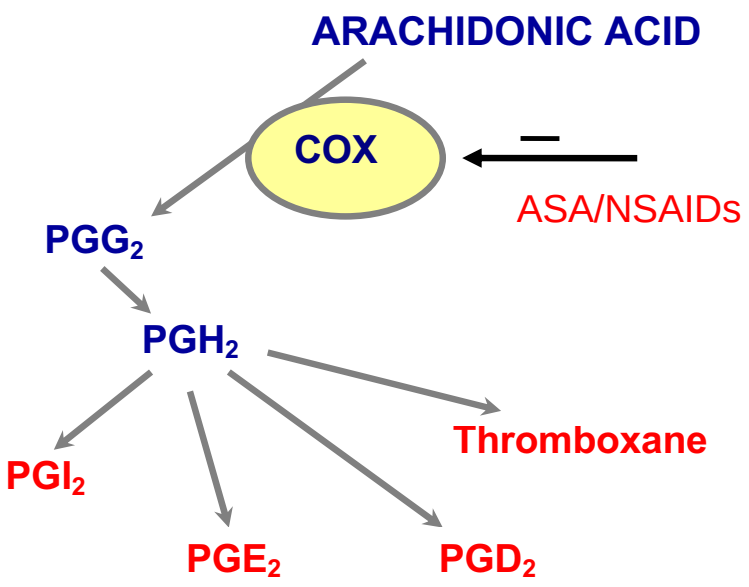


Figure 1. Schematic representation of the biosynthesis of prostanoids and its inhibition by ASA/NSAIDs. COX = cyclooxygenase, PG = prostaglandin

As mentioned above, there exist at least two isoforms of COX, i.e., COX-1 and COX-2. ASA and indometacin inhibit COX-1 more potently than COX-2 and cause asthma attacks in AIA patients. In contrast, nimeluside and meloxicam, which inhibit COX-2 to a greater extent than COX-1, are usually tolerated well by AIA patients in low doses, but can cause symptoms of airway obstruction and rhinnorrhea if taken in higher doses [56, 57].

Celecoxib is approximately 375-fold more potent in inhibiting COX-2 relative to its inhibition of COX-1 [58]. Thus, at the doses employed clinically, celecoxib does not inhibit COX-1, which is why it is considered selective for COX-2. It became of obvious interest to challenge patients of the AIA phenotype with this new type of compound. With the dual purposes of 1) discovering mechanistic information and understanding one fundamental step in the intolerance reaction and 2) identifying a desirable alternative treatment for alleviation of prostaglandin-mediated pain in patients with AIA, the study described in **Paper I** was initiated.

1.6 THE INVOLVEMENT OF LEUKOTRIENES IN ASTHMA

Discovered in 1979 [59, 60] the leukotrienes (LT), are one class of the substances derived from metabolism of the arachidonic acid present in the cell membranes of bone marrow derived cells. Their designation as leukotrienes reflects the fact that they are produced by leukocytes and contain three double bonds, in a conjugated triene structure. The biosynthesis of leukotrienes (Figure 2) has been described in detail by Samuelsson and co-workers [61] and will be described only briefly here.

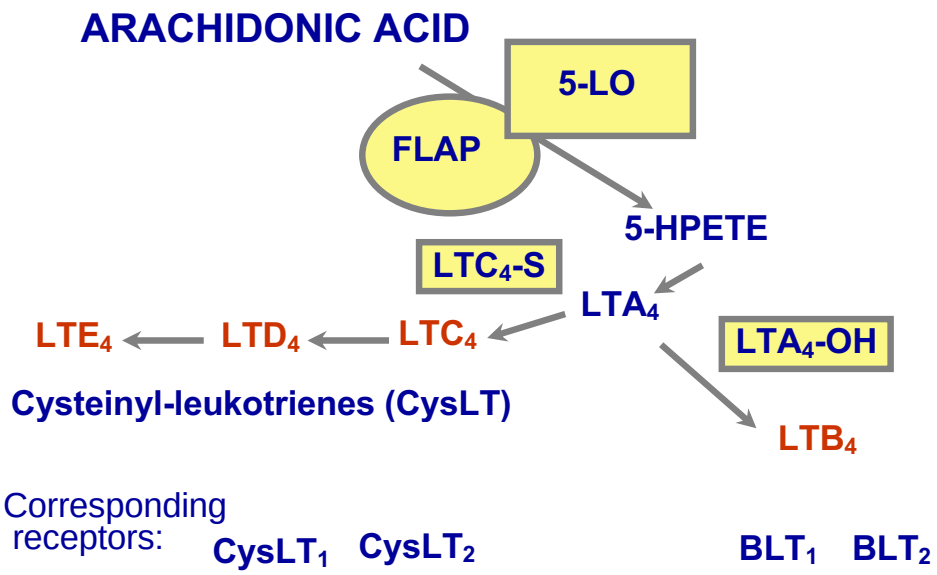


Figure 2. Schematic representation of the biosynthesis of leukotrienes.
5-LO = 5-lipoxygenase; FLAP = 5-lipoxygenase-activating protein;
5-HPETE = 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid;
LT = leukotriene; LTC_4-S = leukotriene C_4-synthase;
LTA_4-OH = leukotriene A_4-hydrolase.

Through the catalytic activity of the key enzyme 5-lipoxygenase (5-LO) in combination with its activating protein (FLAP), arachidonic acid is first transformed to LTA_4, an unstable intermediate and that is converted by LTA_4-hydrolase or LTC_4-synthase into LTB_4 or LTC_4, respectively. In mast cells and eosinophils, LTC_4 is metabolized further by γ-glutamyl transferase, to produce LTD_4, which is finally transformed into LTE_4 by cystein-glycine dipeptidase.

Neutrophils and monocytes produce the largest amount of LTB_4 [62, 63], which upon binding to its receptors BLT_1 and BLT_2 acts as a potent chemokine for neutrophils [64]. However at present the contribution of this mediator to asthmatic inflammation is uncertain. Bronchial challenge of either patients with asthma or in healthy individuals [65] with LTB_4 did not alter pulmonary functions or bronchial responsiveness to histamine.

LTC_4, LTD_4 and LTE_4, now referred to as cysteinyl leukotrienes (CysLTs), were previously, called the slow reacting substances of anaphylaxis (SRS-A) on the basis of their physiological effects. The CysLTs are produced primarily by mast cells and eosinophils. Via trans-cellular metabolism, other types of cells may contribute to their production [66], but only cells derived from the bone marrow contain the key enzyme 5-LO [67].

Once released from their producing cell the CysLTs bind to specific $CysLT_1$ and Cys-LT_2 receptors. In humans $CysLT_1$ is expressed in bronchi [68] and $CysLT_2$ in pulmonary veins [69]. In individuals with asthma, most of the effects of CysLTs appears to be mediated by the $CysLT_1$ receptor. For example, bronchoconstriction and the ventilation-perfusion mismatch caused by inhalation of LTD_4 in patients with asthma can be blocked completely by montelukast [70]. Depending on the type of cell on which it is located the $CysLT_1$ receptor mediates various effects of significance in connection with asthma.

In the first place, CysLTs act as potent bronchoconstrictors, both in patients with asthma [71] and in healthy individuals [72]. Secondly, These substances activate eosinophils and recruits these cells to the airways [73]. Once within the mucosa, eosinophils can release more CysLTs, and in a paracrine manner recruit additional eosinophils, but they can also degrade proteolytic enzymes such as the Major Basic Protein (MBP) and Eosinophilic Cationic Protein (ECP), which are responsible at least in part for the destruction of the respiratory epithelium and bronchial mucosa in individuals with asthma [74]. Another important mechanism by which the CysLTs mediate inflammation involves their ability to cause the endothelium to contract [75], thereby giving rice to plasma leakage and edema [76]. Although not yet extensively studied CysLTs can also stimulate mucus secretion [77-79] and may aggravate asthmatic symptoms by inhibiting the mucus ciliary transport [80].

1.7 ANTILEUKOTRIENES

For the past ten years, i.e., approximately 25 years after the discovery of leukotrienes, antileukotrienes, drugs which inhibit the formation or actions of leukotrienes, have been used in developed countries as a novel treatment for asthma [81, 82]. The effects of leukotrienes can be blocked in two principle ways: First certain drugs attenuate the biosynthesis of leukotrienes by inhibiting the enzymes 5-LO or FLAP, thereby inhibiting production of both LTB_4 and the CysLTs. Secondly, other drugs that bind to the $CysLT_1$ receptor can competitively inhibit the binding of CysLTs.

The three antagonists of CysLT receptors that are currently available (i.e., montelukast, zafirlukast and pranlukast) are all specific for the $CysLT_1$ receptor. These antagonists were developed primarily on the basis of functional studies with smooth muscle preparations *in vitro*, in which context the guinea-pig trachea and human bronchus are especially good predictors of the therapeutic effects of anti-leukotrienes in human subjects. Other *in vitro* investigations [83] involving LTC_4 and LTD_4 together with selective and unselective antagonists of CysLTs have indicated the existence of two major subgroups of receptors. Antagonists of the $CysLT_1$ receptor produce clinical effects similar to those obtained by inhibition of 5-LO with zileuton [81] but these antagonists have the advantage that they can be taken once or twice daily with excellent tolerance.

1.7.1 Mechanistic studies in humans

One of the most extensively studied and well documented effects of antileukotrienes is the protection that these drugs provide against induction of bronchoconstriction by various factors in patients with different phenotypes of asthma. In the case of allergic asthma, challenge of pulmonary tissue with the appropriate antigen elicits bronchial contraction that is correlated with the release of cysteinyl-leukotrienes [84] and can be attenuated by antileukotrienes [20, 85-87]. Antileukotrienes have also been demonstrated to prevent bronchoconstriction precipitated by exercise [88-90], as well as by other factors that mimic exercise in this respect such as cold air [91], eucapnic hyperpnea [92] and mannitol [93]. In the case

of AIA where mast cell degranulation plays an important role [94] antileukotrienes attenuate the intolerance reaction produced by exposure to aspirin [95, 96].

1.7.2 Clinical trials

Antileukotrienes are superior compared to placebo in terms of improving pulmonary function and maintaining control in patients with many different phenotypes of asthma [97-103]. Antagonists of the $CysLT_1$ receptor also improve the stability of asthmatic patients that remain unstable even when administered moderate-to-high doses of inhaled corticosteroids (ICS) [104, 105]. Moreover, these antagonists also prevent exacerbation of asthma and help maintain pulmonary functions in connection with reduction of medium-to-high doses of ICS [106, 107].

Investigations designed to compare antagonists of the $CysLT_1$-receptor and inhalation of corticosteroids as the first-line treatment for asthma have been evaluated by the Cochrane collaboration [108]. In most trials, the benefit effects of daily administration of ICS (400 μg of beclomethasone or the equivalent) were superior to those of anti-leukotrienes (10 mg of montelukast or the equivalent). In addition, the subjects receiving montelukast alone were more likely to suffer exacerbation that required systemic administration of steroids. Other significant advantages of ICS involved more pronounced amelioration of symptoms, fewer nocturnal awakenings, less need for rescue medication, a larger number of symptom-free days and better asthma related quality of life. Asthmatic subjects not controlled with inhaled corticosteroids alone benefited from also using either a Long-Acting β_2 Agonist (LABA) [109] or an antagonist of the $CysLT_1$ receptor antagonist [104], both of which appeared to prevent the exacerbation of asthma equally well [110].

1.7.3 Responders vs. non-responders to antileukotriene therapy

Despite all of the evidence concerning the beneficial effects of antileukotrienes, adequate application of these drugs is problematic due to a fundamental lack of understanding of which patients will benefit most from such treatment. From the large trials involving treatment with different antileukotriene drugs referred to above [99, 101, 111] as well as ten years of clinical experience, it is evident that responsiveness to antileukotrienes varies, which has led certain investigators to distinguish between responders and non-responders. However the clinical response among individuals is probably normally, rather than bimodally distributed. The success or failure of antileukotriene treatment may be more related to the extent to which leukotrienes are important mediators of the phenotype of an individual with asthma.

An indicator that aids the physician in predicting whether a particular subject with asthma will respond well to antileukotrienes is needed. Patients with aspirin-intolerant asthma [103, 112] and exercise-induced asthma [113] are generally considered to respond particularly well to such treatment. However, since asthmatic patients with similar clinical symptoms may have developed airway obstruction via different underlying mechanism, asthma of different phenotypes or involving trigger [11] may be leukotriene dependent.

One reason why individuals with AIA appear to respond well to antileukotrienes could be related to the fact that their basal rate of CysLT biosynthesis is higher than to aspirin-tolerant asthmatics [32, 33]. Moreover it has been claimed that the clinical response to pranlukast in patient with stable extrinsic and intrinsic asthma is correlated to the extent of release of CysLTs by blood leukocytes stimulated *ex vivo* [114]. However only 3 of the 16 subjects that responded and 4 of the 15 non-responders in that particular investigation were aspirin-intolerant (information provided by the author via e-mail). In addition in the case of the group with AIA involved in the clinical trial of montelukast, there was no correlation between the level of LTE_4 in urine and response to treatment [112]. In order to make sense of these confusing findings, attempts have been made to correlate the response to treatment with polymorphisms in

the genes coding for 5-lipoxygenase [115] or leukotriene C_4-synthase (Figure2) [116, 117] but the results obtained so far have been contradictory.

In the study performed by Drazen and coworkers [115], 114 subjects with asthma not controlled on β_2-agonists alone were treated with ABT-761, a selective inhibitor of 5-LO, (300 mg daily for 12 weeks). The individuals with a wild-type (64) or heterozygous (40) genotype at the 5-LO locus (at chromosome 10q11.2) demonstrated improvements of 18.8 and 23.3%, respectively in their FEV_1 values. In contrast, those patients with a mutant genotype (10) at the 5-LO locus did not benefit from the treatment, as reflected in an average change of -1.2% in their FEV_1. However, only approximately 5% of individuals with asthma carry this mutant genotype, which therefore cannot adequately account for the relatively large subgroup of asthmatics who respond poorly to antileukotrienes.

In the investigation by Sampson and colleagues [117], 23 subjects with severe asthma were treated with Zafirlukast (20 mg twice daily for 2 weeks), in addition to inhaled corticosteroids and β_2-agonists. Thirteen asthmatic patients with the variant LTC_4 synthase genotype increased their average FEV_1 and FVC values by 9 and 15%, respectively; whereas the corresponding values for 10 patients with the wild-type genotype decreased by an average of 12 and 18% respectively. In this same article eosinophils isolated from healthy individuals with the variant LTC_4 genotype and stimulated *ex vivo* by exposure to Indometacin were reported to produce significantly larger quantities of LTC_4 than eosinophils from individuals with the wild-type genotype. Unfortunately the corresponding information concerning the asthmatic subjects was not provided.

Finally, a retrospective meta-analysis [116] involving subjects with mild-to-moderate asthma has been performed in attempt to determine whether variations in the attenuation of bronchial responsiveness to adenosine 5'-monophosphate (AMP) and methacholine (MCh) by antagonists of the leukotriene receptors are related to genetic polymorphism with respect to leukotriene C_4 synthase. In all of the studies evaluated, the antagonist was superior to a placebo in attenuating the response to either AMP or MCh, but this response was independent of polymorphism in the LTC_4 synthase gene.

All of these attempts to identify responders to antileukotriene treatment have focused on the individual's ability to synthesize leukotrienes, either directly or indirectly. However it remains unclear whether the beneficial effect of antileukotrienes is correlated to the extent of leukotriene production. We reasoned that the responsiveness of the airways to leukotrienes must also be taken into consideration, and it is not known whether bronchial responsiveness to LTD_4 is correlated to the level of general indicators of leukotriene production. This question was addressed in **Paper II.**

1.8 PREVENTING EXACERBATION OF ALLERGIC ASTHMA DUE TO ALLERGEN

Subjects with asthma develop a number of different symptoms that they must cope with, including coughing, dyspnoea, variation in pulmonary function, bronchial hyperresponsiveness, ahma exacerbations and the asthma related reduction in quality of life. Exacerbation of asthma results from inadequate control of the associated inflammation in the airways, which may be symptomatic or asymptomatic. The time-course with which well-controlled asymptomatic inflammation becomes uncontrolled and symptomatic in patients with asthma has been difficult to monitor. One promising model system in this context is repeated challenges with low dose of allergen, a situation that mimics natural exposure to air-borne allergens [118].

Measurement of exhaled nitric oxide (F_{ENO}) is a novel, noninvasive and promising approach to assess airway inflammation. NO produced predominately by inducible NO synthases in the epithelial cells of the bronchial wall [119], is the major source of the elevated values of F_{ENO} demonstrated by individuals with asthma [120]. In connection with an exhalation, NO diffuses from the bronchial wall into the airways, thereby increasing concentration of this mediator in the exhaled air. At a standardized flow rate of 50 mL/s healthy adults usually exhibit F_{ENO} values between 10 and 25 ppb [121]. The elevated F_{ENO} values associated with asthma can be reduced by glucocorticosteroids [122] and antileukotrienes [123].

Another surrogate marker of inflammation is the urinary level of LTE_4, (which reflects the whole-body production of CysLTs) in combination with urinary levels of the prostaglandin D_2 metabolite, 9α11ß-prostaglandin F_2 (PGF_2) (a specific indicator of mast cell activation) [124]. The early (EAR) and late asthmatic responses (LAR)

elicited by conventional challenge with a high-dose allergen are associated with an increase in the urinary level of 9α11ß-PGF_2 [125]. While activation of eosinophils is known to occur in connection with a low-dose challenge [126-128], possible activation of mast cells under these conditions has not been explored as extensively.

The aim of the investigation described in **Paper III** was to assess the usefulness of repeated measurements of F_{ENO} for early discover of deterioration of asthma provoked by allergen prior to symptomatic exacerbation. The primary goal was to establish whether there is any relationship between F_{ENO} values and alteration in airway responsiveness associated with inflammation caused by repeated exposure to low-dose allergen. We had an additional hypothesis that priming of mast cells occurs in connection with the development of airway hyperresponsiveness and performed measurements of urinary excretion of 9α11ß-PGF_2 in order to test this hypothesis.

In another attempt to determine whether mast cells are activated, challenges with adenosine 5'-monophosphate (AMP) were also carried out. AMP act indirectly as a bronchoconstrictor primarily by stimulating mast cells to release of bronchoconstrictive mediators [129-131]. Apparently degranulation in response to stimulation of adenosine A_{2B} receptors on the surface of human pulmonary mast cells is the primary trigger of adenosine-induced limitation in airflow [132, 133], although the relative importance of the various subtypes of adenosine receptors in patients with asthma remains to be elucidated in detail. Such responsiveness to AMP has been proposed to be more closely associated with inflammation of airways than are the response to bronchoconstrictors that act directly such as MCh [134].

1.9 INTERACTIONS BETWEEN CORTICOSTEROIDS AND LEUKOTRIENES

Although the potent anti-inflammatory effect of glucocorticoids was recognised immediately after their discovery and has been appreciated clinically ever since [135], the mechanism underlying this effect has not yet been elucidated fully. One potential mode for this action involves alterations in arachidonic acid metabolism. And indeed, eicosanoid biosynthesis by cells *in vitro* is reduced by exposure to corticosteroids [136-138]. However, in several *in vivo* studies on healthy individuals [139, 140] and patients

with asthma [141-143] inhalation or oral administration of corticosteroids did not reduce the production of eicosanoids and, in particular, of CysLT (as reflected in the urinary levels of LTE_4).

Corticosteroids also attenuate bronchial hyperresponsiveness to histamine and MCh [144]. In the case of allergic asthma, this reduction can be explained at least in part, by decreases in the number of cells recruited, the expression and release of adhesion molecules, airway permeability and the production of cytokines involved in airway immunity or remodeling [145]. Furthermore, potent antagonists of the $CysLT_1$ receptor prevent the obstruction of airways which constitutes the major component of the EAR and LAR induced by allergens [20, 146]. Moreover, administration of the potent glucocorticosteroid fluticasone propionate (FP) for two weeks also results in pronounced attenuation of the EAR and LAR, without influencing leukotriene production, (as assessed by measurement of urinary LTE_4) [142] a finding in line with the studies involving treatment with glucocorticoids discussed above [139-141].

It is intriguing that ICSs exert no effect on allergen-induced formation of cysteinyl-leukotrienes, despite the fact that these substances are the major mediators of bronchoconstriction induced by allergen [20, 146] The hypothesis tested in **Paper IV** was that treatment with an ICS (FP) would diminish bronchial responsiveness to inhaled LTD_4, which might explain the inhibitory effect of these drugs on the EAR and LAR in individuals with asthma. In fact, the overall influence of antileukotrienes and ICSs on these responses is similar [147].

1.10 BRONCHIAL CHALLENGE TESTS

In connection with this thesis work bronchial challenge tests were the most important method employed. In the clinic bronchial hyperresponsiveness (BHR) to histamine or MCh are often determined in order to diagnose (exclude) asthma or to monitor the severity of the disease. Although BHR is not totally specific for asthma, such hyperresponsiveness constitutes one of the major pathophysiological features of this condition [8].

Bronchoconstrictors may limit airflow either directly or indirectly. Direct bronchoconstrictors usually act on the smooth muscle cells via specific receptors. Indirect bronchoconstrictive stimuli, on the other hand, act on intermediary cells such as inflammatory cells, bronchial epithelial cells and/or neuronal cells, thereby stimulating the release of pro-inflammatory mediators and/or neurotransmitters, that in turn interact with smooth muscle cells to produce limitation of airflow [148]. In my investigations I have challenged subjects with three different bronchoconstrictors that act directly (i.e., histamine, methacholine and LTD_4) as well as two that act indirectly (AMP and allergen).

The bronchial challenge is the most conclusive approach for examining bronchial constriction in individuals suffering from asthma of different phenotypes. Use of a dosimeter-controlled jet nebulizer allows subjects to inhale reasonably well-defined doses of various compounds and several minutes later their pulmonary function can be evaluated with a spirometer and their responsiveness to these different compounds calculated. This procedure can be employed to evaluate the effect of a certain treatment as in **Paper IV** or of a certain kind of provocation as in **Paper III**. Performance of bronchial challenges is like working in the laboratory, with all the associated advantages such as accuracy and at the same time examining the disease in lungs of a breathing patient.

2 AIMS

The general objective of this project was to elucidate molecular and cellular mechanisms underlying asthma of different phenotypes, with a focus on leukotrienes and other eicosanoids and the ultimate goal of improving the monitoring and management of patients with different types of asthma. More specifically, the studies documented here were designed to answer the following questions:

Can individuals with a typical history of AIA use celecoxib, a novel NSAID selective for COX-2 without experiencing serious side-effects? Is the intolerance reaction associated with this type of asthma caused by inhibition of COX-1?

Can individual's capacity to synthesize CysLTs be helpful in predicting his/her bronchial responsiveness to LTD_4? Is there a significant correlation in subjects with mild chronic bronchial asthma between responsiveness to inhaled LTD_4 and two general markers of leukotriene production, i.e., formation of LTB_4 by whole blood upon stimulation *ex vivo* and the urinary level of LTE_4?

Can repeated measurement of FE_{NO} allow early detection of disease exacerbation in individuals with allergic asthma? Are the levels of NO in the air exhaled by allergic asthmatics elevated in response to repeated challenges with low doses of allergen prior to the appearance of symptoms?

Is there a rationale for the benefit from combined treatment with ICS and antileukotrienes in patients with allergic asthma? Does inhalation of corticosteroids attenuate bronchial responsiveness to inhaled leukotriene D_4 in such patients?

3 METHODOLOGICAL CONSIDERATIONS

3.1 SUBJECTS

In the study described in **Paper I** 33 subjects (21 females and 12 males) diagnosed as suffering from AIA were recruited from university hospitals in Stockholm (n=12), Krakow (n=11) and Nashville (n=10). Their asthma was in a stable state with no exacerbation having occurred during the previous three months. A criterion for inclusion was that the subjects had responded to a challenge by inhaled or orally administered aspirin within the nine-month period prior to the initiation of the investigation.

The study documented in **paper II** involved 20 non-smoking subjects with intermittent-to-mild asthma recruited from a general practitioner's clinic in Stockholm. Ten of these subjects were taking ICS (budesonide at a median daily dose of 400 μg) together with short-acting ß2 agonist as a rescue drug and ten were using only short acting ß2 agonist. All were documented as exhibiting airway hyperresponsiveness (with a MCh PD_{20} FEV_1 of ≤ 45,282 nmol) and 10 non-smoking healthy individuals recruited through advertisements were included as controls. Of the 20 asthmatic subjects, 16 (half of whom were using ICS) demonstrated a positive reaction to a skin prick test with at least one air-borne allergen.

The study in **paper III** involved 8 non-smoking subjects with stable, mild allergic asthma, a positive response to bronchial challenge with allergen, treatment only with a short-acting β_2-agonist as a rescue drug and established bronchial hyperresponsiveness (with a histamine PD_{20} FEV_1 of ≤ 2090 μg) as well as 8 age and sex-matched healthy controls. The subjects with asthma were recruited from the allergy clinic at our department and the healthy volunteers through advertisements.

Finally for the study shown in **paper IV** 13 non-smoking subjects with stable, mild allergic asthma being treated only with a short-acting β_2-agonist as a rescue drug, and demonstrating established bronchial hyperresponsiveness (with a MCh PD_{20} FEV_1 of ≤ 5579 nmol) were recruited from the allergy clinic at our department.

The baseline characteristics of all of the subjects involved in this project are shown in Table 1.

Table 1. Baseline characteristics of our subjects

Paper	Subjects Number and disease	Age in years, mean (range)	Gender F/M	FEV_1 in L/min mean (range)	FEV_1 as % predicted mean (range)
I	33 AIA	43 (20-70)	21/12	2.7 (1.5-4.4)	85 (71-100)
II	10 Healthy	35 (23-54)	5/5	4.1 (3.3-5.1)	110 (92-132)
	10* ICS	31 (23-42)	6/4	3.3 (2.2-5.6)	91 (72-124)
	10* no ICS	32 (21-44)	5/5	3.2 (2.6-4.4)	90 (74-104)
III	8 *no ICS	32 25-41)	7/1	3.1 (2.7-4.7)	94 (84-109)
	8 Healthy	33 (24-43)	7/1	3.6 (3.2-4.0)	104 (88-127)
IV	13* no ICS	31 19-45	10/3	3.9 3.1-5.7	101 (88-118)

* Intermittent-to-mild asthma

3.2 ETHICAL CONSIDERATIONS

Oral and written informed consent were obtained from all subjects and pre-approval from the local ethics committee at the Karolinska University Hospital in Solna was given for each study (Nrs. 99-243, 00-267, 98-248 and 02-207).

3.3 DESIGN OF THE STUDIES

In the case of **Paper I** the investigation was conducted on three different days separated by 7-day intervals. The subjects first underwent a double-blind, randomized two-period cross-over oral challenge with increasing doses of celecoxib and the placebo (5, 10, 30, and 100 mg in suspension). Thereafter, on the third day an open-label challenge involving 200 mg of celecoxib in oral suspension followed 2 hour later with administration of 200 mg of celecoxib administered as an oral capsule.

Paper II describes a cross-sectional study that required the subjects to visit the Department of respiratory Disease on four separate occasions. In connection with the screening visit, the subject's medical history was taken and a skin prick test and challenge with MCh was performed. During the second visit F_{ENO} was measured and dynamic spirometry and chest X-ray (in preparation for bronchoscopy) were performed. In the case of the asthmatic subjects, asthma specific quality of life (QoL) was also evaluated. During the third visit the subjects were examined by bronchoscopy and biopsies and bronchoalveolar lavage (BAL) fluid were obtained (the results of which are not discussed here). Finally in connection with the fourth visit, a bronchial challenge with LTD_4 was conducted. In connection with all visits, blood samples for analysis of *ex vivo* stimulated formation of LTB_4 and urinary samples for measurements of LTE_4 were taken upon arrival. In addition following bronchoscopy (visit 3) and bronchial challenge with LTD_4 (visit 4) new blood and urine samples were gathered.

Paper III describes a two-period, single-blind study of cross-over design. Subjects allergic to pollen or animal dander and exhibiting no current symptoms of asthma were exposed by inhalation to low doses of the allergen PD_5 or diluent alone (placebo) once daily for 7 consecutive weekdays. Bronchial responsiveness to histamine and AMP were assessed prior to and after these challenges on two consecutive days. Urinary levels of metabolites of mediators were sampled on four days and of F_{ENO} were measured daily during the entire period. The control group consisting of eight healthy individuals was subjected to challenge with diluent only, but otherwise the same protocol was used in this case.

Paper IV reports on a double-blind, randomised, placebo-controlled study with a cross-over design. Here the subjects received either fluticasone propionate (500 μg twice daily during two periods of 14 days each) or an appropriate placebo, delivered via a Diskus® powder inhaler. A wash out period of at least 21 days was allowed to elapse between the two periods of treatment. Bronchial provocation with MCh and LTD_4 were performed on consecutive days prior the initiation of each treatment period, as well as on the 13^{th} and 14^{th} days of treatment respectively. In connection with each visit measurement of F_{ENO}, sampling of urine for analysis of baseline levels of U-LTE_4, and dynamic spirometry were performed in that order.

3.4 CHALLENGES

3.4.1 Oral challenge with celecoxib

Celecoxib and its placebo formulation were provided by Pharmacia Corp (Chicago, USA) in bottles containing 5, 10, 30, or 100 mg in a form suitable for preparation of a fine suspension for oral administration. The powders were suspended in a solution of Tween 80 in ethanol by sonication, diluted with apple juice, and then administered to the patients under direct supervision. For the open-label challenge, celecoxib was supplied in bottles containing 200 mg in a suspension suitable for oral administration as well as 200-mg capsules.

The challenges were always performed in the morning. Pulmonary function was assessed as the FEV_1 value, determined in duplicate with a spirometer, and the baseline value was required to be ≥70% of the predicted normal value in order for the patients to receive the medication, which was administered at two-hour intervals. During the double-blind phase, escalating doses (5, 10, 30, and 100 mg in solutions) of celecoxib or placebo was administered orally. In the case of the-open label challenge suspension containing 200 mg and then a 200-mg capsule were ingested. These challenges were performed under close observation with resuscitative equipment readily available. The spirometric data and vital signs were recorded 0.5, 1 and 2 hours following administration of each dose. Nasal symptoms were scored (0-3) and signs of conjunctivitis, dermal flush, gastrointestinal symptoms and urticaria/angioedema were assessed prior to and 1 and 2 hours after administration of each dose.

3.4.2 Challenges involving Inhalation

All challenges involving inhalation were performed employing a dosimeter-controlled jet nebulizer (Spira Elektro 2; Intramedic, Bålsta, Sweden). In **Paper II** and **III** pulmonary function was assessed on the basis of the FEV_1 value determined by spirometry (Vitalograph MDI Compact; Förbandsmaterial, Stockholm, Sweden) while in **Paper IV** a different spirometer was utilized (MasterScope, Erich Jaeger GmbH, Hoechberg, Germany). As stated above the best baseline value obtained in three separate recordings had to be ≥ 70% of the predicted value. The challenges always began with inhalation of diluent. Provided that the FEV_1 value did not decrease by

more than 10%, increasing doses of the test substance were administered until this value was reduced by at least 20% from the highest baseline value obtained following inhalation of diluent.

3.4.2.1 Challenge by inhalation of methacholine

In connection with the challenge involving inhalation of methacholine chloride the dose being administered was doubled every third minute and single spirometric measurements were performed before these increases. 2, 4 and 8 breaths of preparations containing three different concentrations (i.e., 6.24, 50 and 400 mM, prepared at Norrlands University Hospital Pharmacy) were taken to achieve the desired dose (from 89 to 45,282 nmol).

Table 2 Protocol for dosing methacholine (M Wt = 160.24 g)

Methacholine concentration (mg/mL)	No. of breaths	Dose (µg)	Cumulative dose (µg)
1	2	14.2	14.2
1	4	28.4	42.6
1	8	56.8	99.4
8	2	114	213
8	4	227	440
8	8	454	894
64	2	909	1803
64	4	1818	3621
64	8	3635	7256

3.4.2.2 Challenge by inhalation of histamine

Bronchial responsiveness to histamine (histamine diphosphate, prepared by the Karolinska Hospital Pharmacy) was also assessed by increasing the dose every third minute and performing single spirometric measurements between every consecutive administrations. Two concentrations (1.6 $mg \cdot mL^{-1}$ and 16 $mg \cdot mL^{-1}$) and a variable number of breaths were used to achieve the desired doses (from 11 to 2090 µg) of histamine.

Table 3 Protocol for dosing histamine (M Wt = 325.2 g)

Histamine concentration (mg/ml)	No. of breaths	Dose (μg)	Cumulative dose (μg)
1.6	1	11	11
1.6	2	22	33
1.6	7	77	110
16	2	220	330
16	7	770	1100
16	9	990	2090

3.4.2.3 Challenge by inhalation of leukotriene D_4

In order to obtain approximately half-logaritmic increments in the cumulative dose (i.e., 3, 10, 34 pmol, etc) every 10 minutes (the dose range being 3-335,780 pmol), six solutions of LTD_4 Good Manufacturing Practice Grade (Cascade Biochemicals, Reading, UK) with concentrations differing by ten-fold (i.e., from 4.2×10^{-8} to 4.2×10^{-3} M) and a varying number of inhalations (1-7) of a given solution were employed. Spirometry was performed at 5 and at 10 minutes following administration of each dose and the PD_{20} value calculated on the basis of the maximal reduction observed.

Table 4 Protocol for dosing LTD_4

LTD_4 concentration (μM)	No. of breaths	Dose pmol	Cumulative dose (pmol)
0.42	1	3.4	3
0.42	2	6.7	10
0.42	7	23.4	34
4.2	2	67.5	100
4.2	7	235	336
42	2	672	1008
42	7	2350	3360
420	2	6720	10,080
420	7	23,500	33,580
4200	2	67,200	107,800
4200	7	235,000	335,780

3.4.2.4 Challenge by inhalation of adenosine (AMP)

The challenges with adenosine 5'-monophosphate (AMP) involved doubling the concentration of the solution inhaled every 5 minutes (from 1.56–400 $mg \cdot mL^{-1}$; Sigma Chemical Co., St Louis, MO, USA). Spirometry was performed 1 and 3 minutes after administration of each dose and the PC_{20} value calculated on the basis of the maximal reduction observed.

Table 5 Protocol for dosing AMP

AMP concentration (mg/ml)	No. of breaths
1.56	5
3.125	5
6.25	5
12.5	5
25.0	5
50.0	5
100.0	5
200.0	5
400.0	5
400.0	10
400.0	20

3.4.2.5 Challenge by inhalation of a low dose (PD_5) of allergen

First, a screening challenge was performed on each individual employing the allergen considered to be of most clinical significance for him or her. From this screening challenge the dose of allergen that resulted in an early fall in the FEV_1 value of approximately 5% (PD_5) was determined for each individual. The PD_5 dose of the allergen was then administered once daily for 7 successive weekdays, with a break over the weekend. The asthmatic subjects inhaled the same number of breaths of diluent as of allergen, whereas the control individuals inhaled three breaths of the diluent. Spirometric recordings were made prior to and 15 minutes after inhalation.

3.5 ENZYME IMMUNOASSAY OF EICOSANOIDS

3.5.1 Urinary levels of LTE_4 and 9α11β-PGF_2

In order to monitor endogenous production of cysteinylleukotrienes, urinary levels of the ultimate metabolite (U-LTE_4) were analysed in **Papers I-IV**. In addition, urinary 9α11β-PGF_2, a metabolite of PGD_2, was monitored in **Paper III** as a marker of mast cell activation *in vivo* [94]. (For details concerning sampling conditions, see the individual articles).

Using a validated method, previously described [94, 149], enzyme immunoassay was performed with rabbit polyclonal antisera and acetyl-cholinesterase-linked LTE_4 or 9α11β-PGF_2 tracer. 96-well microtitre plates were coated with a solution of mouse monoclonal anti-rabbit IgG in 0.05 M potassium phosphate buffer (200 µl per well). After incubation for 18 hours at RT, 100 µl of saturation buffer (200mg sodium azide and 2 g of BSA/l of EIA buffer (100mg sodium azide, 23.4g sodium chloride, 370 mg tetrasodium EDTA and 1g BSA/l of 0.1 M potassium phosphate buffer pH 7.4)) was added to each well. The plates were covered with plastic film and were ready to use after 18 hours at +4°C. Solutions of LTE_4 or 9α11β-PGF_2 standard, LTE_4 or 9α11β-PGF_2 acetylcholinesterase tracer and LTE_4 or 9α11β-PGF_2 antiserum were prepared in EIA buffer.

Before use, the saturation buffer was removed from the precoated plates. To each well was then added 50 µl each of antiserum (except for total activity, non-specific binding and blank wells), LTE_4 or 9α11β-PGF_2 acetylcholinesterase tracer (except for total activity and blank wells), standards or unknowns in several (at least three) dilutions in duplicates. Non-specific binding and maximum binding wells were supplied with up to 150 µl of EIA buffer. The plates were then incubated overnight in darkness at RT. After washing the enzyme substrate (Ellman's reagent, 200 µl per well) was added to the LTE_4 or 9α11β-PGF_2 antibody-bound ligand attached to the monoclonal antibody surface. Tracer was added to the total activity wells and absorbance was measured at 414 nm. With a set of standards ranging from 0.4 to 50 pg per well and % B/B_0 from 20 to 80%, the detection limit was around 8 pg/ml in

unknown samples. Rabbit polyclonal antisera against LTE_4 or 9α11β-PGF_2 were supplied by Cayman Chemical Company, Ann Arbor, MI, USA. Automization of the EIA procedure was performed with a pipetting robot, MultiProbe 104 (Canberra Packard, Merideen, CT, USA). Creatinine was measured in all urine samples with a colorimetric assay by using an alkaline picrate method previously described [149], and the results were expressed as nanograms of LTE_4 or 9α11β-PGF_2 per millimole of creatinine.

3.5.2 *Ex vivo* formation of LTB_4 by whole blood

Blood samples were obtained by venipuncture into heparinized vaccutainer tubes in **Paper II**. *Ex vivo* stimulation of freshly drawn peripheral whole blood, was performed with a modified version of previously described protocols [150, 151]. The blood was kept in room temperature for 1 hour prior to incubation in order to minimize fluctuations in values due to decreased capacity for leukotriene formation within the first hour of blood collection [151]. The calcium ionophore ionomycin was dissolved in 95% ethanol to a stock concentration of 10 mM. The stock solution and vehicle (95% ethanol) were diluted 10 times with autologous plasma. Aliquots of blood (1 ml) were preincubated at 37ºC for 2 min, followed by addition of vehicle or ionomycin in 50 µl of autologous plasma. The final concentration of ionomycin was 50 µM. Incubations were continued for 15 min at 37ºC and interrupted on ice. Plasma was obtained by centrifugation at 714 x *g* for 5 min at 4ºC, and stored at -70ºC until assayed for LTB_4 by enzyme immunoassay as described above (Cayman Chemical, Ann Arbor, MI, USA).

3.6 MEASUREMENT OF NITRIC OXIDE IN EXHALED AIR

The values for F_{ENO} reported in **Papers II, III** and **IV** were obtained online according to the recommendations of the American Thoracic Society [152]. For this purpose the subjects were asked to rinse their mouths with water prior to measurement in attempt to minimize interference from food. In the case of **Paper III**, an Aerocrine prototype NO system (Aerocrine AB, Stockholm, Sweden), including a CLD 77 AM

chemiluminescence analyser (Eco Physics AG, Durnten, Switzerland; sensitivity 0.1 ppb NO; rise time 0–90% <0.1 s; sample flow rate 110 mL·min^{-1}; lag time from mouthpiece 0.7 s) was used for online measurements of NO and a pneumotachygraph for monitoring flow and pressure. The rate of exhalation through a linear flow resistor (Hans Rudolph Inc., Kansas City, KS, USA) at a pressure of 5 cm H_2O was maintained constant at 250 mL·s^{-1} through visual feedback. A two-point calibration was performed prior to each session utilization mass-flow controlled dilutions of certified calibration gas (stock concentration 2 ppm NO in N_2; AGA AB, Älvsjö, Sweden). In the studies reported in **Papers II** and **IV,** a NIOX instrument (Aerocrine AB, Stockholm, Sweden) was used for online measurements of NO.

3.7 STATISTICAL ANALYSES

Paper I

Statistical analysis of the proportion of subjects that reacted to celecoxib proved to be unnecessary, for obvious reasons. Student's paired T-test was used to analyze differences in the mean U-LTE_4 values obtained prior to and following a positive screening challenge with aspirin.

Paper II

The provocative doses that led to reductions of 10, 15 and 20% in the FEV_1 value (i.e., PD_{10}, PD_{15} and PD_{20}) were derived from the log of the cumulative dose-versus-response curves by linear interpolation. Calculations of geometric mean values were performed on logarithmically transformed raw data. The urinary levels of LTE_4 and *ex vivo* concentrations of LTB_4 are presented as median values with ranges. Relationships between different bronchial challenges were analyzed employing the Pearson product moment correlation and all other comparisons made with the Spearman rank order correlation. The Mann-Whitney rank sum test was used to compare the values for different groups and Kruskal-Wallis one-way analysis of variance on ranks to assess the variability in the baseline values for *ex vivo* LTB_4 and U-LTE_4.

Paper III

In this investigation the choice of a sample consisting of 8 subjects was based on measurements in our own laboratory that showed that a group of this size allows detection of a 50% increase in F_{ENO} with 80% power and $\alpha = 0.05$. This conclusion is in agreement with the evaluation of the reproducibility of F_{ENO} measurements and estimations of required sample size performed by Kharitonov *et al.* [153].

The geometric means of the PD_{20}, PD_{10} and PD_5 values were calculated used logarithmically transformed raw data. The measures of pulmonary function, F_{ENO} values and urinary levels of mediators were found to be normally distributed, ANOVA and student's paired t-tests were utilized to compare the different periods and groups.

Paper IV

Baseline FEV_1 values were analyzed with One-Way Repeated Measures Analysis of Variance. The PD_{20} measurements were transformed logarithmically prior calculation and are presented as geometric means. Student's paired t-test was applied for comparison of the effects of different treatments on the values for LTD_4 PD_{20}, MCh PD_{20} and U-LTE_4, and the Wilcoxon signed rank test for changes in F_{ENO}. Period and carry-over effects of the drug treatments were analyzed by the procedure of Hills and Armitage [154] and correlations examined with the Pearson Product Moment Correlation.

In all of the studies, statistical analysis was carried out with Sigma Stat 3.00, SPSS, and p-values of < 0.05 considered to be statistically significant.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Subjects with a typical history of AIA tolerate celecoxib, a novel COX-2-selective NSAID well. Thus, the intolerance reaction demonstrated by patients with AIA appears to be caused by inhibition of COX-1.

Subjects with proven sensitivity to aspirin were challenged with celecoxib, first in a blinded fashion with a placebo control and a cross-over design. No one was considered to exhibit a true intolerance reaction to celecoxib, so all of our subjects went on to participate in the open challenge with therapeutic doses of this drug (200 + 200 mg) (Figure 3). Again, the highest recommended daily dose of celecoxib was tolerated well, with no symptoms or development of changes in pulmonary function.

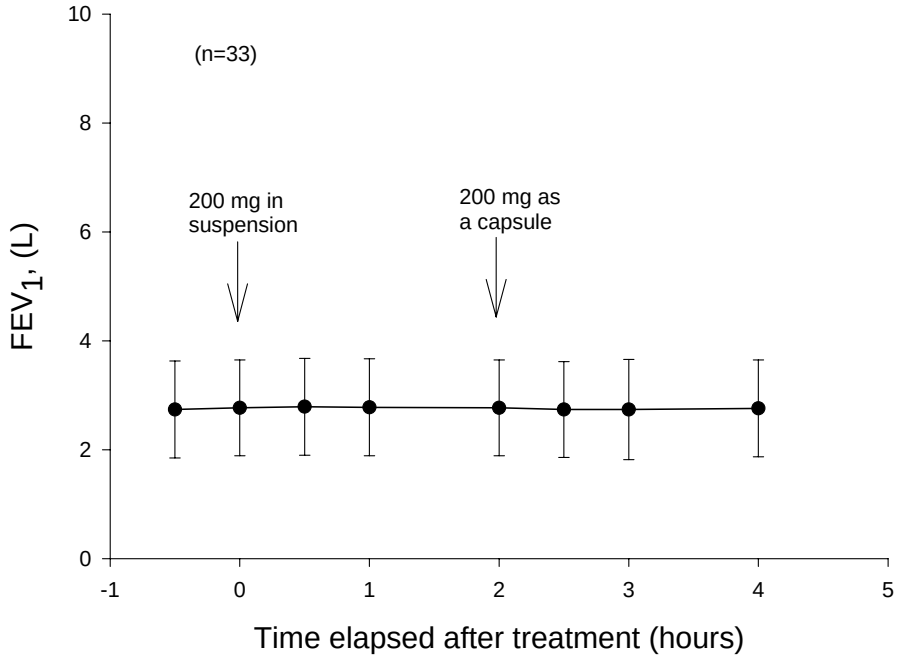


Figure 3. Pulmonary function (measured as FEV_1, in liters) during the open label challenge with 200 mg of celecoxib in suspension, followed 2 hours later by ingestion of an additional 200 mg in capsule form. The values shown are mean ± SD (n=33).

These findings also confirmed an earlier report [32] following challenge of subjects with AIA with aspirin, their urinary levels of LTE_4 are significantly elevated. In our case this increase was approximately 4-fold 124±112 to 408±376 LTE_4 ng/mmol creatinine (mean ± SD; n=19; $p<0.001$). In contrast when our subjects with AIA were challenged openly with a total dose of 400 mg celecoxib, the levels of LTE_4 in their urine was not altered i.e., 95±61 and 135±107 (ng/mmol creatinine pre- and two hours postchallenge; n=33, $p>0.05$).

Thus since celecoxib, a selective inhibitor of COX-2 is tolerated well by these patients it appears that the intolerance reaction leading to bronchoconstriction in individuals with AIA is due to inhibition of COX-1. However, why inhibition of COX-1 triggers an intolerance reaction in only a limited number of asthmatic patients, remains to be explained.

Patients with AIA sometimes require medication to alleviate the pain caused by prostaglandin-mediated inflammation, for example headache, myalgia, dysmenorrhoea or even rheumatoid arthritis or osteoarthritis. Earlier, pain in these patients has often been treated with weak NSAIDs such as paracetamol (acetaminophen), sometimes with inadequate alleviation or with morphine-like drugs or glucocorticosteriods with their undesired side-effects. The results presented in **Paper I** indicate that now for the fist time, specific inhibitors of COX-2 offer a rational choice for treatment of prostaglandin-mediated pain and inflammation in these individuals. Here and in other similar placebo-controlled studies, a total of more than 200 subjects with AIA have been shown to tolerate an acute challenge with a specific inhibitor of COX-2 well [155-161].

To date, no formal studies have examined whether long-term usage of these drugs is tolerated by patients with AIA. However, several of the subjects documented in Paper I have now been administered celecoxib for treatment of lumbago or headaches without adverse reactions. Clearly the recommendation that asthmatic patients of all phenotypes should avoid using specific inhibitors of COX-2 must be reconsidered.

One problem that remains to be solved is that a relatively large proportion of adult asthmatics do not know whether they suffer from AIA or not. This lack of information

is probably due to the fact that asthmatics of all phenotypes are often advised by physicians and pharmacists not to take NSAIDs. This dilemma could be remedied by development of a convenient diagnostic test for AIA. Safe and reliable challenge procedures are available, but clinics that offer these tests cannot meet the actual need.

Assuming that 8% of the Swedish population suffer from asthma and that 5% of Swedish asthmatics above the age of 18 have AIA, it can be calculated that 28,000 individuals in this country have AIA. However, this number might be an underestimation, since recent investigations have revealed that 5-10% of patients with asthma exhibit the AIA phenotype [25-27]. Undiagnosed aspirin intolerance can also have a serious negative impact on the safety of asthmatic patients. In one report approximately 20% of asthmatic patients who were hospitalized because they required acute mechanical ventilation for the first time were later found to have AIA [162].

To summarize, patients with AIA tolerate an acute challenge with celecoxib a selective inhibitor of COX-2 well. Thus, the intolerance reaction associated with AIA appears to involve inhibition of COX-1. Consequently, selective inhibitors of COX-2 would seem to offer a safe means of treating prostaglandin mediated pain in all individuals with asthma.

4.2 PAPER II

The capacity of individual's to synthesize CysLTs cannot be used to predict his/her bronchial responsiveness to LTD_4. In subjects with mild chronic bronchial asthma, there was no significant correlation between responsiveness to inhaled LTD_4 and two general markers of leukotriene production (i.e., formation of LTB_4 by whole blood stimulated *ex vivo* and urinary levels of LTE_4).

There was no correlation between the responsiveness of airways to LTD_4 and whole blood *ex vivo* stimulated formation of LTB_4 generation on the day of provocation either in the case of subjects with asthma ($n = 20$, $r = 0.36$, $p = 0.12$) or healthy individuals (n

= 7, r = –0.12, p = 0.80) (Figure 4A). Likewise, there was no correlation between this responsiveness and baseline urinary concentrations of LTE_4 in subjects with asthma (n = 20, r = –0.22, p = 0.36) or healthy individuals (n = 7, r = –0.17, p = 0.71; figure 4B). However in the asthmatic subjects airway responsiveness to LTD_4 was significantly correlated with responsiveness to MCh (r = 0.73, p<0.001). Interestingly, LTD_4 was relatively less potent as a bronchoconstrictor in the asthmatic subjects who were most responsive to inhalation of MCh and more potent in the patients least responsive to inhaled MCh. In fact a linear relation between the responsiveness of airways to MCh and the ratio of the dose of MCh to that of LTD_4 (r = 0.6, p<0.01) could be observed.

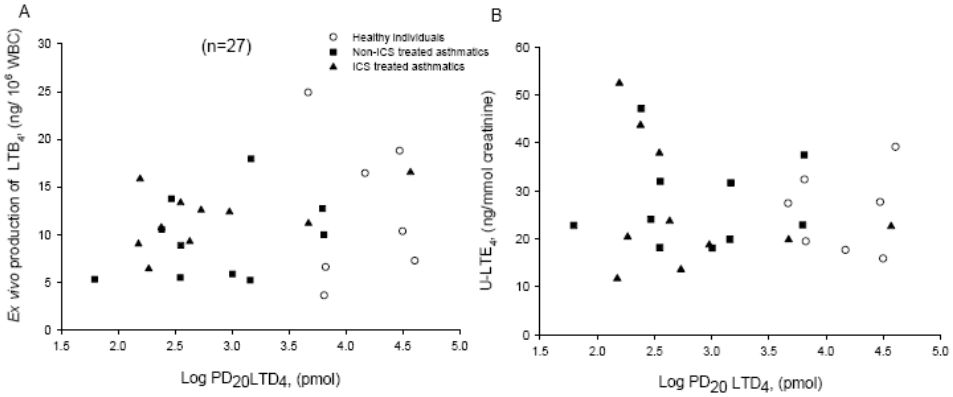


Figure 4. Lack of any relationship between the responsiveness of airways to inhaled LTD_4 and two general markers of leukotriene production in subjects with asthma or in healthy individuals (A) LTB_4 production of whole blood stimulated *ex vivo*; (B) the baseline concentration of LTE_4 in urine. These plots were analyzed employing the Spearman rank order correlation (n=27).

An agonist can elicit either positive or negative feedback that alters the expression of the receptor to which it binds. Therefore it's logical that the capacity for biosynthesis of CysLTs can influence responsiveness involving the $CysLT_1$ receptor. Since LTD_4 provokes the bronchoconstriction via activation $CysLT_1$ receptors coupled to G-protein in the smooth muscle of airways, it has been proposed that responsiveness to LTD_4 might be related to the endogenous levels of leukotrienes. However, no relationship between two general measures of leukotriene biosynthesis and bronchial responsiveness to inhaled LTD_4 was detected here. Although baseline values (including asthma-specific quality of life) indicated that the severity of disease in our subjects with asthma

was relatively similar, their responsiveness to LTD_4 varied almost 1000-fold (with PD_{20} values ranging from 60 pmol (30 ng) to 40 nmol (20 μg)).

On the other hand, the asthmatic patients who were most responsive to MCh demonstrated the lowest responsiveness of the airways to LTD_4, relative to their response to MCh. Although the relationship between responsiveness to LTD_4 and standard direct bronchoconstrictors has been debated[163-165], the relationship between responsiveness to MCh and LTD_4 observed in our studies supports previous findings [71, 166]. Ädelroth and coworkers have proposed that asthmatic subjects with more severe airway inflammation and more pronounced responsiveness to MCh somehow develop a specific tachyphylaxis towards inhaled CysLTs, possibly as a result of enhanced local biosynthesis of these compounds. Interestingly, Ketchell and colleagues reported that bronchial responsiveness to MCh, but not to LTD_4 was increased following a challenge with allergen, a finding that indirectly are in line with Ädelroth's hypothesis and support our findings [167]. Thus, although MCh and LTD_4 both act directly as bronchoconstrictors, the difference in the relative potencies of these two classes of bronchoconstrictors in patients with asthma exhibiting varying degrees of hyperresponsiveness indicates that each bronchoconstrictor exerts unique effects on asthmatic airways. Our findings are consistent with the hypothesis [166] that subjects with asthma and pronounced airway responsiveness to MCh develop tachyphylaxis towards LTD_4, as a consequence of the elevated endogenous level of this mediator present in their inflamed airways.

Direct and indirect indicators of general leukotriene production provide very little information concerning the responsiveness of subjects with asthma to antileukotrienes [112, 115-117]. Since the range of sensitivity to inhaled LTD_4 is considerably greater than the variation in biosynthesis (as reflected in the urinary level of LTE_4), it seems more likely that differences in responsiveness to leukotrienes may better reflect a variation in responsiveness to antileukotriene treatment. However, local production of leukotrienes within the respiratory tract leading to down-regulation of the $CysLT_1$ receptor [168, 169] may be characteristic of responders to antileukotriene treatment. Although we detected no such relationship in Paper II, our observation that asthmatic subjects who were most responsive to MCh exhibited the lowest relative bronchial responsiveness to LTD_4 provides indirect support for this concept.

In summary, this investigation refutes the hypothesis that the responsiveness of airways to LTD_4 is related to the individual's global propensity to synthesize leukotrienes. Either there is no direct relationship between the level of this agonist and its receptors or the methods employed here are too crude and indirect to provide reliable information concerning the production and action of LTs in the airways. In attempt to predict which patient will respond to antileukotriene treatment, further studies within the respiratory tract measuring production capacity of leukotrienes and responsiveness coupled to the receptors of leukotrienes are ongoing.

4.3 PAPER III

Repeated measurement of F_{ENO} allows early detection of disease exacerbation in subjects with allergic asthma. Following repeated challenges with low doses of allergen, elevated levels of NO can be detected in the air exhaled by patients with allergic asthma before symptoms develop.

In our subjects with asthma the mean(± SEM) F_{ENO} values increased from 8.6±1.4 ppb prior to the period of challenge with allergen to 14.7±2.3 ppb 24 hours after the last inhalation of allergen ($p<0.05$; Figure 5). In contrast, no significant change in F_{ENO} occurred in connection with inhalation of the diluent (9.8±1.7 ppb before *versus* 10.4±1.6 after; $p>0.05$). The stable values for F_{ENO} in the healthy control group were significantly lower than those of the asthmatic subjects levels ($p<0.05$).

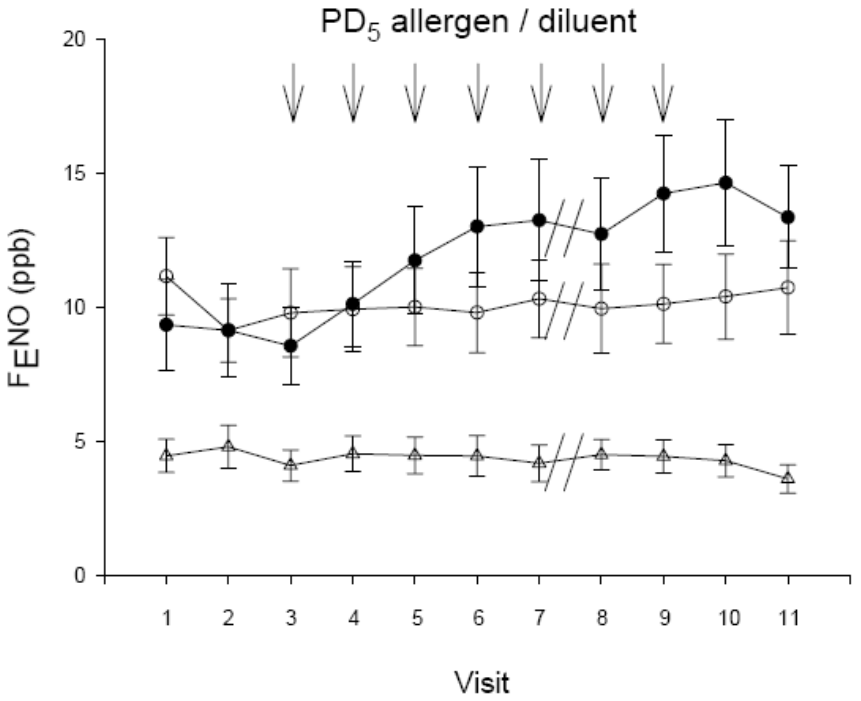


Figure 5. The time course of nitric oxide in exhaled air (F_{ENO}) prior to inhalation of allergen or diluent during the challenge period. The arrows denote the days on which a challenge with allergen or diluent was performed. ●: allergen challenge in asthmatic subjects; ○: diluent challenge in asthmatic subjects; △: diluent challenge in healthy control subjects, (mean± SEM).

During the period of exposure to diluent, none of the subject with asthma reported any symptoms (average symptom score = 0, on a scale of 0-4) whereas during low-dose exposure to allergen, four subjects experienced mild symptoms (average symptom score = 1 for these patients); (group mean±SEM = 0.16±0.07; $p>0.05$). The repeated challenge with a low dose of allergen did not cause any alterations in the baseline values for pulmonary function of the subjects with asthma (Figure 1, Paper III). In addition, none of these subjects suffered an early asthmatic response following this challenge. The mean group change in FEV_1 during the challenges with allergen and diluent were 0.6±1.0% and 0.3±0.7%, respectively. Moreover none of the subjects exhibited any clinically significant late deterioration of pulmonary function, as assessed by measurements of PEFR and reporting of symptoms (not shown).

However, a significant reduction in the geometric mean of the PD_{20} for histamine did occur after exposure to allergen (i.e., from 724 (324–1622) µg to 316 (166–603) µg

corresponding to 2.3 doubling doses $p<0.01$). In contrast, this PD_{20} value was unaffected by repeated doses of the diluent (457 (178–1175) µg before *versus* 562 (302–1047) µg after; $p = 0.48$).

Paper III documents the first controlled examination of the time-course of elevation of F_{ENO} in subjects with mild asthma employing a model that mimics natural exposure to allergen. Increases in F_{ENO} were detected despite the fact that we used here the prototype analyzer with a higher flow-rate that yields lower values. At the same time, the patients exhibited no symptoms of asthma and required no rescue bronchodilator medication and the caliber of their airways was unaffected.

The seven challenges with PD_5 levels of allergen in this protocol spread over a period of nine days, with two days without challenge on the weekend. One can only speculate as to what the outcome would have been if the challenge had been prolonged, in analog to the pollen season or chronic exposure to house-dust mites or pets. Under such conditions the subjects of asthma might have been exacerbated, since the value of F_{ENO}, demonstrated a nearly linear increase throughout the period of challenge employed here.

Allergic asthma is associated with pronounced inflammatory processes in the airways, including elevated numbers of mast cells, neutrophils and lymphocytes and, most strikingly, elevated numbers of eosinophils [170, 171]. In connection with this disease fiberoptic biopsies of the bronchi has become the "gold standard" for assessing inflammation in airway walls, but this invasive procedure is not suitable for routine clinical practice and cannot be repeated often. These limitations have led to the use of induced sputum to detect inflammation, an approach which is relatively reproducible and allows quantification of inflammatory cells and mediators [172]. However, this technique is also somewhat invasive, since it involves inhalation of hypertonic saline, which may induce coughing and bronchoconstriction. Therefore, the possibility of monitoring inflammation in the lungs by examining exhaled gases and condensates has been explored.

Asthmatic subjects consistently exhibit values of F_{ENO} higher than those of healthy subjects [120], was also observed here (Figure 12). In subjects with asthma the F_{ENO} value is correlated to the number of eosinophils in sputum [173-175]. However, the

relationship between the levels of exhaled NO and airway inflammation remains uncertain and in smaller studies no significant relationship between this parameter and the number of eosinophils in bronchial biopsies or bronchoalveolar lavage fluid has been seen [176]. On the other hand, in subjects with mild atopic asthma, inhalation of allergen does cause a significant increase in F_{ENO} [177] as well as elevation in the number of eosinophils in BAL fluid and biopsy samples [178].

Increasing airway eosinophils is a good predictor of exacerbation of asthma, as well as an indicator of such exacerbation during stepwise reduction in the dose of inhaled corticosteroids [179]. In addition a treatment strategy designed to reduce the number of sputum eosinophils of patients with asthma, also attenuated exacerbation [180]. Leuppi has reported that F_{ENO} is not a good predictor of exacerbation of asthma, but in his particular study, sputum and F_{ENO} were analyzed only once a month which might not be frequently enough. Also of great interest is the finding that F_{ENO} was associated with a positive value of 80-90% for predicting and diagnosing loss of asthma control when withdrawing ICS in subjects with mild to moderate asthma. In fact it was as good as induced sputum or airway hyperresponsiveness to saline in monitoring airway inflammation [181].

Recently, measurement of F_{ENO} at 4-8-week intervals has been found to reduce the required dose of inhaled steroids by approximately 40% in comparison to conventional guidelines designed to achieve the same degree of asthma control [182]. In our present investigation an early and progressive rise in F_{ENO} occurred within a few days after initiation of the challenge with allergen. This observation indicates that monitoring F_{ENO} on a daily basis might allow early detection of exacerbation, providing an opportunity to treat such exacerbation before reaching the "point of no return" beyond which no successful treatment is yet available [183].

4.4 PAPER IV

Combination treatment of asthma with ICS and antileukotrienes is scientifically reasonable, since inhaled corticosteroids do not attenuate bronchial responsiveness to inhaled leukotriene D_4 in subjects with mild allergic asthma.

Here, responsiveness of the airways to LTD_4 was found to be unaffected by a 2-week treatment with fluticasone propionate. The logarithm of the mean (±SD) shift in PD_{20} was −0.04 (±0.30) for treatment with fluticasone compared to 0.005 (±0.35) in the case of the placebo (p = 0.75; Figure 6).

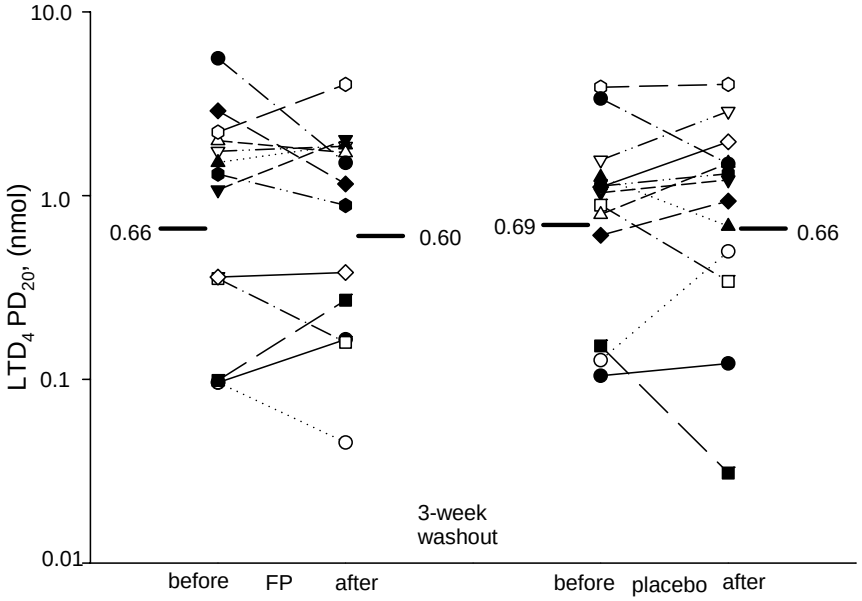


Figure 6. The responsiveness of airways to LTD_4 was not altered by a 2-weeks treatment with fluticasone (500 μg bid) or placebo. The horizontal bars indicate the means (n = 13; p =0 .75 between the treatments). Note the log scale on the y-axis. FP = fluticasone propionate

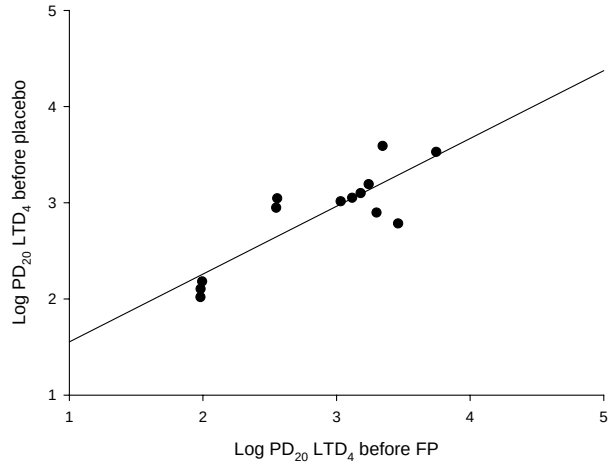
In contrast, airway responsiveness to methacholine was significantly attenuated by this treatment with fluticasone propionate causing a 2.6-fold shift in the PD_{20} value. The logarithm of the mean(±SD) shift in this PD_{20} value was thus 0.41(±0.43) and 0.02(±0.32) from geometric mean baselines of 1148 and 1349 nmol following administration of fluticasone or placebo, respectively ($p<0.05$ for comparison of these treatments). In addition F_{ENO} values were, significantly reduced by treatment with fluticasone propionate, indicating that the subjects took their medication as instructed. The difference in F_{ENO} before and after treatment was 22.0 (range, −1.7 to 154) and 1.8

(range, −7.3 to 24.0) ppb from median baselines of 40.4 and 29.9 after treatment with fluticasone propionate or placebo, respectively (n = 11; $p<0.01$ between treatments).

In agreement with earlier findings, fluticasone treatment did not influence urinary excretion of LTE_4. The change in U-LTE_4 concentrations as a consequence of treatment with fluticasone or placebo were 6.9(±8.0) and 1.4(±7.9) ng/mmol creatinine respectively (mean±SD; $p = 0.15$ between treatments).

At the beginning of the two treatment periods there were no differences in bronchial responsiveness to methacholine or LTD_4 or in baseline $F_{E_{NO}}$ values. Furthermore, no period or carryover effects were observed in this study. The good reproducibility of bronchial provocation with LTD_4 previously shown by Frolund and coworkers [184] was confirm here (Figure 7), even though at least five weeks were allowed to elapse between baseline challenges with LTD_4 in our case. Moreover, repeated challenges with LTD_4 do not cause tachyphylaxis if the intervals between challenges are longer than 2 hours [185].

A



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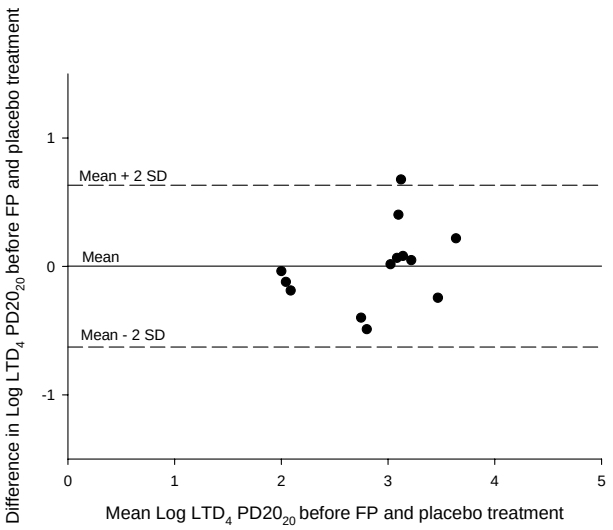

Figure 7. The reproducibility in response to challenge with LTD_4 is illustrated.

A) The logPD_{20} values for LTD_4 prior to treatment with fluticasone (FP) and placebo were significantly Correlated ($r=0.85$ $p<0.0005$).

B) Here, the differences in the log PD_{20} values for LTD_4 associated with the two challenges are plotted against their means according to Bland and Altman. The coefficient of reproducibility for the log PD_{20} of LTD_4 was 0.6 nmol, indicating reproducible responsiveness to the challenges [186].

This investigation is the first to examine whether the bronchial responsiveness of asthmatic subjects to a cysteinyl-leukotriene (LTD_4) is attenuated by two-week treatment with an inhaled corticosteroid (fluticasone propionate, 500μg b i d for two weeks). Despite the fact that the same treatment of our same subjects with asthma reduces the F_{ENO} value and responsiveness to inhaled MCh, there was no evidence that treatment with FP attenuates bronchial responsiveness to LTD_4.

The implication of our finding for the mechanisms underlying allergen-induced EAR and LAR are, not obvious. Cysteinyl-leukotrienes are established as the major mediators of both of these responses [20, 146, 187, 188] and, furthermore, glucocorticosteroids inhibit airway obstruction induced by allergen to a similar, although not identical degree as leukotriene antagonism [147]. Thus, single doses of ICSs exert less effect on the EAR, but more influence on the LAR than do leukotriene antagonists. Nevertheless, employing the same dose (500 μg bid) and duration of fluticasone treatment as in our present study, O'Shaughnessy and colleagues [142] found that both the allergen-induced EAR and LAR were profoundly inhibited and that this inhibition was not associated with a reduction in urinary excretion of LTE_4.

Therefore, our hypothesis was that the protective effect of fluticasone in connection with challenge by allergen might reflect inhibition of the action of cysteinyl-leukotrienes, rather than of their formation. In another investigation involving allergen challenge, Leigh and coworkers [147] demonstrated significant attenuation of the EAR and LAR by the antileukotriene montelukast, as well as by the ICS budesonide, but combination of these two treatments did not result in an additive effect also suggesting a common target of action, such as the leukotriene pathway. The findings in their study, however, appear to argue against the possibility that the effects of ICSs on the EAR and LAR are related at least in part to blockade of the actions of cysteinyl-leukotrienes in the airways.

Prior to our present study, characterization of the manner in which glucocorticosteroids influence the leukotriene pathway have focused on possible inhibition of the biosynthesis. This has led to extensive evidence that glucocorticosteroids do not block the biosynthesis or release of cysteinyl-leukotrienes *in vivo* [112, 139-142, 189], a conclusion supported by our current findings that fluticasone caused no significant reduction in the basal level of urinary excretion of

LTE_4. Since in this same group of subjects with asthma fluticasone did not alter bronchial responsiveness to LTD_4, the leukotriene-dependent aspects of bronchoconstriction and airway inflammation appear to be uniquely resistant to the anti-inflammatory effects of glucocorticosteroids. Indeed, the additional beneficial effects of treating asthmatic patients who are taking glucocorticoids with antileukotrienes as well are well-established [105, 106, 110], and sometimes quite remarkable in the case of subjects with more severe varieties of asthma [112, 190].

It might be argued that the conclusion we have drawn here are only relevant for individuals with mild asthma who do not use ICS on a regular basis. However, clinical trials have provided strong evidence that antileukotrienes exert beneficial effects on patients with asthma of all degrees of severity who are already being treated with ICS [97-101, 104, 105]. Likewise, when the dose of ICS is reduced asthma control can be maintained by treating the patients with antileukotrienes instead [106, 107].

On the basis of all of these observations, we can now conclude that the additive therapeutic effects of ICSs and antileukotrienes reflect a lack of effect of glucocorticosteroids on the leukotriene pathway. Thus, neither the release nor the actions of leukotrienes appear to be sensitive to ICSs, strengthening the rationale for their combination with antileukotrienes in the treatment of allergic asthma.

4.5 ADDITIONAL FINDINGS NOT INCLUDED IN PAPERS I-IV.

Urinary excretion of LTE_4

Baseline urinary levels of LTE_4 were determined in connection with all of the studies described in this thesis and found to be similar in subjects with mild aspirin-tolerant asthma and healthy controls. In fact, these baseline values (median(range)) for healthy individuals, (25.8(14.8-54.6) ng/mmol creatinine), asthmatics not using ICS (32.8(14.4-91.1)) and asthmatics being administered ICS (21.4(10.5-57.8)) were not significantly different ($p>0.05$). However patients with aspirin-intolerant asthma had significantly higher levels of U-LTE_4 (67.5(15.9-421.5)) than did aspirin-tolerant asthmatics and healthy subjects ($p<0.001$) (Figure 8).

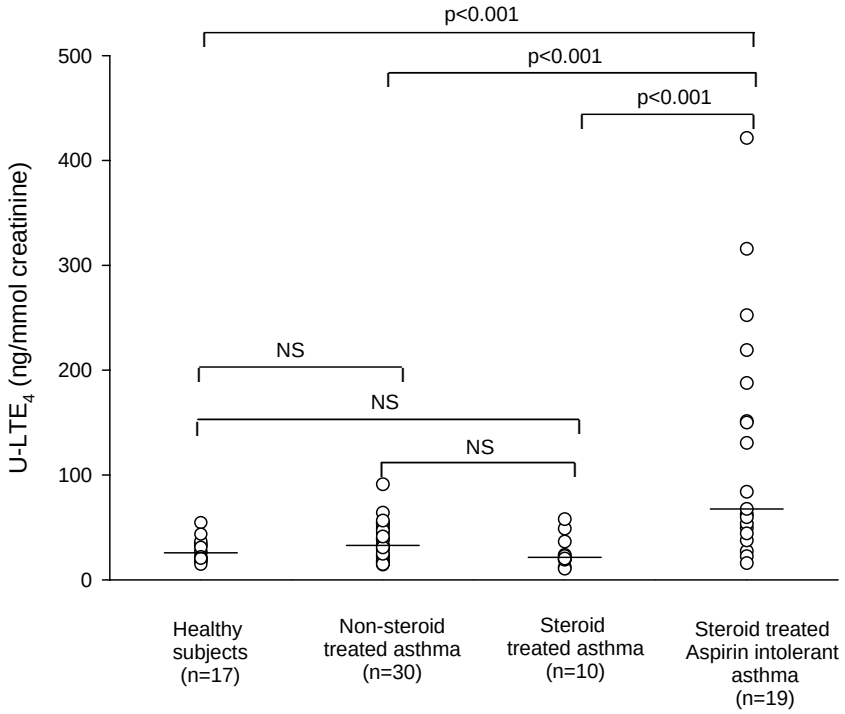

Figure 8. Combined baseline levels of urinary LTE_4 for subjects examined in **Papers I-IV**. The values obtained in connection with the first visit during each study are shown. The subjects with AIA were found to excrete significantly higher levels than those with ATA and healthy control individuals. The bars indicate the median values.

In **Paper III** we found a small, but significant difference in urinary excretion of LTE_4 by healthy subjects and asthmatic not receiving ICS treatment. Compared to the combined analysis described above where cross-sectional data were used, the values in this paper are based on repeated sampling and thereby provide greater power to detect small differences.

In **Paper II**, although the baseline urinary excretion of LTE_4 in all three groups studied was similar, there was a small but significant increase in this excretion by all subjects (n=30) following the bronchoscopy performed during the second visit (20.5(11.9-56.1) (median(range)) before versus 29.3(17.4-65.8) and 33.0(15.6-68.3)

ng/mmol creatinine at 3 and 4 hours after bronchoscopy respectively; $p<0.01$) (Figure 9). This procedure triggers airway an inflammation, although to a much lower extent than that observed, for example, following an allergen challenge [125]. This observation should be taken into account when performing bronchoscopy prior to and after an intervention. Since CysLTs act as potent chemokines for eosinophils [73, 191], this rise in U-LTE_4 may confound the findings of such studies.

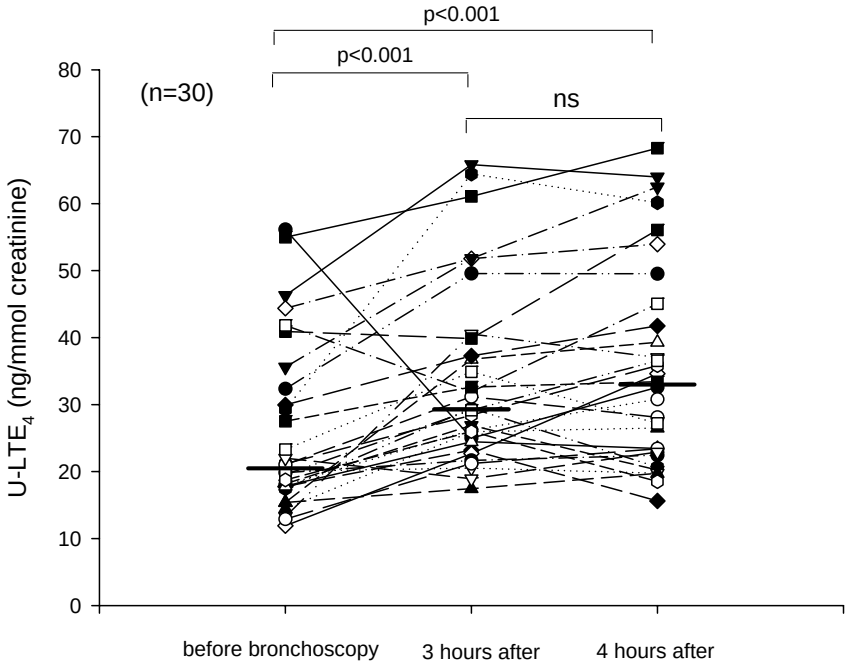

Figure 9. Baseline levels of urinary LTE_4 and the increases observed 3 and 4 hours after completion of bronchoscopy. The bars indicate the median values.

Furthermore the levels of U-LTE_4 in the two samples collected following inhalation challenge with LTD_4 performed during the third visit were significantly elevated. For the group as a whole, the correlation between the cumulative dose of inhaled LTD_4 and post-challenge excretion of U-LTE_4 (i.e., the U-LTE_4 values two hours after maximal reduction, minus the corresponding mean of two baseline samples taken before the challenge) was statistically significant ($p<0.001$)(Figure 10).

Although this is not surprising, such a direct correlation has not been demonstrated previously.

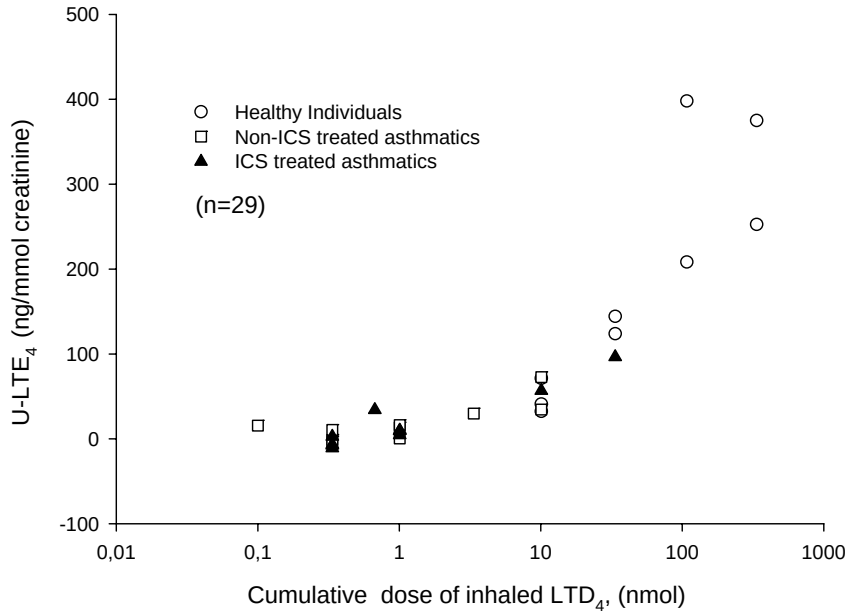

Figure 10. The correlation between the cumulative dose of inhaled LTD_4 and post-challenge excretion of LTE_4 (i.e., the U-LTE_4 value at two hours after maximal reduction, minus the corresponding mean of two baseline samples taken before the challenge) ($r = 0.82$; $p < 0.0000001$).

As a corollary, the excretion of LTE_4 following the challenge with LTD_4 was lower for the asthmatic subjects (10.2(-10.8-96.6) ng/mmol creatinine (median(range)), who were more responsive and therefore received a lower total dose of LTD_4, than in the case of the healthy subjects (median(range): 144.2(32.3-398.0) ng/mmol creatinine; $p < 0.001$) These data support the accuracy of the procedure used to measure U-LTE_4.

Bronchial responsiveness to LTD_4 and methacholine

In **Papers II and IV**, bronchial responsiveness to LTD_4 was related to the corresponding responsiveness to MCh, a standard indicator of such responsiveness. The dose-response relationships observed in this connection for all of the subjects with intermittent-to-mild asthma are depicted in Figure 11 and the mean values for different measures of responsiveness, for the different groups, involving healthy controls, are documented given in Table 6.

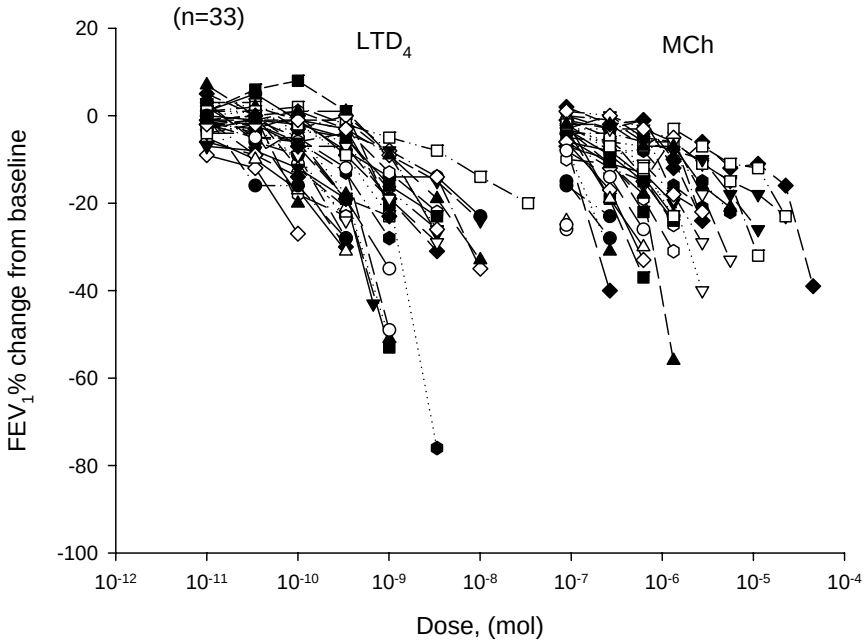

Figure 11. The dose-response curves for LTD_4 (left) and MCh (right) for 33 individual subjects with intermittent-to-mild asthma. In the term of the PD_{20} value, as well as on a molar basis LTD_4 was approximately 1400-fold more potent than MCh.

Table 6. The geometric means (ranges) of measures bronchial responsiveness

	Healthy individuals (n=10) Geometric mean (Range)	**Non-steroid treated asthmatics (n= 23)** Geometric mean (Range)	**ICS treated asthmatics (n=10)** Geometric mean (Range)
$PD_{20}LTD_4$ (nmol)	14.12 (6.47-40.37) (n=7)	0.76 (0.06–6.43)	0.65 (0.15–37.05)
$PD_{20}MCh$ (nmol)	nd	1109 (89-25457)	862 (89-37188)
Ratio $PD_{20}MCh$/ $PD_{20}LTD_4$	nd	1451 (174-17557)	1326 (206-5825)
$PD_{15}LTD_4$ (nmol)	16.50 (2.96–192.07) (n=9)	0.42 (0.03–5.17)	0.41 (0.07–12.27)
$PD_{15}MCh$ (nmol)	nd	727 (32-19316)	582 (89-27298)
Ratio PD_{15} MCh/ PD_{15} LTD_4	nd	1712 (575-30181)	1420 (240-5982)
$PD_{10}LTD_4$ (nmol)	7.14 (1.14–54.24)	0.23 (0.01–1.25)	0.22 (0.04–5.00)
$PD_{10}MCh$ (nmol)	12 974 (3830-47351) (n=7)	322 (0.1-2441)	270 (89-9819)
Ratio $PD_{10}MCh$/ $PD_{10}LTD_4$	1827 (321-7399) (n=7)	1377 (2.9-18743)	1238 (116-4746)

nd = could not be determined

For the subjects with asthma (n=33), there was no significant difference in bronchial responsiveness to LTD_4 and MCh as assessed by the PD_{20} values between those using and not using ICS (Table 6). Based on the first challenges documented in Paper IV the geometric mean (range) of the PD_{20} values for LTD_4 and MCh for all of the subjects with asthma were 0.73 (0.06–37.05) nmol and 1027 (89–37 188) nmol, respectively, of the 10 healthy control subjects involved in Paper II, 7 exhibited PD_{20} values for LTD_4, but none for MCh, with a cumulative dose of 45, 282 nmol. However, this was expected, since a positive response to a MCh challenge was a criteria for exclusion of healthy controls. The geometric mean (range) of the PD_{20} value for LTD_4 for healthy controls was 14.12 (6.47-40.37) nmol.

Thus, in the 20 subjects with intermittent-to-mild asthma, LTD_4 was approximately 1400-fold more potent than MCh on a molar basis (the ratio of PD_{20} values was 1027/0.73 = 1410 and the ratios of the PD_{15} and PD_{10} values were similar). This finding is consistent with the earlier reports that CysLTs are approximately 100-10000 times as potent, on a molar basis, as MCh or histamine in causing constriction of the airways. This has been observed both in bronchoprovocation studies [163-165, 192] and in investigations on isolated human airways [193-195]. In addition, relative to their responsiveness to MCh, healthy individuals are more sensitive to CysLTs than are subjects with asthma [166].

During challenge with allergen, both LTE_4 and methylhistamine are excreted in measurable quantities in the urine. For instance, following challenges of mild asthmatic patients with a dual allergic reaction the urinary levels of LTE_4 and methyl-histamine were increased from approximately 50 to 100 and from 10 to 20 μg/mmol creatinine respectively [125]. Assuming that the provocative concentration of histamine required to reduce the FEV_1 value of stable subjects with asthma by 20% (PC_{20}) correlates closely with the PC_{20} for methacholine [196], it is reasonable to conclude that CysLTs contribute at least as much, if not more than histamine to the contraction of airways in response to an allergen challenge. Indeed, intervention studies with antileukotrienes consistently reveal the involvement of CysLTs in airway obstruction triggered by allergen, whereas antihistamines exert a less pronounced effect. This was demonstrated most clearly by treating subjects with allergic asthma with specific antagonists of both histamine and CysLTs prior to challenge with allergen [20].

Exhaled nitric oxide

The exhaled level of nitric oxide was determined in **Papers II - IV.** In **Paper III** F_{ENO} was analysed with a prototype NIOX analyser, using a higher flow rate of exhaled air**.** The subjects with asthma exhibited significantly higher baseline values of F_{ENO} than healthy individuals. These values (median(range)) were 34.2(6.5-198.6) ppb; for the asthmatic subjects not using ICS ($p<0.001$), 17.2(6.1-86.5) ppb for the asthmatics being treated with ICS ($p<0.05$) and 11.0(6.3-19.7) ppb for healthy

individuals. There was no significant difference in the FE_{NO} values for asthmatics using and not using ICS (Figure 12).

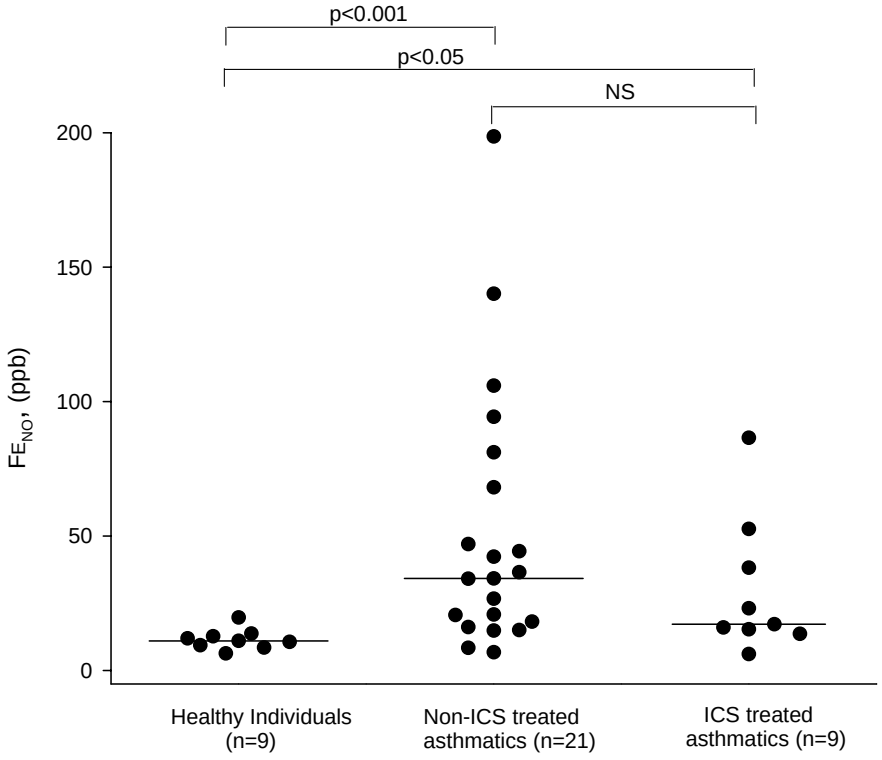

Figure 12. Baseline FE_{NO} values for the subjects described in **Paper II and IV**. The FE_{NO} values (obtained in connection with the first visit in Paper IV) for subjects with asthma were significantly higher than those of healthy individuals. The horizontal bars indicate the median values for the different groups.

The FE_{NO} values depicted in Figure 12 represent cross-sectional data from healthy subjects and two groups of patients with asthma. The investigation documented in Paper III revealed that the FE_{NO} value can increase when subjects with allergic asthma are exposed to allergen. On the other hand, we have also shown that the FE_{NO} of asthmatic subjects can be decreased by treatment with ICS (Paper IV). These changes in FE_{NO} can occur within a short period of time, increasing or decreasing within one or two weeks, respectively.

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

This thesis demonstrates that individuals with AIA tolerate an acute challenge by celecoxib well. Since this drug is a specific inhibitor of COX-2, the intolerance reaction associated with AIA patients appears to be mediated via inhibition of COX-1[197]. Why such intolerance to aspirin and other unselective NSAIDs occurs only in a limited number of asthmatic patients remains to be explained.

Prostaglandin E_2 exerts anti-inflammatory effects on the lung, attenuating both the early and late reactions induced in airways by allergens [198]. This attenuation may be achieved by a reduction in the release of CysLTs and PGD_2 [199]. Moreover, PGE_2 also inhibits the release of various mediators from different types of inflammatory cells [200-202]. In the case of subjects with AIA, the mast cell is most probably responsible for the intolerance reaction [94, 203], and PGE_2 can prevent the degranulation of mast cells. Thus, pretreatment with PGE_2 also attenuate the bronchoconstrictive reactions produced by exposure of patients with AIA to aspirin [204]. The reason why the mast cells within the respiratory apparatus are so vulnerable and dependent on PGE_2 is not yet known. It can be speculated that the reduced capacity of nasal polyps [205], fibroblasts [206], and respiratory epithelial cells [207] in patients with of AIA to produce PGE_2 is of relevance in this connection. Alternatively the mast cells of subjects with AIA may require higher concentrations of PGE_2 to prevent their degranulation during inhibition of COX-1, in comparison to the mast cells of individuals with ATA.

It is promising that patient with AIA do tolerate specific inhibitors of COX-2 well. However, recent findings indicate that the risk for cardiovascular disease is elevated by long-term usage of such inhibitors [208-210]. These inhibitors were developed on the basis of the hypothesis that COX-2 is the source of the prostaglandins that mediate inflammation, while COX-1 produces the prostaglandins that protect against peptic ulcers.

The most convincing hypothesis with respect to the enhanced risk for cardiovascular disease caused by specific inhibitors of COX-2 concerns the imbalance between

thromboxane A_2 (TXA_2) and prostaglandin I_2 (PGI_2) that these inhibitors create, in contrast to non-selective COX inhibitors [208]. Whereas the non-selective inhibitors prevent production of both of these substances, the specific inhibitors of COX-2 do not inhibit biosynthesis of TXA_2, since platelets lack COX-2. Moreover, the assumption that synthesis of PGI_2 is catalyzed primarily by COX-1 appears to be incorrect, since the COX-2 expressed by vascular endothelial cells [211] can catalyze the formation of prostaglandin endoperoxide from arachidonic acid. Thus, in subjects who ingest a specific inhibitor of COX-2 production of hemodynamically protective PGI_2 is inhibited while at the same time platelets continues to produce pro-thrombotic TXA_2.

There is now strong evidence that the hypothesis described above has clinical consequences. The (APPROVe) Trial [212] revealed that treatment with rofecoxib increased the relative risk of suffering a thrombotic event almost two-fold compare to the placebo and that this increased risk became apparent after 18 months of such treatment. Similar results were obtained in connection with the adenoma prevention study with celecoxib [213]. Both of these studies involved subjects at a low risk for developing cardiovascular disease. However, short-term (10-day) treatment of patients with a high risk for cardiovascular pathology with the specific COX-2 inhibitors parecoxib and valdecoxib also enhances the risk for such disease [214]. Similar results were obtained in another short-term (14-day) investigation of similar design and with these same drugs, also involving patient undergoing cardiac surgery but in this case with by-pass procedures [215].

Further discussions of this complicated matter lies beyond the purpose of this thesis. Still, there is evidence that long term administration of specific inhibitors of COX-2 to patients with AIA should be avoided. When there is a requirement for inhibition of prostaglandin synthesis in order to reduce pain and inflammation in subjects with AIA, short- or medium-term treatment, with careful monitoring of the patient, should be initiated unless the individuals has a high risk for cardiovascular disease.

The present investigations confirm earlier findings that patients with aspirin-intolerant asthma excreted more LTE_4 in their urine than do subject with ATA or healthy control subjects. To date this is the only abnormal finding consistently

associated with AIA. However, because of the overlap in urinary concentrations of LTE_4, patients with AIA can not be distinguished from subjects with ATA on the basis of this parameter.

However, these high urinary levels LTE_4 are likely to be involved in the chronicity of AIA. From this perspective, patients with AIA should be suitable for testing the proposal that the capacity to produce and respond to leukotrienes in relation to responsiveness to antileukotriene treatment. Indeed, clinical studies have demonstrated that patients with AIA generally respond particularly well to treatment with antileukotrienes[102, 103, 112].

Moreover, in relation to their responsiveness to histamine, these patients are much more responsive to LTE_4 than are individuals with ATA [216, 217]. This observation is not consistent with our hypothesis that elevated production of CysLTs might lead to down-regulation of the expression of the $CysLT_1$ receptor. However, following desensitization with ASA, which presumably involves activation of mast cells, subjects with AIA become less responsive to both ASA and CysLTs [216]. This effect of desensitization may reflect, at least in part selective down-regulation of the $CysLT_1$ receptor in the cells of airways [218].

In connection with the AIA phenotype, biosynthesis of CysLTs, although higher than in patients with ATA, may not normally be high enough to cause such down-regulation. Furthermore the level of CysLT required for feedback regulation of the numbers of $CysLT_1$ receptors may differ in the various phenotypes of asthma. In attempting to identify an asthmatic phenotype(s) that is dependent on leukotrienes, it must be helpful to examine the AIA phenotype more closely with respect to responsiveness of airways to CysLTs and the biosynthetic capacity of the airways of different individuals.

Discrepancies exists between the primary effects of antileukotrienes observed in mechanistic studies [20] and the results of clinical trials [111] or even what happens in real life. At the beginning of clinical trials, the asthmatics patients are usually stable; whereas mechanistic studies are most often connected with exacerbation. Such exacerbation is provoked, e.g., by a challenge with various substances, such as aspirin

or allergen, or by exercise, which lead to activation of mast cells and release of CysLTs as a final common response, despite the different routes by which mast cells are activated. Perhaps under stable conditions little CysLTs is released from mast cells, except in the case of AIA. If so, under stable conditions other mediators than leukotrienes may play an important role in maintaining the inflammation.

However certain evidence does suggest that CysLTs influence structural cells that participate in the remodeling of airways caused by the inflammation associated with allergic asthma. For instance, certain findings indicate that CysLTs may promote the proliferation of smooth muscle cells *in vivo* [219]. Thus, substances that inhibit the production of CysLTs reduce the number of myofibroblasts present following challenges with repeated low doses of allergen [220]. The ability of Cysteinyl leukotrienes to potentiate collagen production by human fetal lung fibroblasts exposed to TGF-β_1 [221] may also be relevant to the remodeling process. This process is probably very slow and may not influence the outcome of most clinical trials.

Thus, there is a considerable difference in the amount of CysLTs detectable in sputum and urine under stable conditions and the 2-5-fold higher levels associated with the activation and degranulation of mast cells induced by allergen, exercise or aspirin in connection with mechanistic studies on asthma [32, 222, 223], also supported by Paper I. Although this discussion is highly speculative it might be a good idea in the near future to perform investigations designed to help develop more effective strategies for treatment of different asthmatic phenotypes with antileukotrienes both under stable conditions and in connection with exacerbation. Antileukotrienes are probably a good candidate for effective treatment of the latter, especially in emergency departments and other locations where patients with exacerbation of asthma are cared for [70, 224, 225].

The investigations described in **Paper III** involved destabilization of asthma control by repeated bronchial challenges with low doses of allergen. However, exposure to allergens is not the most common cause of loss of control or even exacerbation of asthma. Both in children and adults, the majority of asthma exacerbations are caused by respiratory viral infections in which rhinoviruses (RVs) are by far most frequently involved [226, 227]. Thus the pronounced increase in asthma exacerbations that occurs in the fall, winter, and early spring is predominantly virus-related, with RVs dominating [228, 229]. Virus-induced exacerbation of asthma represents a major unmet clinical

need. The mechanism by which viruses cause problems in the lower airways, secondary to the infection of the nose is under debate, but the concept of “united airways” is widely accepted by clinicians.

The next question is whether viral infections can cause a rise in the exhaled level of NO. In a laboratory study involving experimental infection with RV, the F_{ENO} value of subjects with asthma was elevated [230]. Furthermore, bronchial responsiveness to histamine was enhanced significantly by RV infection, and there was a significant correlation between the RV-induced change in levels of exhaled NO and the accompanying decrease in the PC_{20} value for histamine. Thus, the greater the increase in the level of exhaled NO, the smaller the decrease in PC_{20}.

These findings suggest that viral induction of nitric oxide synthase within the airways may play a protective role in connection with exacerbation of asthma. The levels of exhaled NO from the lower airways of healthy subjects suffering from natural viral infections of unknown etiology is also elevated [231]. Accordingly it would be highly interesting to examine whether it might be possible, at least in part to predict and thus prevent all kinds of exacerbation of asthma by monitoring F_{ENO} on a daily basis at home. Exacerbations of asthma are expensive [232] and minimizing these will save considerable resources, as well as improve the quality of many lives.

Since neither the release nor the actions of leukotrienes appear to be influenced by ICSs, there is now a mechanistic rationale for using glucocorticosteroids and antileukotrienes in combination to treat asthma. Indeed, 17% of subject with mild asthma (age 6-17), benefit from being administered both ICS and antileukotrienes [233]. Moreover, both ICSs [234, 235] and antileukotrienes [236, 237] attenuate the F_{ENO} value. Thus, another approach to finding a way to identify responders to antileukotrienes in advance would be to treat subjects who despite administration of ICS, have uncontrolled asthma and elevated F_{ENO} levels with antileukotrienes as well. This study should probably involve patients with mild-to-moderate asthma, since addition of an antileukotriene to existing high-dose corticosteroid therapy of asthmatic subjects with elevated numbers of eosinophils in their sputum does not reduce this eosinophilia any further [238].

In summary, this thesis is based on four investigations on mechanisms of importance in connection with different phenotypes of asthma. The information obtained is of value for monitoring and managing patients with asthma. These mechanistic studies should now be followed up by clinical trials involving a larger number of subjects preferably of the same phenotype as those examined here, (to prevent flattened result) designed to confirm and extend our mechanistic findings.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund

Astma är en mångfacetterad sjukdom. Den beror på en underliggande inflammation som leder till luftvägssammandragningar, hosta, slemproduktion och varierande grad av andnöd. Det finns olika typer av astmasjukdom (fenotyper), t ex allergisk och icke allergisk astma, en del individer får besvär företrädelsevis i samband med fysisk ansträngning. Luftrörskänsligheten för cigarettrök, parfymer och luftföroreningar är ofta ökad, men olika individer är olika känsliga. De allra flesta patienter med astma försämras av luftvägsinfektioner, och det är också den vanligaste orsaken till att sjukdomen försämras tillfälligt. Patienter med olika typer av astma drar nytta av astmamediciner på ett varierande sätt. Ytterliggare ett exempel på sjukdomens många olika skepnader är den aspirin intoleranta astman. Patienter med denna fenotyp av astma riskerar livshotande reaktioner om de av misstag får i sig en tablett innehållande aspirin eller annan värktablett med liknande vekningsmekanism. Med anledning av ovanstående exempel på olika typer av astma är det mer ändamålsenligt att prata om astma som ett syndrom, än som en enhetlig sjukdom. Det är i klinisk praxis en fördel att diskriminera de olika typerna av astma för att därigenom åstadkomma mer skräddarsydd behandling som förhoppningsvis leder till ett bättre utfall för den enskilde patienten.

I denna avhandling har vi undersökt patienter med 2 olika fenotyper av astma; allergisk- och Aspirin Intolerant Astma (AIA). Avhandlingen har fokuserat på den del av inflammationen som beror på leukotriener. De bildas i de vita blodkropparna (leukocyter) och innehåller tre närliggande dubbelbindningar som kemiskt kallas för en ”triene”, där av deras namn.

I **delarbete I** har vi undersökt patienter med AIA. Det är ca 5-10% av vuxna astmatiker som har denna fenotyp. Överkänslighetsreaktionen mot aspirin beror på att denna typ av läkemedel hämmar enzymet cyclooxygenas (COX), som är nyckelenzymet vid biosyntesen av prostaglandiner. Patienter med AIA har också en ökad basal produktion av leukotriener jämfört med astmatiker som tolererar aspirin.

Sedan en tid tillbaka känner man till att det finns mer än ett COX. COX-1 är ett enzym som finns i alla kroppens celler och som ser till att kroppen bland annat bildar prostaglandiner som har livsviktiga funktioner i alla kroppens celler. COX-2 är ett annat enzym som uttrycks fram för allt i inflammatoriska celler och som vid cellskada och eller inflammation bildas i allt större omfattning. Detta har fått läkemedelsindustrin att tillverka särskilda COX-2 selektiva hämmare för behandling av inflammation och värk.

33 patienter med verifierad AIA provocerades med den COX-2 selektiva hämmaren celecoxib. Vid de 2 första besöken fick försökspersonerna med 2 timmars mellanrum dricka en lösning av ökade doser (5, 10, 30 och 100 mg) av celecoxib eller placebo. Vare sig försökspersonerna eller försökspersonalen var informerad om vid vilket tillfälle som aktiv substans respektive placebo gavs.

Resultat: Då inte någon av de 33 försökspersonerna utvecklade någon form av överkänslighetsreaktion vid de besök då celecoxib gavs kunde alla försökspersonerna vid ett tredje besök öppet få den högsta dagliga rekommenderade dosen 200 + 200 mg av celecoxib med 2 timmars mellanrum. Inte heller då var det någon som reagerade med någon överkänslighetsreaktion.

Diskussion: Patienter med AIA tolererar en akut provokation med den COX-2 selektiva hämmaren celecoxib. Överkänslighetsreaktionen vid AIA är sannolikt medierad via en hämning av COX-1. Detta innebär att patienter med AIA för första gången nu kan erbjudas en ändamålsenlig behandling mot prostaglandin-mediterad inflammation och smärta.

I **delarbete II** har vi kartlagt om det finns något samband hos patienter med lindrig astma och friska kontroller, mellan deras förmåga att bilda leukotriener, analyserat från blod och urin, och deras luftvägskänslighet för inandat leukotrien D_4, en leukotriene av betydelse vid astma.

När man behandlar astmatiker med antileukotriener har det visat sig att patienterna svarar olika bra på behandlingen. Detta är frustrerande då det inte går att förutse en bra

behandlingseffekt hos alla patienter. Som ett led till att ta reda på vilka patienter med astma som har en särskilt leukotrieneberoende astmatisk inflammation undersöktes hur förmågan att producera leukotriener mätt i blod och urin korrelerade med känsligheten i luftvägarna för inandat leukotrien D_4.

10 försökspersoner med lindrig kortisonbehandlad astma, 10 försökspersoner med lindrig astma utan kortisonbehandling samt 10 friska kontroller undersöktes i en tvärsnittsstudie med blodprover, urinprover, luftvägsprovokationer med metakolin samt leukotrien D_4 och utandat kväveoxid. Kväveoxid bildas i luftvägarna och tros spegla den underliggande inflammationen vid astma.

Resultat: Vi hittade inga samband vare sig hos försökspersoner med astma eller hos de friska kontrollerna mellan förmågan att producera leukotriener analyserat från blod och urin och luftrörskänsligheten för inandat leukotrien D_4. De försökspersoner med astma som hade störts känslighet för inandat metakolin hade i relation till sin metakolin-känslighet lägst luftvägskänslighet för leukotrien D_4. Kväveoxid nivåerna var högre hos patienterna med astma jämfört med de friska kontrollerna.

Diskussion: Det är rimligt att tänka sig att produktionsförmågan för leukotriener eller känsligheten för samma substans i luftvägarna har betydelse för om man har en särskilt leukotrieneberoende astma. Generella mått på produktionsförmåga av leukotriener i blod och urin kan inte förutse luftvägskänslighet av leukotrien D_4. Vi kommer att gå vidare och undersöka om det finns något samband mellan produktionsförmåga kontra luftvägskänslighet för leukotriener lokalt i lungan, men denna undersökning är inte klar för närvarande. Dock har vi kunnat påvisa att astmatikerna med störts känslighet för inandat metakolin har i relation till sin metakolikänslighet den lägsta känsligheten för inandat leukotrien D_4. Det kan bero på att dessa patienter har en ökad mängd leukotriener lokalt i lungan som där bidrar till en nedreglering av receptorn för leukotrien D_4.

I **delarbete III** undersökte vi 8 lindrigt sjuka allergiska astmatiker. De fick genomgå luftvägsprovokationer med histamin och adenosin före och efter det att de provocerats

med inandat allergen (t ex pollen el. pälsdjur) enligt en särskilt lågdosmodell. Under 7 på varandra följande vardagar, med avbrott för lördag och söndag fick astmatikerna dagligen andas in en dos av allergen som motsvarar den som gav en sänkning av lungfunktionen (FEV_1) med 5%. Varje dag mättes lungfunktion, symptom och kväveoxid i utandningsluften. Astmatikerna var sina egna kontroller och allergenprovokationerna var placebokontrollerade. Även 8 friska försökspersoner undersöktes, men dessa fick enbart andas in placebo (den lösning som allergenet var löst i) vid de sju besöken.

Resultat: Under allergen-provokationsserien steg kväveoxid i utandningsluften hos astmatikerna, men utan att de fick astmasymptom eller att deras lungfunktion påverkades. Efter allergen-provokationsserien var astmatikerna mer känsliga för histamin jämförda med före. Ingen skillnad noterades avseende adenosin-provokationerna för och efter allergen-provokationsserien, men enbart hälften av astmatikerna var känsliga för adenosin redan före. Placebo hade ingen effekt på utandat kväveoxid eller känsligheten för histamin hos vare sig astmatikerna eller de friska kontrollerna.

Diskussion: Delarbete III visade tidsförloppet för hur kväveoxid stiger i luftvägarna hos allergiska astmatiker då de påverkas av allergen i en modell som strävar efter att efterlikna den naturliga exponeringen av allergen. Kväveoxidnivåerna steg i utandningsluften utan att astmatikerna fick mer besvär eller att dimensionen på deras luftvägar påverkades. Då utandat kväveoxid antas spegla den underliggande inflammationen i luftvägarna, kan man tänka sig att det går att förhindra en del försämringsperioder av allergisk astma genom att låta astmatikerna dagligen mäta kväveoxid i utandningsluften i hemmet, och vid förhöjda nivåer ta extra astmamedicin.

I **delarbete IV** undersöktes 13 patienter med lindrig allergisk astma med bronkialprovokationer med metakolin och leukotrien D_4 före och efter 2 veckors behandling med inandat kortison (flutikason 500 µg morgon och kväll). Nivåer av utandat kväveoxid och urinnivåer av leukotrien E_4 (en annan leukotriene av betydelse vid astma), i urin mättes också före varje bronkialprovokation. Studien var

placebokontrollerad, varje försöksperson undersöktes med totalt 8 stycken bronkialprovokationer, (metakolin och leukotrien D_4) före och efter behandling med både kortison och placebo.

Resultat: Inandat kortison sänkte känsligheten i luftvägarna för metakolin samt minskade nivåerna av utandat kväveoxid, utan att leukotrien E_4 i urinen eller känsligheten för inandat leukotrien D_4 påverkades.

Diskussion: Resultaten visar att kortison inte påverkar vare sig bildningen eller effekten av leukotriener i luftvägarna. De förefaller som om kortison och antileukotriener påverkar den astmatiska inflammationen på olika nivåer. Studien ger en mekanistisk förklaring till de tidigare behandlingsstudier som har visat att man får en tilläggseffekt då man adderar antileukotriener till kortison behandlingen hor patienter med allergisk astma.

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