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**CLINICAL STUDIES ON THE  
ROLE OF EICOSANOIDS IN  
THE ASTHMATIC AIRWAY  
INFLAMMATION**

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TO MY FAMILY



## ABSTRACT

The underlying mechanisms in the asthmatic airway inflammation involve the interaction between different cells and mediators that consequently result in different clinical phenotypes. The aim of this thesis was to investigate the impact of inflammatory mediators, with emphasis on eicosanoids, on the inflammatory and functional airway responses under basal and triggered conditions in subjects with asthma, in particular ASA/NSAID-intolerant and allergic phenotypes. In the studies included in this thesis, we investigated the possibility of finding new phenotype-specific biomarkers of asthma in connection with mechanistic pathways of eicosanoid biosynthesis. The studies were possible because of careful and extensive characterizations of the patients.

Eleven aspirin-sensitive asthmatics had, in comparison with ten aspirin-tolerant asthmatics, higher exhaled nitric oxide levels and higher baseline levels of CysLTs in saliva, sputum, blood *ex vivo* and urine. Levels of urinary LTE<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> increased after aspirin provocation whereas leukotriene levels in saliva and *ex vivo* stimulated blood did not increase. These findings support a selective CysLT-overproduction in this distinct clinical syndrome. CysLTs in saliva should be explored as a new and clinically convenient biomarker of AIA and other diseases associated with increased production of leukotrienes.

In an explorative study, the capacity of eosinophils to produce 15-LO pathway products and their *ex vivo* responsiveness to COX inhibition was studied in the peripheral blood drawn from healthy volunteers and three asthma groups. In the absence or presence of lysine-aspirin, eosinophils were stimulated with arachidonic acid and calcium ionophore to trigger the 15-lipoxygenase-1 (15-LO) and 5-lipoxygenase (5-LO) pathways, respectively. The results displayed an increased release of the recently discovered lipid mediator eoxin C<sub>4</sub> (EXC<sub>4</sub>) as well as the main indicator of 15-LO activity, 15-HETE, in activated eosinophils from severe and aspirin-intolerant asthmatics. Eosinophils from AIA subjects also showed elevated EXC<sub>4</sub> and LTC<sub>4</sub> formation after cellular activation in the presence of lysine-aspirin. This higher biosynthetic activity of 15-LO pathway in AIA is in part due to increased numbers of eosinophils, but the data also support enhanced eosinophil function, possibly involving transcellular interactions with platelets. The findings support contribution of 15-LO pathway in the pathophysiology of severe and aspirin-intolerant asthma.

This thesis also aimed at evaluating the role of COX-1 and COX-2 in the biosynthesis of the pro-inflammatory prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) and bronchoprotective prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) under basal conditions and during heightened airway inflammation and responses after inhaled allergen provocation. Eighteen subjects with asthma and six healthy controls participated in a cross-over study where a selective COX-2 inhibitor, celecoxib 200 mg, or placebo were given b.i.d. on 3 consecutive days following 2 untreated baseline days.

Celecoxib treatment inhibited urinary excretion of the tetranor metabolite of PGE<sub>2</sub>, PGEM, by 50% or more in asthmatic subjects and healthy controls, whereas there was no significant change in the excretion of the tetranor metabolite of PGD<sub>2</sub>, PGDM. In addition, celecoxib did not cause any significant changes in FEV<sub>1</sub> or F<sub>E</sub>NO. In comparison with the healthy controls, the subjects with asthma had higher baseline levels of urinary PGDM but not of PGEM. These findings indicate that biosynthesis of

PGD<sub>2</sub> is catalysed predominantly by COX-1 and that COX-2 contributes substantially to the biosynthesis of PGE<sub>2</sub>. The asymmetric impact of COX-2 inhibition on prostanoid formation raises the possibility of long-term adverse consequences of COX-2 inhibition on airway homeostasis by the decreased formation of PGE<sub>2</sub> and maintained production of increased levels of PGD<sub>2</sub> in asthmatics.

Therefore, the effect of selective COX-2 inhibition on induced asthmatic airway obstruction and inflammation was investigated in 16 subjects with mild atopic asthma who underwent rising dose inhalation challenges with allergen and methacholine (MCh) to determine the provocative dose causing a 20% drop in FEV<sub>1</sub> (PD<sub>20</sub>) during a control study period and following 10-13 days of treatment with etoricoxib (90 mg once daily). Study periods were randomized with at least 2 weeks washout between and induced sputum cells and exhaled nitric oxide levels (F<sub>E</sub>NO) were used to assess airway inflammation. Blood assays for COX-1 and COX-2 activity to determine biochemical efficacy were performed and urinary excretion of lipid mediators was measured by mass-spectrometry. The intervention with COX-2 inhibitor in provoked asthma was not found to have any negative effects on allergen-induced airflow obstruction and sputum eosinophils, basal lung function or methacholine responsiveness. The study suggests that short-term use of COX-2 inhibitors is safe in asthmatics.

In summary: 1) The higher baseline LTE<sub>4</sub> levels found in three body matrices lends further support to CysLT-overproduction in AIA and the higher salivary levels should be explored as a new and clinically convenient biomarker of AIA and other diseases with increased CysLT-production. 2) The increased release of the 15-LO products, EXC<sub>4</sub>, and 15-HETE, in activated eosinophils from severe asthma and AIA patients, and the elevated EXC<sub>4</sub> and LTC<sub>4</sub> formation in activated eosinophils from AIA subjects in the presence of ASA support a pathophysiological role of the 15-LO pathway in AIA and severe asthma. 3) Basal biosynthesis of PGD<sub>2</sub> is increased in subjects with asthma and its formation is catalysed predominantly by COX-1. By contrast, COX-2 contributes substantially to the biosynthesis of PGE<sub>2</sub>. 4) COX-2 inhibition in provoked asthma is found to have no negative effects on allergen-induced airflow obstruction and sputum eosinophils, basal lung function or MCh responsiveness suggesting that short-term use of COX-2 inhibitors is safe in asthmatics.

## LIST OF PUBLICATIONS

This thesis is based on the following papers. The papers will be referred to by their Roman numerals (I-IV)

- I            Gaber F, **Daham K**, Higashi A, Higashi N, Gülich A, Delin I, James A, Skedinger M, Gyllfors P, Nord M, Dahlén SE, Kumlin M, Dahlén B.  
Increased levels of cysteinyl-leukotrienes in saliva, induced sputum, urine and blood from patients with aspirin-intolerant asthma.  
Thorax. 2008 Dec;63(12):1076-82.
- II            James A\*, **Daham K\***, Backman L, Brunnström Å, Tingvall T, Kumlin M, Edenius C, Dahlen S-E, Dahlen B and Claesson H-E.  
The influence of aspirin on release of eoxin C<sub>4</sub>, leukotriene C<sub>4</sub> and 15-HETE, in eosinophilic granulocytes isolated from patients with asthma.  
Int Arch Allergy Immunol (accepted)
- III            **Daham K**, Song WL, Lawson JA, Kupczyk M, Gülich A, Dahlén SE, FitzGerald GA, Dahlén B.  
Effects of celecoxib on major prostaglandins in asthma.  
Clin Exp Allergy. 2011 Jan;41(1):36-45.
- IV            **Daham K**, James A, Balgoma D, Kupczyk M, Billing B, Lindeberg A, Henriksson E, FitzGerald G A, Wheelock G, Dahlén S-E, and Dahlén B.  
Effects of selective COX-2 inhibition on allergen-induced bronchoconstriction and airway inflammation in asthma.  
(In manuscript)

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## POPULÄRVETENSKAPLING SAMMANFATTNING

Astma kännetecknas av inflammation i luftrören och leder till luftvägsbesvär i form av hosta, ökad slemproduktion och varierande grad av andnöd. Luftvägsinflammationen karakteriseras av inblandning av celler såsom eosinofiler, mastceller och neutrofiler samt en rad inflammationsförmedlande produkter. I huvudsak finns två typer av astma; allergisk och icke allergisk astma. Astma kan utlösas eller försämrats av en eller flera faktorer, t ex vid exponering för allergener, kall luft, fysisk ansträngning, luftburna kemiska ämnen och läkemedel.

Det dominerande inflammationsmönstret kan eventuellt förklara de olika typer och svårighetsgrader av astma. En special typ av astma är den aspirin-intoleranta där patienterna får luftvägsbesvär som oftast är av svårare art och ibland livshotande när de tar värktabletter som innehåller aspirin eller andra smärtstillande och inflammationsdämpande läkemedel med samma verkningsmekanism. För att med säkerhet ställa diagnosen ASA/NSAID-intolerant astma (AIA) krävs provokation med acetylsalicylsyra (ASA) som är tidskrävande och görs på specialist-kliniker med erfarenhet inom fältet. Det är en fördel att inom det kliniska arbetet hitta specifika inflammationsmarkörer. Detta gör det möjligt att särskilja de olika typerna och erbjuda de mest effektiva terapeutiska möjligheterna. Sådana biomarkörer har dock inte kommit till bredare vardagligt kliniskt arbete.

I denna avhandling har den astmatiska inflammationen och de funktionella luftvägssvaren studerats, under basala förhållanden och vid kontrollerade astmaattacker utlösta vid det kliniska laboratoriet. Avhandlingen är koncentrerad på att utreda betydelsen av nyckelmolekyler inom arakidonsyrafamiljen, dvs prostaglandiner (PG), leukotriener (LT) och härmed besläktade föreningar. Vi har studerat patienter med olika typer och svårighetsgrader av astma, i synnerhet aspirin-intoleranta och allergiska astmatiker.

I **delarbete I**, genomfördes en jämförelse mellan aspirin-toleranta och intoleranta avseende CysLT- och leukotrien (LT) $B_4$ -nivåer i saliv, sputum och *ex vivo* stimulerat blod under basala och triggade förhållanden efter bronkial provokation med ASA. Inducerat sputum, saliv, blod och urin samlades från 21 astma patienter. Elva av dessa patienter visade sig ha AIA som verifierades under studien med en positiv inhalerad ASA-provokation och de resterande tio patienter var ASA-toleranta. Urin undersöktes för CysLT,  $LTB_4$  och  $PGD_2$ -metaboliten  $9\alpha,11\beta$ - $PGF_2$ . Utandad kväveoxid mättes i utandningsluften.

**Resultat:** I jämförelse med ASA-toleranta astmatiker, hade AIA patienter högre värden av utandad kväveoxid och högre nivåer av CysLT i saliv, sputum och *ex vivo* stimulerat blod.  $LTB_4$ -nivåerna mellan de båda astma-typerna visade dock ingen skillnad. Medan  $LTE_4$  och  $9\alpha,11\beta$ - $PGF_2$  i urin ökade efter ASA provokationen, visade dessa lipidmarkörer ingen signifikant ökning i saliv eller *ex vivo* stimulerat blod.

**Diskussion:** Den högre basala  $LTE_4$  hos aspirin-känsliga astmatiker i inducerat sputum, saliv och *ex vivo* stimulerat blod stödjer den ökade CysLT-produktionen vid AIA och att den högre basala  $LTE_4$ -nivån i saliv kan vara en kliniskt användbar markör för AIA och andra sjukdomar med CysLT-



överproduktion.

I **delarbete II**, genomfördes en explorativ studie för att undersöka om en typ av vita blodkroppar, eosinofilerna, via enzymet 15-LO, har kapaciteten att bilda bioaktiva lipider som kan användas som biomarkörer för AIA. Trots den karakteristiska eosinofilin vid AIA, har man inte studerat hur ASA, i eosinofiler, påverkar syntesen av arakidonsyra metaboiter via 5- och 15-LO. Perifert blod togs från friska personer och tre astma-grupper med lindrig, svår och aspirin-intoleranta astma. Med eller utan aspirin, stimulerades de isolerade eosinofilerna med arakidonsyra och calcium ionophore för att trigga 15-lipoxygens respektive 5-lipoxygenas. 15-HETE och eoxiner mättes som 15-LO produkter och LTC<sub>4</sub> som 5-LO-produkt.

**Resultat:** Aktiverade eosinofiler från aspirin-intoleranta och patienter med svår astma producerade fem gånger så hög nivå av 15-HETE som friska och patienter med lindrig astma. Eosinofiler isolerade från samtliga astmagrupper genererade högre nivå av 15-HETE vid inkubation med ASA. Eosinofiler från aspirin-känsliga astmatiker, i närvaro av ASA, producerade signifikant högre nivåer eoxin (EX) C<sub>4</sub> och LTC<sub>4</sub>. Under samma förhållanden genererade eosinofiler från aspirin-känsliga och patienter med svår astma högre nivåer av LTC<sub>4</sub> and 15-HETE.

**Diskussion:** Aktiverade eosinofiler från patienter med AIA har kapaciteten att bilda EXC<sub>4</sub> och produktionen ökar när eosinofilerna stimuleras i närvaro av ASA. Den observerade högre produktionen av 15-HETE i eosinofiler isolerade från patienter med AIA är i linje med tidigare studier. Den ökade 15-LO-aktiviteten kan bero på ett större antal eosinofiler hos patienter med AIA men kan också bero på ökad eosinofil-funktion som möjligen förklaras av en transcellulär interaktion med blodplättarna. Fynden i denna studie stödjer en roll av 15-LO i luftvägsinflammationen vid aspirin-intolerant och svår astma

I **delarbete III**, undersöktes rollen som cyclooxygenas (COX)-1 och COX-2 spelar i biosyntesen av prostaglandin(PG) D<sub>2</sub> och PGE<sub>2</sub> under basala förhållanden. Aderton patienter med astma och sex friska personer deltog i en "cross-over" studie. En selektiv COX-2 hämmare, celecoxib 200 mg, eller placebo gavs två gånger dagligen under tre sammanhängande dagar. Lungfunktion och utandat kväveoxid mättes och urin samlades för eicosanoid-metaboliter såväl basalt som under behandlingsperioden.

**Resultat:** Celecoxib hämmade utsöndring av tetranor PGE<sub>2</sub>-metaboliten, PGEM, med 50% eller mer hos astma patienterna och de friska personerna. Däremot visade tetranor PGD<sub>2</sub>-metaboliten, PGDM, i urin ingen signifikant ändring. I jämförelse med de friska personerna, hade astmatikerna basalt högre PGDM i urin. Ingen signifikant ändring kunde ses i lungfunktionen eller utandat kväveoxid-värdena efter behandling med celecoxib.

**Diskussion:** Patienter med astma demonstrerade ökad basal biosyntes av PGD<sub>2</sub> som katalyseras huvudsakligen av COX-1. Däremot bidrar COX-2 väsentligen till biosyntesen av PGE<sub>2</sub>. Den kraftiga hämningen av biosyntesen av det bronkprotektiva PGE<sub>2</sub> och den bibehållna höga basala produktionen av det pro-inflammatoriska PGD<sub>2</sub> kan öka möjligheten för negativa långsiktiga konsekvenser på luftvägarna av selektiv COX-2 hämning.

I **delarbete IV**, deltog 16 patienter med lindrig atopisk astma i en "cross-over" studie (en behandlad och en obehandlad period) för att undersöka effekten av

selektiv COX-2 hämning på allergen-inducerad bronkkonstriktion. Patienterna genomgick inhalationsprovokationer med allergen och metakolin i början och i slutet av behandlingsperioden. Den provokativa dosen som orsakar en sänkning av FEV<sub>1</sub> med 20% följdes under den behandlade perioden (etoricoxib 90 mg en gång dagligen under 10-13 dagar) och under den obehandlade perioden. Studie-perioderna var randomiserade och separerade med minst två veckor (washout). Antalet celler i inducerat sputum och utandat kväveoxid användes som luftvägsinflammations-markörer. Blod togs för mätning av COX-1 och COX-2 aktivitet. Urin samlades för mätning av eicosanoid-metaboliter.

**Resultat:** Etoricoxib orsakade inga signifikanta förändringar i den basala lungfunktionen, allergen- eller metakolin-luftvägssvaret. Den allergen-inducerade ökningen i antalet sputum-eosinofiler visade ingen signifikant skillnad mellan de behandlade och obehandlade perioderna. Utandat kväveoxid visade heller ingen skillnad mellan perioderna. Biokemiska blodprover och den hämmande effekten på utsöndring av tetranor PGE<sub>2</sub> i urin bekräftade den selektiva COX-2 hämningen.

**Diskussion:** Denna första studie av COX-2 hämning hos allergen-provocerade patienter med lindrig atopisk astma visade inga negativa effekter av etoricoxib på allergen-inducerad luftvägsobstruktion, sputum-eosinofiler, basal lungfunktion eller metakolin-luftvägssvaret. Fynden i denna studie talar för att korttidsbehandling med COX-2 hämmare kan vara säker hos patienter med lindrig atopisk astma.

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## LIST OF ABBREVIATIONS

AA	Arachidonic acid
AHR	Airway hyperresponsiveness
AIA	ASA/NSAID-intolerant asthma, aspirin-intolerant asthma
ASA	Acetylsalicylic acid
ATA	ASA/NSAID-tolerant asthma, aspirin-tolerant asthma
BAL	Bronchoalveolar lavage
COX	Cyclooxygenase
CysLT	Cysteinyl leukotriene
EAR	Early allergic reaction, early asthma response
EIA	Enzyme immunoassay
EX	Eoxin
F <sub>E</sub> NO	Fraction of exhaled nitric oxide
FEV <sub>1</sub>	Forced expiratory flow in one second
FLAP	Five lipoxygenase-activating protein
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage colony-stimulating factor
15-HETE	15-hydroxyeicosa-5Z, 8Z, 11Z, 13E-tetraenoic acid
HV	Healthy volunteers
ICS	Inhaled corticosteroid
IgE	Immunoglobulin E
IL	Interleukin
IQR	Interquartile range
LAR	Late allergic reaction, late asthma response
LC/MS/MS	Liquid chromatography/tandem mass spectrometry
LT	Leukotriene
LO	Lipoxygenase
LPS	Lipopolysaccharide
MA	Mild asthmatics
MCh	Methacholine
NO	Nitric oxide
NSAID	Non-steroid antiinflammatory drug
PD <sub>20</sub>	Provocative dose causing a 20% fall in FEV <sub>1</sub>
PG	Prostaglandin
SA	Severe asthmatics
SD	Standard deviation
SEM	Standard error of the mean
TNF- $\alpha$	Tumor necrosis factor $\alpha$
TX	Thromboxane
VC	Vital capacity



# **1. Background**

## **1.1 Asthma – A considerable burden**

Asthma is one of the most common chronic disorders affecting both children and adults with a prevalence varying widely around the world probably due to gene-by-environment interactions. The increase in asthma among children and adolescents has recently leveled off in several westernized countries(1-3). However, diverging and opposite trends in Germany and United Kingdom have been pointed out(3-5).

In Sweden, asthma is still highly prevalent with a current prevalence between 8-10%(6). In a more recent study, the prevalence of obstructive airway symptoms common in asthma did not increase in Swedish young adults from 1990 to 2008 suggesting the previous upward trend in asthma has recently reached a plateau(7). Asthma burdens the healthcare system and the society every year because of its considerable contribution to lowered quality of life and lost productivity(6-8).

## **1.2 Definition of asthma**

The Global Strategy for Asthma Management and Prevention developed by GINA, Global Initiative of asthma, defines asthma, based on its clinical, physiological and pathological characteristics, as recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. Wide-spread, but variable airflow obstruction within the lungs is associated with these episodes which are often reversible spontaneously or with treatment. Many cells and cellular elements play a role in this chronic inflammatory disorder and the associated airway hyperresponsiveness(8).

## **1.3 Asthmatic Inflammation**

The airway inflammation in asthma, which is associated with an exaggerated contractile response of the airways to a variety of stimuli, reflects a distortion of the balance normally found between immune cells, the epithelium and the host immune response. Asthma appears to presume both exposure to appropriate stimuli and a genetic predisposition.

There is substantial evidence that human mast cells (MC) contribute to the pathophysiology of asthma via formation and release of an array of pro-inflammatory mediators and cytokines. The mast cells exhibit a tailored pathogen- and antigen-specific immune responses, i.e. the pattern of this MC contribution varies depending on the stimulus(9). Mast cells in normal human lungs are usually found in close association with blood vessels in the lamina propria. In asthmatic subjects, mast cells are observed in the airway epithelium(10), mucous glands, and the airway smooth muscle(11-13).

Mast cell precursors, derived from hematopoietic stem cells, migrate to the peripheral tissues, complete their differentiation and maturation and take up residence(14). When activated by specific antigens and IgE through FcεRI or by other endogenous or exogenous substances or stimuli, mast cells rapidly generate and release newly formed eicosanoids which can initiate, heighten or dampen inflammatory responses and cause broncho-constriction(10,15-17).

Multiple lines of evidence suggest an important immunoregulatory role of eosinophils in asthma. Eosinophil counts in the blood and eosinophilic infiltration of the lungs have long been correlated with asthma severity(18). Interleukin (IL)-5 is known to have a central role in eosinophil differentiation and survival(19). Recent studies of eosinophil depletion with anti-IL-5 therapy have shown clinical improvement in subjects with refractory asthma whose selection for the treatment was based on finding of eosinophils in sputum. Eosinophils have the capacity of elaborating lipid mediators derived from arachidonic acid via both 5- and 15-LO pathways. In eosinophils, LTC<sub>4</sub> synthase catalyzes the biosynthesis of LTC<sub>4</sub> from LTA<sub>4</sub> (20); alternatively, 15-HETE and eoxins are formed via 15-LO. Eosinophils also produce oxygen radicals, numerous cytokines e.g., IL-4, IL-5 IL-13 and TNF- $\alpha$  as well as chemokines(21). In addition to mast cells and eosinophils, the inflammatory process in asthma involves other cells like neutrophils and CD4<sup>+</sup>T lymphocytes.

Neuronal mechanisms contribute also to the pathogenesis of asthma. In addition to control of airway smooth muscle tone and gland secretion, evidence has mounted for a bidirectional interaction between inflammatory cells and airway innervation; the neuronal chemotactic activity in the lungs leads to recruitment of inflammatory cells which in turn results in release of neurotransmitters that affect not only contractility of airway smooth muscles, but also inflammatory responses(22-25).

In my research studies, the focus has been put on investigating the role of different lipid mediators, eicosanoids, on the asthmatic airway responses and the associated inflammation.

#### **1.4 Asthma phenotypes**

Different phenotypes of asthma have been recognized since many years with the focus initially put on the clinical and physiological characteristics. However, the heterogeneity/complexity of asthma requires a more precise identification of the phenotypes with the necessity to link biomarkers to phenotype(26).

In this thesis, allergic and ASA/NSAID-intolerant asthma (AIA) were studied, as eosinophils and mast cells with their inflammatory mediators are known to be involved in the pathogenesis of these two asthmatic phenotypes. The study of the role of lipid mediators released by these cells, as possible determinants of phenotypic differences, may lead to the unraveling of novel characteristic biomarkers.

##### *1.4.1 ASA/NSAID-intolerant asthma*

ASA/NSAID-intolerant asthma (AIA) is a distinct clinical syndrome characterised by chronic non-allergic asthma associated with chronic hyperplastic rhinosinusitis that is acutely precipitated/exacerbated by ingestion of ASA and related non-steroidal anti-inflammatory drugs (NSAIDs)(27). A few years after that aspirin was marketed by Bayer 1898, serious respiratory symptoms attributed to this substance were reported(28). In 1922, Widal documented the association of ASA sensitivity, asthma and nasal polyposis, and further, the first ASA challenges and desensitization were pioneered(29). This clear-cut syndrome runs an intractable course of inflammation in both upper and lower respiratory tract with an average age of onset around the third decade of life and with women being more affected than men(30,31). In 1968, Samter and Beers described a syndrome consisting of asthma, aspirin sensitivity, and nasal polyps, which came to be known as Samter's triad(32).



Components of AIA usually develop over a period of years(31,33). The majority of patients initially develop refractory rhinitis (often following viral infection) usually established by early thirties. This is followed by the development of chronic hypertrophic eosinophilic rhinosinusitis, characterized by anosmia and nasal polyposis. The reaction is not an allergy, but is triggered by the pharmacological effect of cyclooxygenase-1 (COX-1) inhibitors, whereas specific COX-2 inhibitors, so called coxibs, are generally tolerated by subjects with this asthma phenotype(34,35). The number of eosinophils in the blood and bronchial mucosa of subjects with AIA is higher in comparison with ASA-tolerant asthmatics(36,37). AIA is also characterized by overproduction of cysteinyl leukotrienes (CysLTs) at baseline and further elevation occurs after exposure to aspirin(38).

Estimates of the prevalence of ASA/NSAID-intolerant asthma reported from different parts of the world exhibit a considerable variation depending on whether the diagnosis is based on medical history alone or in combination with ASA challenge tests(39). To date, there is no *in vitro* diagnostic test for this asthmatic phenotype which is often severe and sometimes even life-threatening. A higher number of asthmatics may suffer from this intolerance reaction urging the necessity of improved diagnostic measures. Conversely, many subjects with asthma are unnecessarily warned against taking ASA and NSAIDs which are important therapeutics in treating pain and inflammatory diseases and as a prophylactic measure in cardiovascular diseases.

#### *1.4.2 Allergic asthma*

Approximately 50% of all adult asthmatics have allergic asthma which frequently coexist with allergic rhinitis(40,41). Allergic asthma is primarily an airway inflammation associated with involvement of T helper type 2 (T<sub>H</sub>2) cells that promote IgE production and recruitment of mast cells and eosinophils. T<sub>H</sub>2-type cytokines orchestrate the inflammatory cascade in allergic asthma, including T<sub>H</sub>2 cell survival (regulated by IL-4), B cell isotype switching to IgE synthesis (IL-4 and IL-13), mast-cell differentiation and maturation (IL-3, IL-9 and IL-13), eosinophil maturation and survival (IL-3, IL-5 and GM-CSF) and basophil recruitment (IL-3 and GM-CSF).

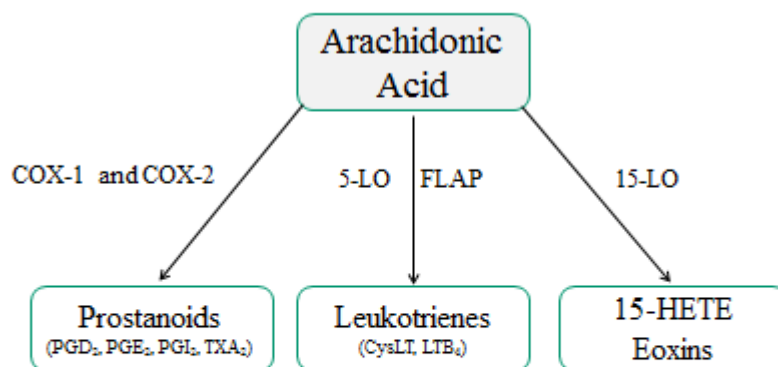
The allergic airway inflammation involves allergen-specific immunoglobulins (IgE), that bind to high-affinity Fcε receptors on the surfaces of basophils and mast cells present in the subepithelial layer of the airways leading to release of inflammatory mediators such as leukotrienes, prostaglandins, and histamine that possess the capacity to cause contraction of airway smooth muscle cells and induce edema and mucus secretion(42).

Sensitized subjects that inhale a relevant allergen develop airway constriction usually within 10 minutes of exposure. This early appearing reaction, the early asthmatic reaction (EAR), reaches a maximum within 30 minutes and resolves in general within 1-3 hours. In some subjects, the airway obstruction may persist or recur after 3-4 hours, developing into the late asthmatic reaction (LAR), to reach a maximum within 8-12 hours and lasting up to 24 hours or more(43).

### **1.5 Eicosanoids**

Eicosanoids are diverse lipid mediators of inflammation derived from the cell membrane bound polyunsaturated fatty acid precursor arachidonic acid and consist of prostanoids (prostaglandins, thromboxane and prostacyclin), leukotrienes, lipoxins and a number of additional metabolites (figure 1). These biologically active lipids serve

regulatory and homeostatic functions in inflammation and have several roles in the pathogenesis of asthma. In response to various inflammatory stimuli, the complex interplay of eicosanoids can differently influence the nature, intensity and duration of airway responses in asthma(44,45).



**Figure 1.** Schematic representation of eicosanoid pathways

In 1971, Vane demonstrated that the pharmacological actions of aspirin and related drugs were due to the inhibition of biosynthesis of prostaglandins(46). A few years later, Szczeklik and colleagues proposed a non-allergic mechanism underlying precipitation of asthmatic exacerbation by compounds sharing aspirin-like activity that inhibited cyclooxygenase enzyme in sensitive patients(47,48). In the COX pathway, arachidonic acid (AA) in cell membranes serves as a precursor for prostanoids(49).

Cyclooxygenase enzyme exists at least as two isoforms, COX-1 and COX-2(50). COX-1 is constitutively expressed in most tissues and is responsible for the basal production of prostanoids involved in “housekeeping” functions, whereas COX-2 is undetectable in most tissues, but highly inducible and can be up-regulated during inflammatory conditions(51-53).

Aspirin and related NSAIDs show different potencies in inhibiting the respective isoenzymes(54) and a positive correlation has been found between *in vitro* potency prostaglandin biosynthesis inhibition by a drug and its risk for precipitating aspirin-induced asthma symptoms(47). Thus, aspirin and NSAIDs like indomethacin and piroxicam that are more potent inhibitors of COX-1 than COX-2 isoenzyme, always precipitate asthma attacks in AIA patients.

Contrary to nonselective NSAIDs, drugs preferentially inhibiting COX-2, such as nimesulide and meloxicam, are usually well tolerated by AIA patients at therapeutic doses in these patients(35,55-57). Furthermore, there is a strong body of evidence that highly selective COX-2 inhibitors, so called coxibs, are well tolerated by patients with

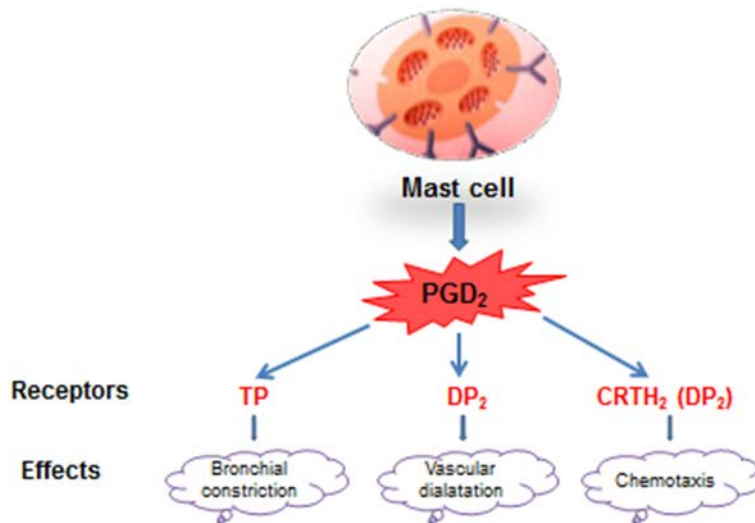
ASA/NSAID-intolerant asthma(35,58-60). Selective inhibitors of COX-2 were introduced in 1999 and celecoxib was one of the first selective COX-2 inhibitors with a selectivity estimated by the human whole blood assay, in favor of COX-2, i.e. a potent inhibitor of COX-2 and weak inhibitor of COX-1(61). Second generation selective COX-2 inhibitors have now been developed with higher selectivities for COX-2, e.g. etoricoxib with the highest selectivity in favor of COX-2(61).

Recent trials have shown a higher incidence of cardiovascular events, including myocardial infarction, in patients treated with selective COX-2 inhibitors. Biosynthesis of the anti-thrombotic prostacyclin is prevented by the selective COX-2 inhibition, while formation of the pro-thrombotic thromboxane in platelets is left unopposed(62). Lower potency against COX-1 and higher selectivity for COX-2 is in favor of lower incidence of adverse events related to stomach bleedings(63-65). Colon cancer cells synthesize prostaglandins derived via the COX-2 pathway, PGE<sub>2</sub> and PGI<sub>2</sub>. PGE<sub>2</sub> has been implicated in cancer cell proliferation and survival and PGI<sub>2</sub> in protecting cancer cells from apoptosis. COX-2 inhibitors are reported to induce cancer cell apoptosis(66,67).

In order to understand the pathophysiological effects of prostanoids in asthma, it is important to assess the endogenous formation of these lipid mediators synthesized via different pathway. In humans, metabolites of prostanoids are excreted to body fluids, such as plasma and urine. Analysis of the more abundant tetranor metabolites of prostanoids in urine by liquid chromatography-tandem mass spectrometry reflects modulated biosynthesis and will complement the use of pharmacological interventions in the further elucidation of the mechanistic pathways of these lipid mediators *in vivo*(68).

#### 1.5.1 Prostaglandin D<sub>2</sub>

Prostaglandin (PG) D<sub>2</sub> is the most abundant lipid mediator produced by mast cells that exerts its inflammatory effects through activation of three different receptors (Figure 2). The D Prostanoid 1 receptor (DP<sub>1</sub>), expressed by vascular smooth muscle and platelets is mediating vasodilatation(69,70) and inhibition of platelet aggregation(71), and the chemoattractant-homologous receptor (CRTH2), expressed preferentially by Th2 lymphocytes, eosinophils and basophils(72,73) which mediate chemotactic responses of these cells to PGD<sub>2</sub>(74). In addition, PGD<sub>2</sub> is also known to act via the receptor for thromboxane A<sub>2</sub> (TXA<sub>2</sub>), the TP receptor(75). The TP receptors are expressed on bronchial and vascular smooth muscle cells, blood platelets and myofibroblasts(76,77) and are known to mediate a strong and long-lasting contraction in these tissues(78,79).



**Figure 2.** Prostaglandin D<sub>2</sub> receptors and effects

In general, PGD<sub>2</sub> is thought to influence the asthmatic airway causing bronchoconstriction, vasodilation, increased vascular permeability and mucous formation(70,80-84). Howarth and colleagues investigated the effect of a potent TP receptor antagonist on the bronchoconstriction induced by inhaled PGD<sub>2</sub> in atopic asthmatics and found only partial protection suggesting that the vascular DP receptor may play a more important role in PGD<sub>2</sub>-induced lower airway constriction than has previously been recognized(85).

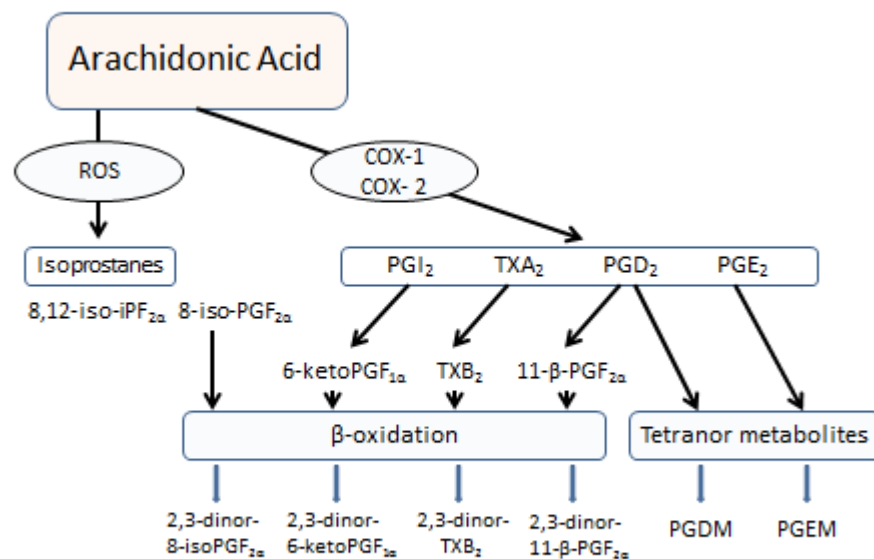
Allergen challenge has been shown to lead to rapid production of PGD<sub>2</sub> in the airways of asthmatics(17) and the nasal mucosa of allergic rhinitis(86). The ASA-induced bronchoconstriction in patients with aspirin-intolerant asthma (AIA) is followed by a significant dose-dependent increase in the urinary excretion of the early appearing PGD<sub>2</sub> metabolite, 9 $\alpha$ ,11 $\beta$  PGF<sub>2</sub>(87,88).

Measurement of PGD<sub>2</sub> and its metabolites in asthmatic subjects has mostly been performed in urine, bronchoalveolar lavage fluid, induced sputum and plasma(17,89-91). The amounts of PGD<sub>2</sub> produced by eosinophils, platelets, macrophages and Th2 lymphocytes is 100-1000 times lower than those produced by activated mast cells. Thus, the urinary PGD<sub>2</sub> metabolites serve as useful markers of mast cell activation(92-94). The “F-ring” PGD<sub>2</sub> metabolites in urine, 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> and 2,3-dinor-9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub>, has been used in the clinical studies related to asthmatic airway inflammation(93-95). Recently, the most abundant “D-ring” PGD<sub>2</sub> metabolite in urine, 11,15-dioxo-9 $\alpha$ -hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor-PGDM) was identified(68), (figure 3).

### 1.5.2 Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)

Prostaglandin E<sub>2</sub> plays an important role in regulating inflammatory processes and through four E-prostanoid (EP) receptors, EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, and EP<sub>4</sub> evokes diverse actions in humans(96,97). In the airways, the epithelial and endothelial cells, the airway smooth muscle, and the monocytes/macrophages are the main sources of PGE<sub>2</sub>

production(98). PGE<sub>2</sub> is generally thought to have proinflammatory properties in several inflammatory conditions, e.g. in rheumatoid arthritis(99). However, PGE<sub>2</sub> in respiratory tract is presumed to be bronchoprotective(100-103). O'Byrne and colleagues demonstrated that inhaled PGE<sub>2</sub> in asthmatic subjects markedly attenuated exercise bronchoconstriction which was not thought to occur through functional antagonism of airway smooth muscle(104). Furthermore, PGE<sub>2</sub> has been shown to provide almost complete protection against aspirin-induced bronchoconstriction in subjects with known AIA with inhibition of the increase in urinary LTE<sub>4</sub> following lysine-ASA bronchoprovocation(105). In atopic asthmatics, inhaled PGE<sub>2</sub> before allergen challenge prevented the decline in airflow associated with EAR and LAR(106). Following inhalation of PGE<sub>2</sub>, the increase in AHR seen during LAR was attenuated as were the number of eosinophils recovered in sputum(103).



**Figure 3.** COX pathway metabolites and isoprostanes

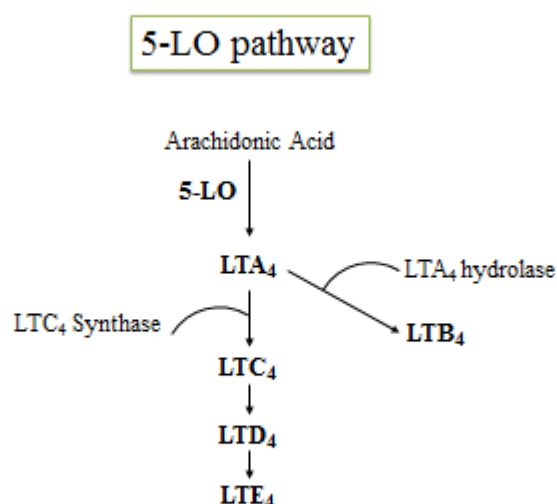
In animals, many *in vitro* studies have reported airway relaxation induced by prostaglandin E<sub>2</sub>(107,108). Early studies with unselective receptor antagonists suggested involvement of the receptor EP<sub>2</sub> in the bronchial relaxation induced by PGE<sub>2</sub> in human bronchial preparations(109). Recently, PGE<sub>2</sub>-induced bronchodilation of human bronchial was shown to be significantly blocked by a selective EP<sub>4</sub> receptor antagonist. In addition, selective EP<sub>4</sub> receptor agonist, but not selective EP<sub>2</sub> receptor agonist, resulted in relaxation of bronchial preparations pre-contracted with histamine(110). Reduced synthesis of PGE<sub>2</sub> and lowered EP<sub>2</sub> receptor expression has been suggested to provoke heightened airway inflammation in asthmatic subjects(111).

In mice, PGE<sub>2</sub>-mediated airway constriction is dependent on expression of the EP<sub>1</sub> and EP<sub>3</sub> receptors(106). It is unclear which of the PGE<sub>2</sub> receptors have constrictive effects on the human airways. However, there are ongoing studies investigating the role of TP, EP<sub>1</sub> and EP<sub>3</sub> receptors in this respect. The bronchodilatory benefits of inhaled PGE<sub>2</sub> are associated with irritancy of the upper airway resulting in a reflex cough which

is suggested to be initiated by stimulation of sensory afferent nerve endings in the airways(103). In animal models, the PGE<sub>2</sub>-induced cough is thought to be caused mainly, if not solely, by activation of the EP<sub>3</sub> receptor, e.g. EP<sub>3</sub> receptor antagonist in Guinea pigs has been shown to attenuate PGE<sub>2</sub>-induced cough *in vivo*(112).

### 1.5.3 Cysteinyl leukotrienes

The studies of the metabolism of arachidonic acid performed in late 1970's by Samuelsson and co-workers led to the discovery of the 5-lipoxygenase (5-LO) pathway and biosynthesis of leukotrienes in leukocytes(113), (figure 4). Leukotrienes are potent lipid mediators in the pathogenesis of asthma(114). Cysteinyl leukotrienes (CysLTs), LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>, appear to exert their effects through at least 2 receptors, CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors(115,116). In response to activation, CysLTs are generated by eosinophils, basophils, mast cells, macrophages, and myeloid dendritic cells(45). The gene that codes LTC<sub>4</sub> synthase is located on human chromosome 5q in a region linked with asthma and atopy(117). CysLTs are important bronchoconstrictors with LTC<sub>4</sub> being as potent as LTD<sub>4</sub> in this regard(118).



**Figure 4.** Biosynthesis of leukotrienes via 5-Lipoxygenase pathway

In AIA, bronchoconstriction following ASA challenge appears to be due to the overproduction of CysLT at baseline and after provocation(119). Asthmatic airways have been shown to be relatively more sensitive to inhaled LTE<sub>4</sub> compared to healthy individuals(120), and the inhalation of LTE<sub>4</sub> was found to increase the numbers of eosinophils in the airways(121). Clinical and *in vitro* studies have shown that CysLTs are implicated in increased mucous secretion, contraction of vascular smooth muscle and likely in extravascular leakage(122-124). While LTC<sub>4</sub> and LTD<sub>4</sub> are known to have a short half-life in the tissue, LTE<sub>4</sub> is the most stable CysLT with the longest half-life in the circulation before being excreted into the bile and urine(125). In asthmatics, CysLTs and leukotriene (LT)B<sub>4</sub> are both formed from LTA<sub>4</sub> and can be measured in body fluids, e.g. bronchoalveolar lavage (BAL) fluid, urine and blood. In humans with

asthma, the CysLTs are increased in BAL fluid and urine after allergen and aspirin provocations(126,127) and urinary release has been demonstrated in association with airway obstruction after challenge with exercise, adenosine and mannitol.

The leukotriene pathway can be inhibited via inhibition of the biosynthesis or blocking the receptors. Zafirlukast, a very potent and selective CysLT<sub>1</sub> receptor antagonist (CysLTR<sub>A</sub>), administered before allergen challenge resulted in inhibition of the immediate and the late response by approximately 80% and 50%, respectively(128). A 4-week treatment with montelukast, a potent CysLTR<sub>A</sub>, resulted in a significant reduction in the number of sputum eosinophils(129). Furthermore, montelukast, given in 16-18 hours before exercise, demonstrated sustained protection against exercise induced bronchospasm(130). Zafirlukast when combined with the antihistamine, loratadine, inhibited both EAR and LAR following allergen challenge by about 75%, and the combination was significantly more effective than either drug alone during the LAR(131). Zafirlukast has also demonstrated a beneficial effect in exercise-induced asthma and inhibited the bronchoconstrictive response to exercise by 57%(132). A specific leukotriene receptor antagonist given to ASA/NSAID-intolerant asthmatics resulted in a significant improvement in basal lung function with an average peak increase in FEV<sub>1</sub> of 18% lending support to drugs that block the action of leukotrienes as a therapeutic alternative in subjects with AIA(133).

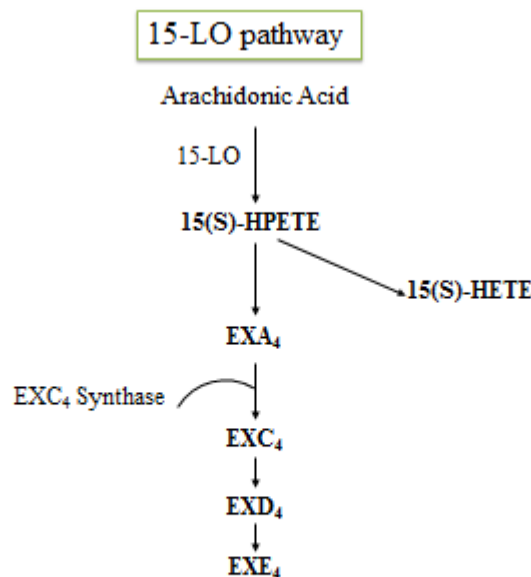
In subjects with AIA who were on regular treatment with medium to high doses of inhaled or oral glucocorticosteroids, addition of a leukotriene pathway inhibitor, zileuton, resulted in improved basal lung function, diminished nasal dysfunction with remarkable return of smell, less rhinorrhea and a trend for less stuffiness and higher nasal inspiratory. Moreover, zileuton led to a small but distinct reduction of AHR to histamine, inhibited aspirin-induced bronchoconstriction and inhibited urinary excretion of LTE<sub>4</sub>(134). These clinical trials indicate that leukotrienes are important mediators of persistent airway obstruction.

#### *1.5.4 Mediators of 15-lipoxygenase pathway*

Little is known about the biological functions of human 15-LO. Abundant amounts of 15-LO-1 exist in human airway epithelial cells, eosinophils and subsets of mast cells and dendritic cells(135-139,139). Several studies indicate a high level expression of the 15-LO in human airways(138,140,141) and asthmatics in particular express a higher number of 15-LO expressing cells that produce significantly higher amounts of 15-HETE(142).

As a major metabolite of arachidonic acid produced via the 15-LO pathway (figure 5), 15-HETE was identified by Hamberg and colleagues in lung tissue from an asthmatic subject(143). In a subsequent study, mono-HETEs, especially 15-HETE, were found to make up the bulk of arachidonic acid metabolites identified in the lungs of allergic asthmatics irrespective of whether the lung was challenged with specific allergen or calcium ionophore(144). Kumlin and colleagues also have demonstrated that airway epithelium appears to be the major source of 15-HETE in the human lung and that the significantly higher 15-HETE found in bronchi from asthmatic subjects would lend support to involvement of 15-HETE in asthmatic airway inflammation(145). Increased formation of 15-HETE is seen after inhaled allergen challenge in atopic subjects supporting involvement of 15-LO in the allergic airway inflammation(144). Furthermore, pre-inhaled 15-HETE increased the EAR significantly, whereas the LAR was not influenced(146). However, conflicting results

in this context, with both lack of effects and increased airway responses have been reported(135).



**Figure 5.** Biosynthesis of 15-HETE and eoxins via 15-Lipoxygenase pathway

Eoxins (EX), EXC<sub>4</sub>, EXD<sub>4</sub>, and EXE<sub>4</sub>, are pro-inflammatory mediators also formed via 15-LO-1 pathway in human eosinophils and mast cells(147). Eosinophils challenged with calcium ionophore produced almost exclusively LTs, whereas EXC<sub>4</sub> formation was favoured over LTC<sub>4</sub> when the eosinophils were incubated with arachidonic acid(147). Eoxins appears to increase the vascular endothelial cell permeability leading to formation of edema, a feature of inflammation(147,148). In essence, several lines of evidence indicate an increased 15-lipoxygenase activity in the lungs and airways. However, it is unclear which role 15-LO pathway plays in the asthmatic airway inflammation.

### 1.5.5 Lipoxins and Resolvins

Lipoxins, resulting mainly from the interaction between 5- and 15-LO pathways, are anti-inflammatory endogenous lipid mediators involved in the resolution of inflammation and are present in the airways of asthmatic patients. Diminished biosynthesis of these counter-regulatory mediators has been identified in severe forms of human respiratory illness, including aspirin-intolerant asthma(149) and severe steroid-dependent asthma(150). Lipoxins generated in mouse models of asthma are potent regulators of airway inflammation and hyper-responsiveness. Furthermore, lipoxins block oedema formation and reduce the levels of pro-inflammatory mediators IL-5, IL-13, prostanoids and cysteinyl leukotriene(151).

Resolvins were so-named as they were proved to be potent regulators of resolution. Resolvin E<sub>1</sub> is produced in healthy individuals and is increased in the plasma of individuals taking aspirin(152). It is possible that disruption of formation of



these pro-resolution mediators by either COX or lipoxygenase inhibitors gives rise to delayed resolution and prolonged inflammation(153).

### **1.6 Airway hyperresponsiveness**

Airway hyperresponsiveness (AHR) is an abnormal increase in airflow limitation which may vary over time, often increase during exacerbations and decrease after treatment with anti-inflammatory medications(123,154). AHR is a characteristic feature of asthma and comprises two components; a persistent and a variable AHR. The persistent component is largely attributed to structural changes in the airways collectively referred to as airway remodelling. The variable or episodic component is related to inflammatory cells and mediators influenced by numerous environmental events, i.e. allergens, respiratory tract infections and therapies(155,156).

Airway responsiveness is quantified as the provocative concentration (PC) or the provocative dose (PD) of the stimuli that cause a given fall in forced expiratory volume in one second (FEV<sub>1</sub>)(123,157). The variability in AHR provides insight into mechanisms that regulate the airway responses(155). In order to measure AHR, the provocative stimuli are differentiated into direct and indirect. The two commonly used direct stimuli, histamine and methacholine, act predominantly on the airway smooth muscle receptors, histamine 1 (H<sub>1</sub>) and muscarinic receptors, respectively(156). By contrast, indirect provocative stimuli such as exercise, mannitol, allergen, adenosine and ASA cause airflow limitation upon stimulation of inflammatory and neuronal cells with subsequent release of endogenous mediators that provoke contraction of airway smooth muscles(157).

### **1.7 Airway challenge tests**

Bronchial provocation tests used in the investigation of asthma are now well-standardized and can offer key information on the therapeutic potential of new agents and their anti-inflammatory effects on the airways. Standardized challenge tests, performed by experienced investigators, are safe and do not result in risks of persistent worsening in asthma or pulmonary function changes. In addition, such interventions expand the knowledge about the mechanistic pathways of development and persistence of airway inflammation. In the research field, inhaled allergen challenge in subjects with mild atopic asthma has gained credibility for assessment of the impact of different therapeutics with a very high negative and a reasonable positive predictive value(158).

#### *1.7.1 Methacholine challenge*

Methacholine, a muscarinic agonist, has become widely used clinically to help assess the presence and the magnitude of AHR in patients with symptoms consistent with asthma who have a normal baseline lung function(159). Methacholine has an excellent sensitivity but mediocre positive predictive value for asthma. Thus, a negative methacholine challenge excludes current asthma with a high degree of certainty. However, a positive methacholine associated with symptoms similar to those which occur naturally documents the presence of airway dysfunction and provides a basis for asthma therapy(70).

Several caveats must be considered when interpreting methacholine provocations. The most important of these are that the symptoms are current, the resting expiratory flow rate is normal and the medications which may affect the airway responsiveness are withheld for their biological duration of action prior to challenge(70). Challenge with

methacholine is currently more commonly used and is preferred to histamine; the latter being associated with more systemic adverse effects, e.g. headache, flushing, and hoarseness(160).

### *1.7.2 Inhaled lysine-acetylsalicylic acid challenge*

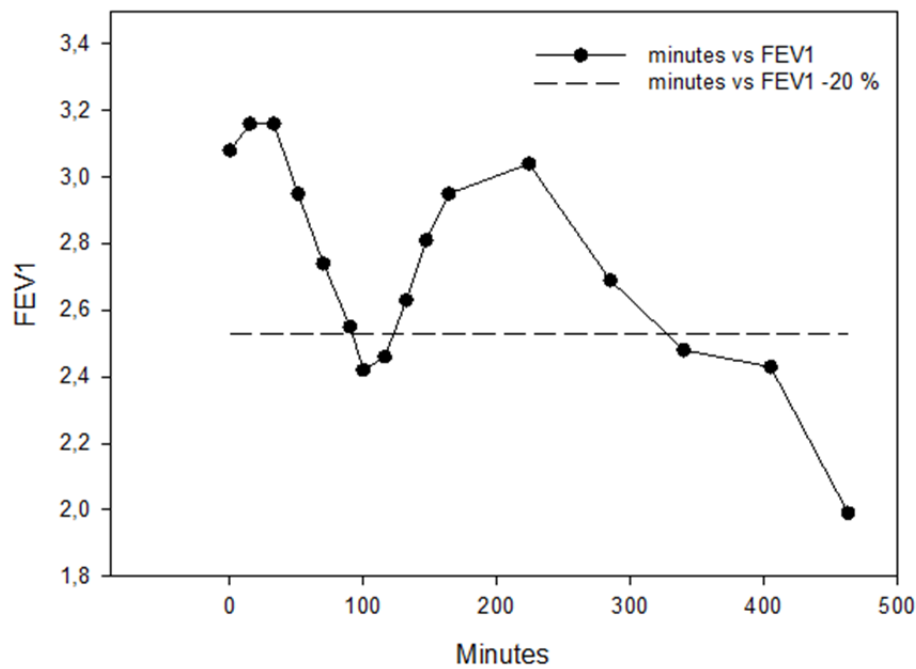
The diagnosis of ASA/NSAID-intolerant asthma is based on a reported history of asthmatic reactions precipitated or exacerbated by ASA or related NSAIDs. In cases without clear history, the diagnosis can be established with certainty only by ASA challenge tests. Oral ASA provocation has been used since the early 1970s to confirm or exclude AIA. However, this procedure is time-consuming and accompanied with the risk of severe bronchial as well as extra-pulmonary and systemic reactions(161). Nasal ASA provocation with lysine-ASA is safe and quick, but with rather low sensitivity and patients with negative nasal provocation results should therefore undergo bronchial or oral ASA challenge tests.

In 1977, Bianco et al. introduced the inhaled ASA challenge for the diagnosis of AIA(162). In a comparative study, the sensitivity of ASA bronchoprovocation has been found to be as high as that of the oral ASA challenge, with respect to detection of airway obstruction(163). The inhaled lysine-ASA challenge produces no systemic reactions and is proved safer as well as quicker to perform than the oral challenge test(161).

### *1.7.3 Allergen challenge*

Sensitized individuals challenged with inhaled allergens can develop either isolated early asthmatic responses (EAR)(43) or dual responses (EAR and LAR)(164). Inhaled allergen challenge, with its excellent reproducibility(165), has become an established tool that provides invaluable insight into the mechanisms of allergen-induced airway responses and inflammation (figure 6).

The fact that airway inflammation is a primary factor in the pathogenesis of allergen-induced asthma has been supported by many observations, e.g. LAR correlates with allergen-induced increase in airway eosinophilia; in bronchoalveolar lavage (BAL)(166) and sputum analyses(167). In addition, AHR itself has been shown to correlate positively with BAL eosinophils and metachromatic cells(168). Consequently, allergen-induced AHR and airway eosinophilia, with or without other markers of airway inflammation, have become the major components of most standardized allergen challenge studies. In standardized protocols for allergen challenge, increasing doses of specific allergen are inhaled until a 20% fall in FEV<sub>1</sub> is observed and PD<sub>20</sub> as the end-point measurement is determined(169).



**Figure 6.** Allergen bronchoprovocation in an atopic asthmatic subject with early and late asthmatic reactions

### 1.8 Sputum induction

Since the introduction of a first standardized sputum induction by Pin et al. in 1992 [187], the method has become applicable as a research and increasingly clinical tool to evaluate the presence, type and extent of the asthmatic airway inflammation. The induction procedure is relatively non-invasive and safe(170-174) with a good short-term repeatability of the induced sputum cell analysis(175-177). The mechanisms whereby inhalation of hypertonic saline results in bronchoconstriction are unknown. However, activation of airway mast cells [192] or sensory nerve endings may be involved(178). Cells obtained from induced sputum have been shown to reflect the findings from bronchial samples (bronchial wash, lavage and to a lesser extent biopsies)(179).

Cell counts in induced sputum samples are usually reported as percentage of non-squamous cells rather than the absolute number of cell. The percentage outcome is preferred due to the variation in techniques (which either use the whole expectorate or selected plugs) and the extent to which saliva may dilute the sputum(179). The extent of sputum eosinophilia is shown to be related to measures of air flow obstruction and AHR(180-182). Furthermore, increasing emphasis on characterization of the eosinophilic and non-eosinophilic asthmatic phenotypes facilitates mechanistic studies of these distinct phenotypes and their therapeutic aspects e.g., the eosinophilic phenotype which is characterized by more subepithelial fibrosis is more responsive to inhaled corticosteroids (ICS)(183-186). In connection with paper I and IV, cells in the induced sputum were studied.

### **1.9 Fractional exhaled nitric oxide (F<sub>E</sub>NO)**

Measurement of fraction of exhaled nitric oxide (F<sub>E</sub>NO), as a noninvasive test and surrogate marker of inflammation, has facilitated the assessment of underlying inflammation in asthma. Nitric oxide, predominantly produced by inducible nitric oxide synthase (iNOS), is elevated in asthmatic subjects(187) and is thought to be primarily due to an increased expression of iNOS in airway epithelial and inflammatory cells(188).

In asthma, numerous studies have demonstrated a close correlation between F<sub>E</sub>NO and eosinophilic airway inflammation measured in BAL, bronchial biopsies and induced sputum(189,190). Elevated F<sub>E</sub>NO has been found to correlate significantly with blood eosinophilia in atopic subjects. Furthermore, levels of F<sub>E</sub>NO have been found to increase when asthma control deteriorates and to significantly decrease when oral or inhaled corticosteroid therapy is administered. In addition, F<sub>E</sub>NO correlates significantly with the changes in AHR and asthma symptoms. After being extensively studied over the last two decades, F<sub>E</sub>NO has evolved from its role as a research method into clinical use in the field of asthma. However, further studies are needed to better define the use of F<sub>E</sub>NO in different clinical settings(191).

## 2. Aims

The general objective of this thesis was to investigate the impact of inflammatory mediators, with emphasis on eicosanoids, on the inflammatory and functional airway responses, under constitutive (baseline) and triggered conditions in subjects with asthma, in particular ASA/NSAID-intolerant and allergic phenotypes. In the studies documented here, several questions were considered to shed light on and to find answers for.

1. In the search for new diagnostic possibilities of AIA, one of the important questions was whether measurements of CysLTs in different body matrices, at baseline and under triggered bronchoconstriction following exposure to ASA, could serve as a new diagnostic opportunity for this distinct asthmatic phenotype. More specifically, the diagnostic potential of measurements of LTE<sub>4</sub> in induced sputum, saliva and *ex vivo* stimulated blood were to be evaluated in comparison with that in urine.
2. Can the capacity of eosinophils to produce 15-LO pathway products be used as a biomarker for AIA? Does the *ex vivo* responsiveness of eosinophils to COX inhibition in subjects with AIA differ from that of eosinophils derived from subjects with other asthma phenotypes and healthy volunteers with regard to the release of key arachidonic acid metabolites, in particular those related to activity of the two major lipoxygenase pathways, 5-LO and 15-LO?
3. Which COX isoenzyme, COX-1 or COX-2, is catalyzing the biosynthesis of the bronchoprotective and bronchoconstrictive/pro-inflammatory prostaglandins in asthma? The thesis, therefore, aimed at evaluating the role of COX-1 and COX-2 in the biosynthesis of PGD<sub>2</sub> and PGE<sub>2</sub> under basal conditions and during heightened airway inflammation and responses after inhaled allergen provocation.
4. Does treatment with selective COX-2 inhibitors impose a risk of causing deterioration of asthma? Are there any consequences of COX-2 inhibition on airway obstruction or airway inflammation during asthma exacerbations?

### 3. Methodological aspects

#### 3.1 Study subjects

The baseline characteristics of all subjects are displayed in table 1 and further details are described in the individual papers. All subjects were never smokers or non-smokers for the last two years prior to the study start with a smoking history of less than five pack years. The asthmatic subjects had stable asthma and had not suffered respiratory infections in the four weeks prior to inclusion.

**Table 1.** Subject characteristics of all papers

<b>Paper</b>	<b>Subjects number, group</b>	<b>Age (year) Mean (range)</b>	<b>Gender F/M</b>	<b>ICS budesonid eqv µg mean (range)</b>	<b>FEV<sub>1</sub>% predicted mean (range)</b>
<b>I</b>	11 AIA	45 (27-56)	8/2	640 (150-1500)	85 (73-98)
	10 ATA	46 (35-63)	6/5	400 (200-400)	97 (84-110)
	8 IA (Atopic)	35 (19-55)	4/4	-	102 (93-112)
<b>II</b>	7 AIA	39 (23-49)	4/3	560 (160-1200)	95 (73-123)
	9 SA	46 (30-60)	5/4	2018 (1280-3200)	76 (40-99)
	8 MA	38 (24-58)	7/1	495 (320-800)	99 (82-122)
	8 Healthy	36 (23-48)	5/3	-	117 (107-133)
<b>III</b>	6 AIA	42 (24-57)	3/3	590 (160-1200)	86 (68-117)
	6 ATA	29 (23-45)	4/2	410 (320-720)	93 (79-108)
	6 IA	30 (23-51)	2/4	-	104 (97-113)
	Healthy	29 (25-39)	3/3	-	-
<b>IV</b>	16 Atopic asthma	34 (23-50)	5/11	-	103 (91-118)

AIA, ASA/NSAID-intolerant asthma; ATA, ASA/NSAID-tolerant asthma; IA, Intermittent asthma; SA, Severe asthma; MA, Mild asthma; F, Female; M, Male, ICS; inhaled corticosteroid

#### 3.2 Ethical aspects

Approval from local ethics committee at Karolinska University Hospital was gained for all four studies documented in this thesis (Dnr 2003 KI syd 518/3, 04-470/1-4, 2007/865-31, 2006/728-31/2 and 2009/959-31-4) Prior to start of each study, oral and written informed consent were obtained from all subjects.

### 3.3 Study design

#### 3.3.1 Paper I

The study comprised a screening and two study visits (Figure 7). During the screening visit, informed consent was obtained followed by clinical assessment and spirometry to determine eligibility of the subjects prior to study enrollment. On a later visit, baseline measurements of F<sub>E</sub>NO and spirometry were followed by collection of urine, saliva and induced sputum. On a further visit (3-10 days after the baseline visit), inhaled lysine-ASA provocation was performed after which asthmatic subjects were assigned to two groups, AIA (*n*=11) and aspirin-tolerant asthma (ATA) (*n*=10). Before the challenge, F<sub>E</sub>NO was measured, saliva and urine were collected and blood was drawn.

During the challenge spirometric measurements were performed and saliva and urine were collected hourly. Blood and saliva samples were taken up to two hours and urine samples were collected up to three hours after the end of the challenge. CysLTs, LTB<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> were measured with enzyme immunoassay (EIA). In a parallel experiment, saliva and urine collected from eight atopic subjects with mild asthma were analyzed to study the impact of inhaled allergen on the excretion of leukotrienes.

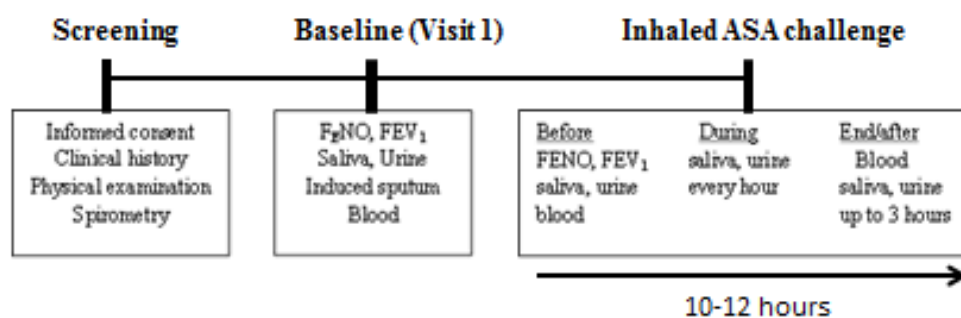


Figure 7. Study design paper 1

### 3.3.2 Paper II

The study described in **paper II** consisted of two clinic visits. During an initial visit, clinical assessment of the subjects was performed, measurements of dynamic spirometry and  $F_{E}NO$  were determined and blood was drawn for differential cell counts. The asthmatic subjects as well as the healthy volunteers underwent skin prick testing (if not previously performed) against common aeroallergens. On the second visit, 100 mL venous blood was drawn for isolation of eosinophilic granulocytes. In highly purified eosinophils, the biosynthesis of key 5- and 15-LO products was studied in presence or absence of ASA.

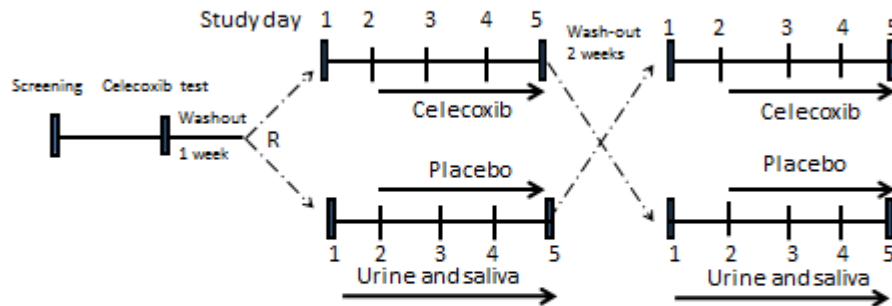
### 3.3.3 Paper III

The study documented here was randomized cross-over and single-blind with a 2-week washout separating two treatment periods (Figure 8). Each period consisted of five clinic visits in the morning on consecutive weekdays. Baseline pre-treatment measurements, done in the mornings of the first 2 study days, were followed by treatment with the study drug, celecoxib 200mg b.i.d. or placebo b.i.d., administered as capsules on study days 2–5 during each period, with the first dose taken immediately after baseline measurements on the day 2.

On an initial screening visit, informed consent was obtained and a clinical assessment including spirometry was done. Blood was drawn for routine haematology and to ensure normal liver and renal function. For safety reasons, tolerance to celecoxib was confirmed (see details in oral and airway challenges) and the test was followed by a 1-week washout. One urine sample from each subject was collected at the unit in the morning (the first morning urine was voided at home). The voided urine volume was measured and the samples were stored at  $-70^{\circ}C$  until assayed. The subjects were instructed not to take any food or beverage, except water, within 1.5 hour before sampling. Subjects were also informed to wash their mouth with water before 5mL of whole saliva was collected into a plastic tube and was stored at  $-70^{\circ}C$  until assayed.

A control group of six healthy individuals were also recruited to participate in the celecoxib study period, but did not receive placebo, to determine the effects of celecoxib on urinary prostaglandins. Measurements of  $FEV_1$  and  $F_{E}NO$  were performed and urine and saliva were collected. The levels of urinary eicosanoid metabolites and salivary  $PGE_2$  were performed by tandem LS/MS/MS and enzyme immunoassay (EIA), respectively.

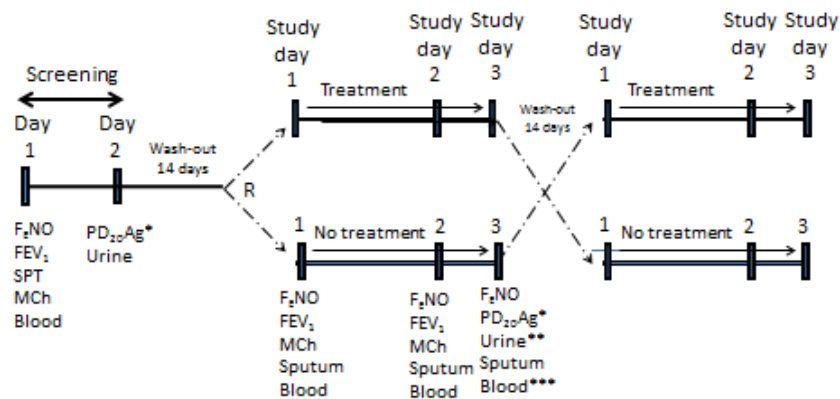




**Figure 8.** Study design **paper III**. Study day 1 and 2 = baseline days, R = Randomization

### 3.3.4 Paper IV

The study described in **paper IV** (figure 9) comprised a screening phase followed by a randomised two-period, cross-over comparison between active treatment with etoricoxib, and an untreated study period with identical design. On screening, baseline characteristics including  $F_{ENO}$ ,  $FEV_1$ , skin prick testing, total and specific IgE for the allergens and current airway sensitivity to methacholine and allergen, were obtained. The allergen challenge tests during this study were followed by a washout period of at least 14 days. Etoricoxib tablets 90 mg were administered once daily for 10-13 days with the first dose taken in the clinic on study day 1, i.e. after baseline assessments on the first day of the treatment period. Methacholine bronchoprovocation was performed on the first and the penultimate day of each period to determine  $PD_{20}FEV_1$ . An allergen inhalation challenge was then performed on the last day of each period to determine the impact of the treatment/no treatment on the airway sensitivity to allergen. Sampling of blood and induced sputum was performed on study day 1, i.e. at baseline, and on the last two days, study day 2 and 3, of each period. Sputum induction was performed one hour and six hours after the maximum  $FEV_1$  fall following methacholine and allergen provocation, respectively. Urine was collected before the start of allergen bronchoprovocation, and one and two hours after the maximum  $FEV_1$  fall.



**Figure 9.** Study design **paper IV**; two randomised periods etoricoxib 90 mg q.d. 10-13 days *vs* no treatment. \* = cumulative allergen challenge, \*\* = Urine, before, 1 and 2 hours after allergen challenge, \*\*\* = Blood before allergen challenge, R = Randomisation

### 3.4 Measurements of lung function

Measurements of lung function using a spirometer (Jaeger Masterscope, Intramedic AB, Bålsta, Sweden) have been a key part of all four studies documented in this thesis. This test provides objective, reproducible and reliable information wherein normal values depend on the height, age, gender and ethnic group of the subjects. It has been an essential tool to screen, define the respiratory impairment, quantify the severity and monitor the changes in the lung function and airway responses as well as follow-up of the subjects. The major measurements comprised the forced expiratory volume in one second ( $FEV_1$ ), vital capacity (VC), forced vital capacity (FVC) and  $FEV_1:FVC$  ratio. The peak expiratory flow rate (PEFR) was measured with a simple handheld device given to the subjects for follow-up purposes. The standards of interpretations have been performed in accordance with the recommendations of the American and European Thoracic Societies(192).

### 3.5 Measurements of fractional exhaled nitric oxide

Subjects included in the studies have undergone standard measurements of  $F_ENO$  according to the guidelines of American and European Thoracic societies to determine nitric oxide (NO) at baseline and the changes along the course of the studies. Using a chemoluminescence analyser (NIOX, Aerocrine AB, Sweden), the subjects inhaled to their total lung capacity and immediately exhale at a constant flow of 50 ml/s (against resistance to exclude possible contamination with nasal NO by means of velum closure) until a 3-second NO plateau was reached at the end of the exhalation(193).

### **3.6 Skin prick testing**

In all four papers, skin prick testing (SPT) has been a part of screening and characterization of the subjects. In paper IV, SPT has been an aiding tool, together with a suggestive clinical history and serum IgE antibodies, to identify the specific allergen used in the airway challenges. Medications were withheld according to standard procedures. The test was performed by introducing a small quantity of allergen into the epidermis by pricking the skin.

Standardized extracts of allergens including *Dermatophagoides pteronyssinus*, *Aspergillus fumigatus*, grass pollen, cat fur, and horse and dog hair (ALK, Sweden) were used as well as positive (histamine hydrochloride) and negative control solutions. The subjects are evaluated for dermographism and the reactions were recorded after 15 minutes. The longest diameter of the wheal was measured and used to assess the positivity of the skin test (41).

### **3.7 Saliva sampling and processing**

In Paper I and II, saliva was collected and studied for the levels of leukotrienes and prostaglandin E<sub>2</sub>, respectively. Subjects were informed not to take any food or beverage, except water, within 1.5 h before sampling. They were also instructed to rinse their mouth with water before collecting 5 mL of whole saliva into a plastic tube which was then stored at -70°C. The samples were thawed prior to assay, centrifuged at 1500g for 10 min (+4°C) and the supernatant was subsequently analyzed.

### **3.8 Sputum induction and processing**

Sputum induction and processing was performed using hypertonic saline and in accordance with the European Thoracic Society guidelines(194,195). Subjects were given salbutamol 0.2 mg and provided that FEV<sub>1</sub> ≥ 70% of predicted, aerosol containing increasing concentrations of sterile saline (3, 4, and 5%) was administered through an ultrasonic nebulizer for seven minutes each, through a mouthpiece without a valve or nose clip (DeVilBiss Ultraneb 3000, Dolema AB, Sweden). For safety reasons, FEV<sub>1</sub> was measured after each period of inhalation. Contamination with saliva and post nasal drip was minimized by rinsing the mouth and blowing the nose. Sputum was expectorated into a sterile container.

There are two methods for processing the expectorate: selecting viscid or dense portions (used in paper IV) and processing the entire expectorate (used in paper I) comprising sputum and variable amounts of saliva. Selected sputum has the advantage of having a squamous cell contamination of less than 5%. Squamous cell contamination of ≥20% of all recovered cells is associated with lower reproducibility of the cell counts(194). Cytospins are prepared from the cell pellet and differential cell counts are established. The cell differential counts are expressed as a percentage of the total number of non-squamous cells. Squamous cells are expressed as a percentage of the total cell number.

### **3.9 Urine collection and correction of dilution**

In paper I, III and IV, urine was collected for measurement of metabolites of eicosanoids. The first morning urine was voided at home and urine samples were collected upon visits according to the study designs. The voided urine volumes were measured and the samples were stored at  $-20^{\circ}\text{C}$  (paper I) and  $-70^{\circ}\text{C}$  (paper III and IV) until assayed. The measurements of leukotrienes and prostanoids were related to creatinine concentration to compensate for the diuresis. The alkaline picrate added to urine reacts with the creatinine resulting in a red color the intensity of which is determined spectrophotometrically at 490 nm. Following acidification, the color changes and the difference in adsorbance before and after adding the acid is proportional with the creatinine concentration which is expressed as mmol/L. The metabolites were then presented as ng/mmol of creatinine.

### **3.10 Oral and bronchial provocations**

The provocations were carried out under direct supervision of experienced physicians skilled in performing provocation. A clinical assessment was done to exclude serious reactions in connection with previous provocations, serious heart, liver or kidney diseases, respiratory tract infection within four weeks prior to challenge, pregnancy and current treatment with  $\beta$ -blockers. Subjects were instructed about the drug withdrawal before the intervention. Conditioned that the subjects were in a stable clinical condition and had a baseline  $\text{FEV}_1$  of at least 70% of predicted value, the challenge was initiated. Equipment for emergency resuscitation was readily available and an intravenous line was attached.

#### *3.10.1 Oral provocation with celecoxib*

In paper III, subjects with AIA were tested for safety reasons with regard to the tolerance of the study medication, celecoxib. Two doses of celecoxib, each of 100 mg, were given 1 hour apart followed by a 2-hour observation. Conditioned that no reaction was observed, the study proper was started after a 1-week washout.

#### *3.10.2 Bronchial challenge with lysine-acetylsalicylic acid*

Inhaled ASA challenge tests were used in the papers I, II and III. Baseline  $\text{FEV}_1$  and PEFR measured as the best of three efforts. If the baseline  $\text{FEV}_1$  was  $>70\%$  of predicted, the test was started with seven breaths of nebulized saline (0.9% sodium chloride). Provided that post-saline  $\text{FEV}_1$  was above 60% of predicted and had not decreased  $>10\%$  after 20 minutes, consecutive increasing doses of lysine-ASA were inhaled through a dosimeter-controlled jet nebulizer (Spira Elektro 2, Respiratory Care Center, Hameenlinna, Finland) every 30 minutes with  $\text{FEV}_1$  measurements every 10 minutes after each ASA-dose (table 2). The provocation was interrupted when  $\text{FEV}_1$  had fallen  $\geq 20\%$  from the post-saline baseline value, or if strong symptoms were seen, as well as when the maximum cumulative ASA-dose was reached. After a positive reaction, spirometry was carried out every 15 min (for at least one hour) until  $\text{FEV}_1$  had returned to within 90% of the post-diluent baseline value. The challenged subjects were advised to record PEFR with a handheld device hourly and in the case of airway symptoms instructed to use rescue medications at predefined level of drop in PEFR or contact the hospital.

**Table 2.** Protocol for lysine-ASA bronchial challenge(161)

Lysine-ASA Conc. (M)	Number of breaths	Dose ( $\mu\text{mol}$ )	Cumulative dose ( $\mu\text{mol}$ )
0.1	1	1	1
0.1	2	2	3
0.1	7	7	10
1.0	2	20	30
1.0	7	70	100
1.0	8	80	180
1.0	12	120	300
1.0	30	300	600

### 3.10.3 Inhaled allergen challenge

In paper IV, all subjects underwent inhaled challenges with a specific allergen, upon an initial screening and in the end of either periods of the study. Determination of post-saline baseline value of FEV<sub>1</sub> was done as mentioned in the inhaled lysine-ASA challenge. Using a dosimeter-controlled jet-nebulizer (Spira Elektro 2, Respiratory Care Center, Hameenlinna, Finland), the challenge was started by inhalation of the lowest dose of allergen followed by incremental doses administered every 20 minutes (table 3). Single spirometric measurement at 18 minutes after each dose increment was obtained. The provocation was terminated when FEV<sub>1</sub> had fallen at least 20% from the post-diluent baseline, or the maximum dose of allergen was reached (7100 SQ). After a positive reaction, spirometry was carried out every 15 min (for at least one hour) until FEV<sub>1</sub> had returned to within 90% of the post-diluent baseline value. Before leaving the clinic, the subjects were provided with a handheld PEFr device and instructed at which predefined level of drop in PEFr or in FEV<sub>1</sub> they should use rescue medication and/or contact the hospital in case of a severe late asthmatic reaction.

**Table 3.** Protocol for allergen bronchoprovocation(196)

Allergen conc. SQ/mL	Number of breaths	Dose SQ units	Cumulated dose SQ units
1000	1	7	7
1000	2	14	21
1000	7	50	71
10000	2	142	213
10000	7	497	710
100000	2	1420	2130
100000	7	4970	7100

### 3.10.4 Methacholine provocation

If the baseline FEV<sub>1</sub>, measured as the best of three efforts, was  $\geq 70\%$  of predicted, the post-diluent baseline was determined and the test was started provided FEV<sub>1</sub> did not deviate by more than 10% from the pre-diluent value. By using increasing number of breaths and different methacholine solutions, doubling increments of the dose of methacholine were administered through a dosimeter-controlled jet-nebulizer (Spira Elektro 2, Medela, Medical AB, Sweden). The methacholine solution was inhaled every three minutes. FEV<sub>1</sub> was obtained as a single measurement at 2.5 minutes after each dose increment (table 4).

The provocation was terminated when FEV<sub>1</sub> had fallen at least 20% from the post-diluent baseline, or the maximum dose of methacholine was reached (3635  $\mu\text{g}$ ). After the challenge the patients were observed until FEV<sub>1</sub> had returned within 90% of baseline, either spontaneously or after inhalation of  $\beta_2$ -agonist.

**Table 4.** Protocol for dosing of methacholine(196)

Methacholine conc. mg/mL	Number of breaths	Dose ( $\mu\text{g}$ )	Cumulated dose ( $\mu\text{g}$ )
1	2	14.2	14.2
1	4	28.4	42.6
1	8	56.7	99.3
8	2	114	213.3
8	4	227	440.3
8	8	454	894.3
64	2	909	1803
64	4	1818	3621
64	8	3635	7256

### 3.11 Enzyme Immunoassay (EIA)

In paper I, measurements of CysLTs, LTB<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> were performed in serially diluted aliquots of the respective samples by enzyme immunoassays (Cayman Chemical, Ann Arbor, Michigan, USA). Concentrations of LTE<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> in urine were given as ng/mmol creatinine. For analyses of LTB<sub>4</sub> and CysLTs in sputum, the same concentration of DTT (0.04%) as in the sputum supernatant was added to the standard curve and enzyme immunoassay buffer. Levels of CC-16 in serum were determined using an enzyme-linked immunosorbent assay for serum CC-16 from BioVendor Laboratory Medicine (Brno, Czech Republic).

In paper II, measurement of PGE<sub>2</sub> in saliva was performed with an enzyme immunoassay (EIA) from Cayman and expressed as pg/mL of saliva. The detection limit was 15pg/mL. The antibody had <0.01% cross-reactivity with other primary PG of the two-series and their immediate metabolites, but 43% cross-reactivity with PGE<sub>3</sub>, 20% with PGE<sub>1</sub> and 1–3% for isoprostanes E<sub>2</sub> and F<sub>2</sub>, respectively.

In paper III, levels of EXC<sub>4</sub>, LTC<sub>4</sub> and 15-HETE were determined using EIA according to the protocol of the manufacturer (EXC<sub>4</sub> and 15-HETE from Cayman Chemicals, LTC<sub>4</sub> from GE Healthcare).

### **3.12 Tandem liquid chromatography/tandem mass spectrometry**

In paper II, III and IV, the metabolites of eicosanoids are measured using tandem liquid chromatography/tandem mass spectrometry (LC/MS/MS). Liquid chromatography coupled to mass spectrometry is the most suitable technique for separation of multiple eicosanoid isomers (e.g, tetranor PGDM and PGEM metabolites) found in urine in low level. Levels of eicosanoid metabolites in urine were normalized to creatinine levels or integrated over time (paper IV) in order to adjust for variations in the urine production(68,197,198).

### **3.13 Isolation and incubation of eosinophils**

An initial centrifugation at 400 x g for 15 min was performed. Thereafter, the upper phase was discarded, and the lower phase was collected and subjected to dextran sedimentation for 30 min at 20<sup>0</sup>C. After sedimentation, the upper phase containing white blood cells was centrifuged once at 400 x g for 10 min. The pellet was suspended in lysis buffer (100 mM NH<sub>4</sub>Cl, 10 mM Tris-HCl, pH 7.4) and incubated for 30 min at room temperature to remove erythrocytes. Density gradient centrifugation using lymphoprep, was performed after incubation. The polymorphonuclear fraction, containing neutrophils and eosinophils was collected and subjected to magnetic cell sorting. Eosinophils were isolated by negative selection using CD16 antibodies conjugated to magnetic microbeads.

In paper II, isolated eosinophils were resuspended in PBS at a concentration of 1 x 10<sup>6</sup> cells per mL and incubated for 5 min, in the presence or absence of lysine-aspirin (200 μM), before further incubation with arachidonic acid (10 μM) or A23187 (1 μM) for 10 min. The reactions were terminated by rapid cool off on ice and centrifugation at 1,200 x g, 5 min. Supernatants were stored at -80°C prior to analysis. Due to the low differential count of eosinophils in healthy subjects, and the order in which the incubations were carried out, insufficient amounts of eosinophils were recovered for comparable analysis of LTC<sub>4</sub> in the group with healthy volunteers.

### **3.14 COX-1 and COX-2 assays**

In paper IV, measurements of thromboxane generation in clotted blood for COX-1 activity, and LPS-induced formation of PGE<sub>2</sub> in leukocytes for COX-2 activity were performed.

Levels of PGE<sub>2</sub> produced in human whole blood following stimulation with LPS were used as an index of the degree of COX-2 inhibition. Following collection into heparin containing tubes, 500μl aliquots of fresh blood were incubated with LPS at a final concentration of 100μg/ml, or the relevant negative control, for 24 hours at 37°C. At the end of the incubation, blood was centrifuged at 1200g for 5 minutes in order to obtain plasma which was stored at -80°C before immunoassay for the measurement of PGE<sub>2</sub>.

To assess COX-1 activity, blood was collected into vacutainers containing no anticoagulants, and 500μL aliquots were allowed to clot for one hour at 37°C. Thereafter, serum was separated by centrifugation and stored at -80°C. Thromboxane (TXB<sub>2</sub>) levels were later measured in the serum by enzyme-immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI, USA).

### 3.15 Statistical analysis

In **paper I**, the non-parametric tests, Mann–Whitney rank-sum and Wilcoxon signed rank, were used in connection with the levels of the mediators. Repeated measures ANOVA were applied to determine the differences between the study groups with regard to the eosinophil counts in induced sputum and data were described as means and interquartile ranges (IQR). The student's *t* test was applied for assessment of the inter-group differences in the baseline  $F_{E}NO$ . The results of  $F_{E}NO$  were expressed as means and standard deviations (SD). Repeatability of  $F_{E}NO$  measurements and levels of mediators in saliva and urine was considered to quantify measurement error using within-group correlation coefficient between the baseline values obtained on visit 1 and on the later visit (visit 2) prior to the challenge.

A p-value of  $< 0.05$  was considered significant. Data in this paper are displayed as medians and 10<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup> and 90<sup>th</sup> percentiles

In **paper II**, the sets of 15-HETE,  $EXC_4$  and  $LTC_4$  data were normally distributed according to the Kolmogorov-Smirnov test. Within-group comparisons and between-group comparisons were performed using Student's paired and unpaired *t*-test, respectively). All correlations were done using the Spearman Rank test as certain clinical characteristics were not normally distributed. A p-value of  $<0.05$  was considered statistically significant.

In **paper III**, the levels of the metabolites on three consecutive days of treatment with celecoxib or placebo were compared with the mean baseline (the 2 preceding baseline days during each period). Mean levels of urinary PGDM during the consecutive days of treatment (days 3–5), expressed either as raw values or in percent of baseline, were used to assess the effect of treatment with celecoxib and placebo. The median (25<sup>th</sup>–75<sup>th</sup> percentiles) percent inhibition of PGEM, PGIM and TXM during the 3-day treatment with celecoxib was compared with the placebo. Mean (SEM)  $FEV_1$  percent predicted and median (25<sup>th</sup> – 75<sup>th</sup> percentiles)  $F_{E}NO$  days 3–5 after treatment with celecoxib were compared with the consecutive treatment days with placebo. For lung function, paired *t*-test was used. Medians and (25<sup>th</sup>-75<sup>th</sup> percentiles) were used to determine the gender differences, with regard to excretion of metabolites at baseline and after treatment. Using the Wilcoxon signed-ranks test for the cross-over periods and the Mann–Whitney rank sum test or Kruskal–Wallis one way analysis of variance on ranks for between-group comparisons, the effects of treatments on urinary metabolites were analyzed.

In **paper IV**,  $PD_{20}FEV_1$  values for methacholine and allergen were logarithmically transformed, paired *t*-test was performed and data were presented as geometric mean and range. Analysis by RM-ANOVA within and between the study arms was performed to determine the changes in the percentage of differential cell counts, and results were presented as mean and SEM. Paired *t*-test was used to determine changes in lung function, blood pressure, urinary metabolites, blood COX-assays, and  $F_{E}NO$  (log transformed values).



## 4. Results and discussions

### 4.1 Paper I

**Baseline levels of CysLTs in saliva, sputum, *ex vivo* stimulated blood and urine are higher in ASA-intolerant asthmatics than in subjects with ASA-tolerant asthma lending further support to a deviated leukotriene metabolism with overproduction of CysLTs (figure 10)**

The clinically well-characterized asthmatic subjects were assigned to either ASA/NSAID-tolerant or intolerant groups based on the outcome of the inhaled ASA challenge test. The two resulting groups, AIA and ATA, demonstrated a high degree of similarity in their baseline characteristics. However, ASA/NSAID-intolerant asthmatics had a propensity for higher eosinophil counts in induced sputum and higher  $F_{E}NO$  values despite having larger doses of inhaled corticosteroids as well as further treatment with leukotriene receptor antagonists known to reduce  $F_{E}NO$  levels(191). This provides further support for the more pronounced eosinophilic inflammation associated with ASA/NSAID-intolerant asthma.

Compared to ASA/NSAID-tolerant asthmatics, subjects with AIA had higher basal  $LTE_4$  in saliva, sputum supernatant and in *ex vivo* stimulated whole blood. Unlike  $LTE_4$ , baseline levels of  $LTB_4$  in those biological matrices were not significantly different between the two groups. In subjects with AIA, the finding of raised baseline CysLT levels in *ex vivo* stimulated whole blood and saliva is new, whereas the observation of increased level of basal  $LTE_4$  in induced sputum replicates the results of only one previously reported study(199). Increased levels of urinary  $LTE_4$  in subjects with AIA confirmed previous data and served to validate this study.

The increased expression of  $LTC_4$  synthase in eosinophils has been suggested to explain the chronic basal CysLT overproduction and impaired baseline lung function in subjects with AIA(37). However, here the basal sputum  $LTE_4$  levels when expressed per million eosinophils, were not significantly different in AIA and ATA subjects suggesting that the raised basal CysLT levels in AIA may be explained by increased number of eosinophils rather than over-activation of eosinophils per se.

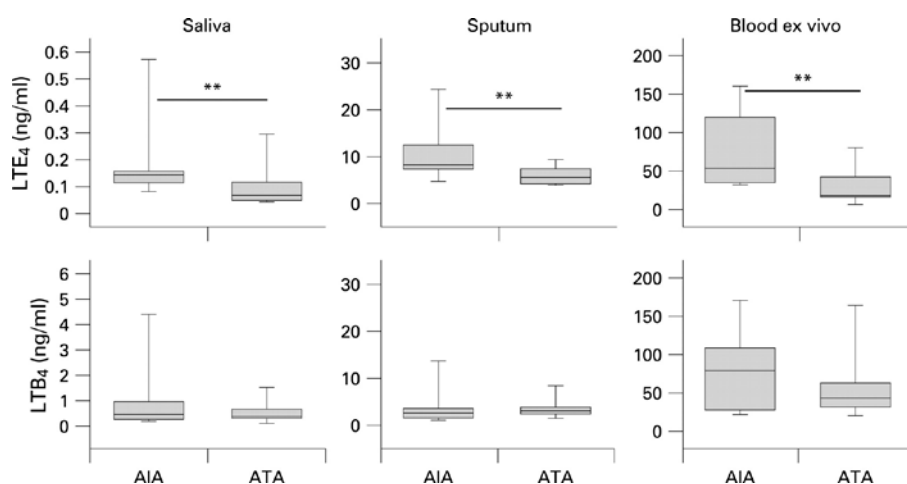
In this study, ASA/NSAID-intolerant asthmatics were for the first time shown to have higher baseline levels of salivary CysLTs than those with ATA. It has been reported previously that inhibition of 5-LO pathway has an inhibitory effect on the salivary leukotriene levels(200). With regard to the leukotriene pathway products, the study results support the proposal that saliva may provide relevant and complementary information to current standards in asthma management as a non-invasive aiding tool for assessment of the inflammatory changes and the response to therapeutics that affect this particular pathway in subjects with AIA.

In a previous study,  $LTB_4$  was detectable in exhaled breath condensate only in the presence of saliva(201) indicating that the salivary leukotrienes may serve as a possible contributory source of these mediators in other biological matrices collected orally. In this study, the possible contribution of saliva to the leukotriene content of sputum in both AIA and ATA subjects was, however, estimated to be equal as squamous cell counts in induced sputum displayed no difference between the two groups. The increments in excretion of  $LTE_4$  in urine that, as expected, followed ASA- and allergen-induced bronchoconstriction were, however, not associated with increased CysLT levels in saliva after either challenge suggesting that the quantified salivary

LTE<sub>4</sub> is unlikely to mirror direct systemic overflow from the bloodstream. Further studies are required to define the cellular source of salivary leukotrienes.

This is also the first time that whole blood from subjects with AIA has been found to display an increased capacity for ionophore-stimulated CysLT production. Measurements of the mast cell-derived PGD<sub>2</sub> metabolite 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> provided further insight into the possible source of the increased baseline production of CysLTs. The basal urinary excretion of the early appearing PGD<sub>2</sub> metabolite, 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub>, was not significantly different between the study groups regardless of sensitivity to ASA/NSAIDs confirming the results of previous studies(87). Eosinophils may serve as a possible contributory source of CysLTs as AIA is characterized by a higher degree of eosinophilia. In contrast, the association between increased urinary excretion of both PGD<sub>2</sub> metabolites and CysLTs after ASA-induced bronchoconstriction supports the mast cell as the source of these mediators during the intolerance reaction. A principal strength of this study was the status of the asthmatic subjects with well-defined characteristics and a diagnosis of AIA verified with an inhaled ASA challenge test at the time of the study.

In conclusion, higher baseline levels of CysLTs were found in saliva, induced sputum, *ex vivo* stimulated blood and urine in AIA than ATA. However, LTB<sub>4</sub> levels exhibited no differences between the two groups. This provides an additional support for a deviated leukotriene metabolism with selective overproduction of CysLTs. The higher salivary CysLT levels in AIA is a new finding that needs to be explored as a clinically convenient biomarker of AIA and other diseases associated with increased production of leukotrienes(202).



**Figure 10.** Baseline levels of LTE<sub>4</sub> and LTB<sub>4</sub> in saliva, induced sputum and plasma (from whole blood stimulated *ex vivo*) from subjects with aspirin-intolerant asthma (AIA) and aspirin-tolerant asthma (ATA).

## 4.2 Paper II

**The 15-LO pathway may contribute to asthma pathogenesis. The eosinophils are likely to be the main source of mediators of the 15-LO pathway, the biosynthetic activity of which is in part attributed to increased numbers of the cells, but also enhanced eosinophil function.**

Despite the characteristic eosinophilia in AIA, lipid mediator biosynthesis in isolated eosinophils have not been characterised before in this particular group of patients. The purpose of this explorative study was to shed light on the capacity of eosinophils to produce 15-LO pathway products and to determine whether such potential eventually could be used as a biomarker for AIA.

In this study, the first assessment of the biosynthesis of key 5- and 15-LO products was performed in highly purified peripheral blood eosinophils from subjects with AIA. Among the four groups of this study, subjects in the group with severe asthma were older than those in the other three groups and had a higher inhaled corticosteroid dose and a lower lung function than the two other asthmatic groups. Levels of F<sub>E</sub>NO and number of eosinophils in peripheral blood were higher in the groups with severe asthma (SA) and AIA than the group with mild asthma (MA). However, no significant difference in the blood eosinophils was found between SA and AIA.

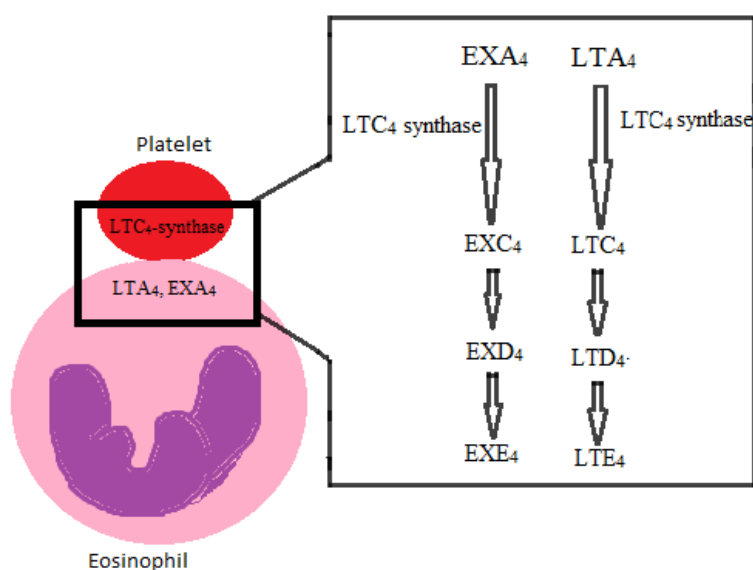
The release of 15-HETE and EXC<sub>4</sub>, both in the presence and absence of lysine-aspirin were significantly correlated with total number of blood eosinophils. Levels of 15-HETE in particular showed significant correlations with the lung function and exhaled NO. Moreover, EXC<sub>4</sub> levels correlated with the lung function when eosinophils were pre-incubated with lysine-ASA. 15-LO-1 is expressed, in addition to eosinophils, airway epithelial cells, alveolar macrophages, dendritic cells and reticulocytes, even in mast cells and might be of importance for the function of mast cells in asthma(137).

Eosinophils in AIA subjects were shown to possess the property to synthesize the novel 15-LO product EXC<sub>4</sub> which exhibited higher levels when the eosinophils were stimulated in the presence of ASA. Under these conditions, the eosinophils in AIA and severe asthma behaved in a similar fashion and also demonstrated an increased release of both LTC<sub>4</sub> and 15-HETE. The similar effects of ASA on arachidonic acid-induced EXC<sub>4</sub> and 15-HETE release on the one hand, and ionophore-induced LTC<sub>4</sub> release on the other hand, are unlikely to be explained by the effects of ASA on the activity of 15-LO pathway. As EXC<sub>4</sub> and LTC<sub>4</sub> release were similarly affected by lysine-ASA in both AIA and SA subjects, it is also unlikely that these results are attributed to the specific intolerance reaction to ASA that occurs in AIA. The most likely explanation for the effect of ASA seems therefore to be the COX inhibition with subsequent abolishment of PGE<sub>2</sub>, known to have a negative regulatory impact on the release of inflammatory mediators(203-205). Furthermore, significant correlations are found between the levels of EXC<sub>4</sub> and 15-HETE, and blood eosinophil counts. The magnitude of the differences in capacity for mediator production between the groups is in fact greater than perceived when levels are expressed per million cells (Figures 1-3). The released amounts of 15-HETE in mild asthmatic or healthy subjects corresponds to 50nM per litre blood compared to 1µM per litre blood in severe or ASA/NSAID-intolerant asthmatics.

In addition to higher eosinophil counts in AIA and SA, increased numbers of hyperactive hypodense eosinophils contribute to this heightened 15-LO activity in these two groups of asthmatics. Blood eosinophilia is known to be associated with greater

proportion of hypodense eosinophils(206) which in turn is increased in asthmatics and related to its severity(207).

With regard to the extended analysis of eicosanoid metabolism in eosinophils from two subjects with AIA, another speculative explanation was raised for the increased release of LTC<sub>4</sub> and EXC<sub>4</sub> observed in severe asthma and AIA. The highest level of any primary COX-pathway product found was TXB<sub>2</sub> which indicates biosynthesis of the platelet-specific arachidonic acid derived mediator TXA<sub>2</sub> (208,209). The finding of high level TXB<sub>2</sub> was unexpected as the eosinophils were highly purified and there were no other cells in the suspension that would primarily express TXA<sub>2</sub> synthase. However, leukocytes are known to carry adherent platelets on their surface(210). Platelets also express LTC-S and have been shown to produce LTC<sub>4</sub> from LTA<sub>4</sub> provided by other cells(211,212) and a similar mechanism could catalyse the formation of EXC<sub>4</sub> from its eoxin intermediate. Eosinophils in AIA subjects have been shown to have a far greater degree of adherent platelets than subjects with ATA(213), and these platelets account for a significant proportion of total 5-LO products. It is possible that the EXC<sub>4</sub> and LTC<sub>4</sub> formed in our suspensions could be derived from eosinophils with adherent platelets. The greater production of LTC<sub>4</sub> and EXC<sub>4</sub> in AIA and severe asthma is explained by an enhanced transcellular metabolism of cysteine-containing products (LTC<sub>4</sub> and EXC<sub>4</sub>) due to platelet-eosinophil interactions (figure 11).

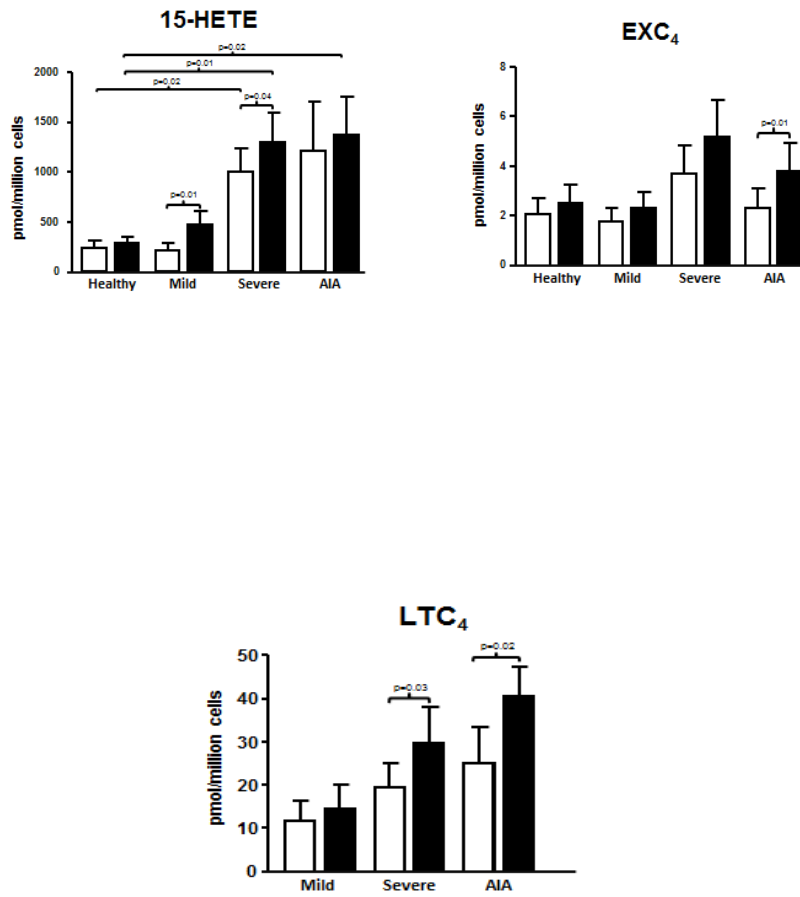


**Figure 11.** 5- and 15-LO activity via transcellular interactions between eosinophils and platelets

This study provides new evidence the 15-LO pathway mediators may contribute to asthma pathogenesis. Significant correlations were found between levels of 15-HETE production and lung function (FEV<sub>1</sub>) as well as exhaled NO. Levels of 15-HETE were high both in severe asthma and AIA. Severe asthmatics had significantly higher steroid doses than those of AIA subjects which are, however, suggested to serve as a marker of asthma severity(214) rather than to have contributed to the increased 15-LO activity. The role for 15-LO products in asthma has been reported previously with 15-HETE as the major metabolite of arachidonic acid in asthmatic lung tissue(143,144). Adult asthmatics display an increased 15-LO activity in the bronchial mucosa compared to control subjects(215). This indicates that the capacity to produce 15-HETE by eosinophils could be used as a biomarker for asthma severity. An increased activity of 15-LO pathway with increased levels of eosins in exhaled breath condensate has also been reported in childhood asthma(216). The ability of 15-HETE to conjugate to phosphatidyletanolamine and stimulate ERK phosphorylation in epithelial cells results in activation of inflammatory pathways in asthmatic lung tissue(217). Overexpression of 15-LO in airway epithelial cells *in vitro* is associated with increased release of proinflammatory cytokines(218). Involvement of 15-HETE has also been reported in mucus secretion in humans(219,220).

In conclusion, the findings in this study suggest that one role of the eosinophils in AIA may be to be a major source of 15-LO products. This higher biosynthetic activity of 15-LO pathway is in part attributed to increased numbers of eosinophils and the data in this study also suggest the contribution of increased numbers of hyperactive hypodense eosinophils and enhanced eosinophil function via transcellular interactions with platelets to this heightened 15-LO activity in AIA and severe.

Moreover, the increased 15-LO activity present in AIA and severe asthmatics was enhanced further following incubation with ASA suggesting that this effect of the latter was not exclusively due to the specific intolerance reaction in AIA, nor was it related to the level of steroid treatment. This documentation of EXC<sub>4</sub> formation in activated eosinophils from asthmatic subjects encourages further explorations of the biological role of this novel lipid mediator (figure 12).

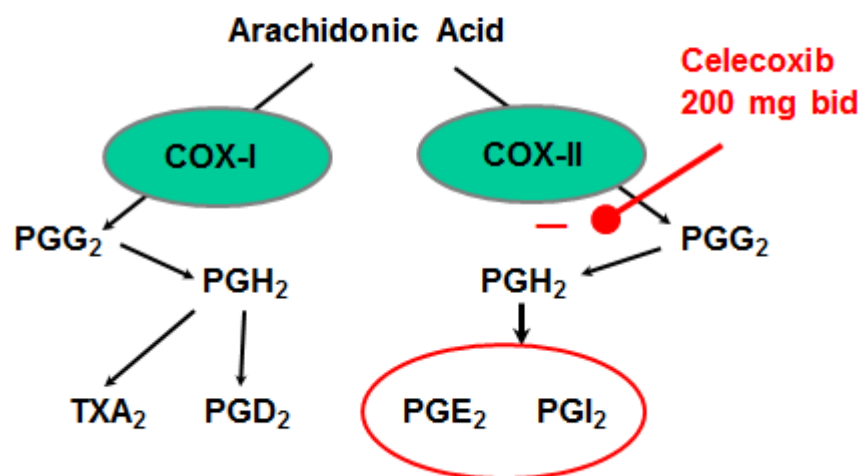


**Figure 12.** Biosynthesis of 15-HETE, EXC<sub>4</sub> and LTC<sub>4</sub> in blood eosinophils.

### 4.3 Paper III

**Basal biosynthesis of PGD<sub>2</sub> in asthmatic subjects is increased and catalyzed by COX-1, whereas whole body formation of PGE<sub>2</sub> predominantly is COX-2 dependent (figures 13, 14 and 15).**

Urinary prostanoid metabolites in the three consecutive days of treatment with celecoxib or placebo were compared with the mean baseline values of the first two untreated days of each period. The baseline values did not differ between the periods. Urinary PGDM and TXM mean levels during treatment (days 3-5) were not affected by treatment with celecoxib. In contrast, urinary PGEM values exhibited a profound inhibition which was progressive during the consecutive treatment days with celecoxib. The excretion of PGIM in urine was also significantly inhibited after treatment with celecoxib. There were higher levels of urinary PGD<sub>2</sub> metabolites in asthmatic subjects. However, there were no significant differences in urinary excretion of the studied metabolites, PGDM, PGEM, PGIM and TXM, between the three asthmatic subgroups: intermittent asthma, persistent ASA/NSAID-tolerant asthma and ASA/NSAID-intolerant asthma, neither with regard to the impact of the COX-2 inhibition nor in baseline levels of the measured urinary metabolites. Compared with the healthy controls, the asthmatic subjects displayed significantly higher basal levels of the urinary tetranor metabolite of PGD<sub>2</sub>, whereas the PGEM levels in urine were not significantly different between the groups.



**Figure 13.** Schematic representation of the conclusions in paper III Showing that biosynthesis of PGD<sub>2</sub> and TXA<sub>2</sub> is COX-1 dependent, Whereas that of PGE<sub>2</sub> and PGI<sub>2</sub> is COX-2 dependent

With regard to the salivary levels of PGE<sub>2</sub>, treatment with celecoxib resulted in a small but significant decrease in the levels of salivary PGE<sub>2</sub>, whereas no changes were seen after placebo. Moreover, there were no gender differences in salivary PGE<sub>2</sub> levels.

Therefore, this study provides new evidence that the constitutive PGD<sub>2</sub> biosynthesis in asthmatics is generated predominantly by the COX-1 isoenzyme. In addition, the study documents elevated PGD<sub>2</sub> metabolites which are 3-5 times higher than those of TXA<sub>2</sub> and PGI<sub>2</sub> and in a similar range as for urinary PGE<sub>2</sub> metabolites. The effects of celecoxib on the urinary PGDM and PGEM did not differ between the healthy controls and the asthmatics. These data are in line with previous studies of the effects of coxibs on urinary excretion of prostanoid metabolites in healthy subjects and patients with cardiovascular diseases(62,68,221,222), as well as the only previous study with asthmatics who were given a single dose of celecoxib with subsequent measurements of only PGE metabolites in urine(223). Taken together, it is concluded that COX-1, with regard to *in vivo* biosynthesis of PGD<sub>2</sub>, is the quantitatively dominant pathway that presumably apply to the mast cells as the major or almost the exclusive source of PGD<sub>2</sub> (93). Our *in vivo* data thus contrast with published data in cultured human and murine mast cells where both COX-1 and COX-2 isoenzymes contribute to the biosynthesis of PGD<sub>2</sub> (224,225).

Although urinary excretion of PGD<sub>2</sub> measures whole body biosynthesis, it is well established that the increase following challenges reflects activation of the pulmonary mast cells. Accordingly, the lungs are perfused well and the clearance of prostanoid metabolites into the bloodstream is efficient. The rate of *in vivo* biosynthesis of prostanoids at baseline is low relative to the capacity of the producing cells or tissues upon activation *ex vivo or in vitro* (226), i.e. even minor and transient increases in systemic availability of prostanoids is reflected by the detectable increases in excretion of urinary metabolites. As a result, bronchoconstrictive responses mediated by mast cell activation, are promptly reflected by increased urinary excretion of metabolites of PGD<sub>2</sub> (87,89,227,228) and TXA<sub>2</sub>(38,229). Inhaled sodium cromoglycate, a local mast cell activation stabilizer in the airways, is followed by significantly decreased urinary excretion of PGD<sub>2</sub> metabolites(228,230). Thus, it is likely that urinary PGDM levels do reflect local biosynthesis in the airways and the higher levels in asthma, observed in this study, provide circumstantial support for this argument. The finding of lower PGDM levels in healthy volunteers than in the asthmatic patients may mirror an increased mast cell activation even at baseline in asthma which is consistent with reports of increased numbers of mast cells in the smooth muscle of subjects with asthma(12). The effect of celecoxib in healthy controls and asthmatic subjects was similar suggesting that the asthmatic airway is not associated with deviations in the PGD<sub>2</sub> biosynthetic pathway. The profound reduction in the biosynthesis of the bronchoprotective PGE<sub>2</sub>, caused by the 3-day treatment with celecoxib, was neither associated with any significant changes in baseline lung function nor F<sub>E</sub>NO as a surrogate marker of airway inflammation(231).

The finding that the well characterized AIA subjects tolerated celecoxib adds to previous indications(34,58,59,223) that COX-2 inhibition does not provoke bronchoconstriction in this particular group of patients. The study results also confirm previous indications that the basal levels of PGE metabolites in urine of AIA subjects do not differ from those in non-AIA subjects(224). The tolerance of COX-2 inhibitors in AIA is not explained by the inhibition of PGD<sub>2</sub> as its biosynthesis did not alter after treatment with celecoxib. The study also confirmed previous data that basal levels of

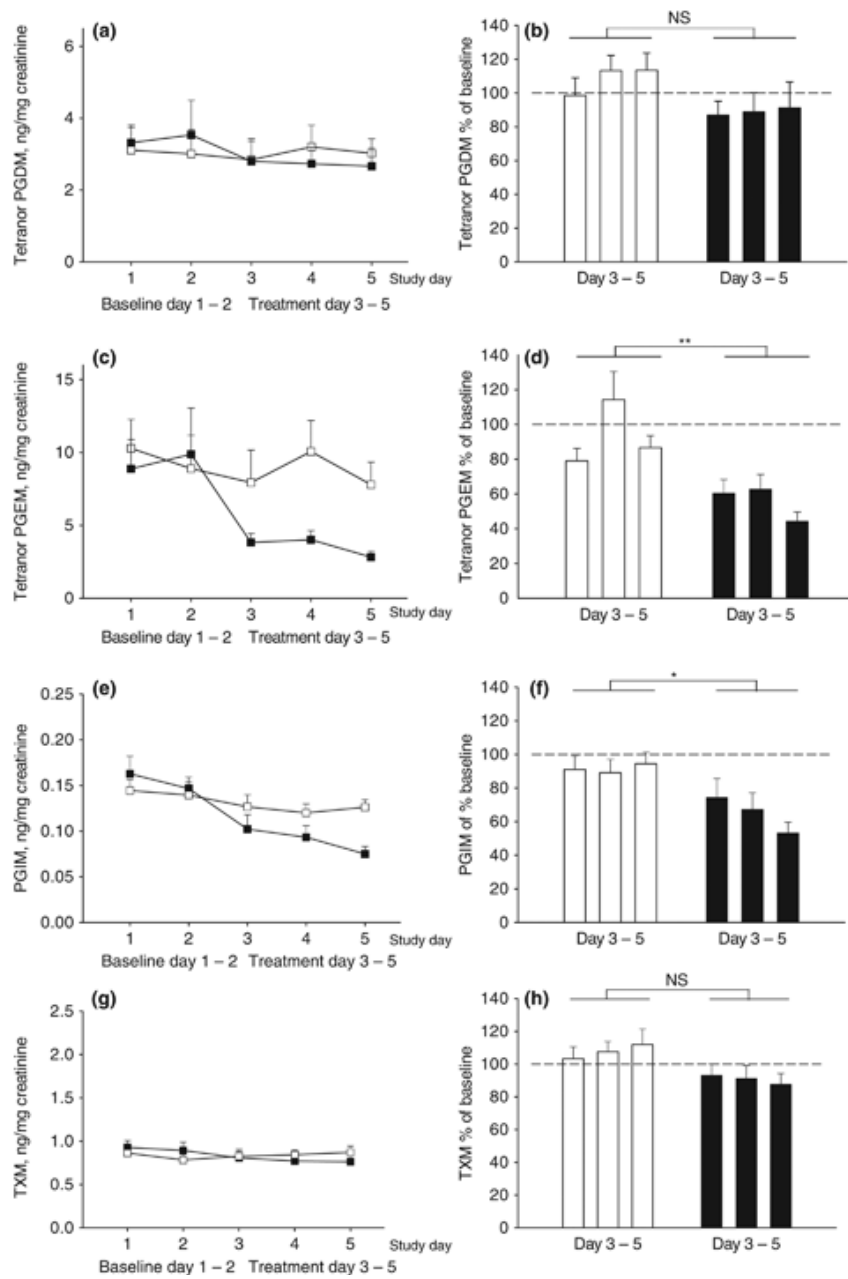


urinary PGD<sub>2</sub> metabolites in subjects with AIA and ATA are not different(12,38,229-231). One of the hypothetical explanations for ASA/NSAID-intolerance is that the stabilization of mast cells in this particular asthmatic phenotype is dependent on the bronchoprotective PGE<sub>2</sub> (161) abolishment of which in intolerance reactions leads to a paradoxical release of PGD<sub>2</sub> (87). However, in this study, the profound reduction in levels of PGE<sub>2</sub> metabolite, caused by celecoxib, was neither associated with bronchoconstriction, nor release of PGD<sub>2</sub>.

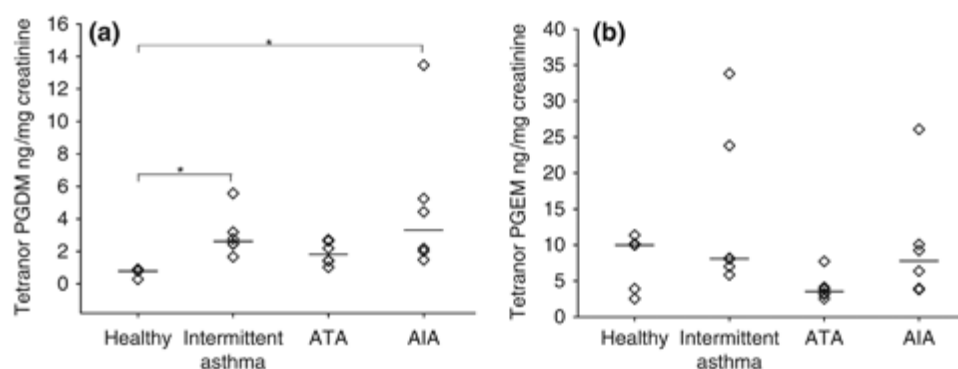
In cultured cells from airways of AIA subjects, Harrington and colleagues have demonstrated that COX-1 is the functionally predominant isoform despite detectable but low levels of COX-2 lending support to that COX-1 catalyzes formation of the PGE<sub>2</sub> in the airways and providing rationale as to why these asthmatics tolerate COX-2 inhibitors(232). The average influence of celecoxib on PGEM was smaller in ICS-treated asthmatics than the intermittent asthma group. This can be interpreted as to be consistent with the steroid-related down-regulation of COX-2(233,234), tilting biosynthesis in subjects maintained on ICS towards COX-1. The difference was, however, small, but is actually consistent with the relatively limited effects of oral steroid treatment on systemic PG formation in healthy subjects(235).

Levels of PGE<sub>2</sub> in saliva were also studied to test possible monitoring of this mediator in this non-invasive matrix using a routine EIA. However, only a minor component of the salivary PGE<sub>2</sub> levels was accounted for by a celecoxib sensitive pathway. No gender differences in the salivary PGE<sub>2</sub> levels were found, whereas an increased urinary excretion of PGE<sub>2</sub> metabolites, as previously reported, were found in males(221,236,237). The reason for or consequence of the higher levels of urinary PGE metabolites in males, observed here and in previous studies, are not clear. The gender difference, with regard to the levels of urinary PGE, is unlikely to be due to biosynthesis in the prostate or other accessory genital glands of males as the metabolites are predominantly formed in the liver. Post-menopausal females have been shown to have PGE levels comparable with those in males(237) suggesting hormonal regulation of systemic production of PGE<sub>2</sub>.

In this study, the findings of increased basal biosynthesis of PGD<sub>2</sub> in asthmatics and the whole body biosynthesis of PGE<sub>2</sub> being predominantly COX-2 dependent warrants future long-term studies to determine the clinical relevance of consequences of asymmetric inhibition of prostanoids, i.e. reduction of the bronchoprotective PGE<sub>2</sub> and maintained production of high levels of the pro-inflammatory PGD<sub>2</sub>.



**Figure 14.** Mean (SEM) urinary concentrations of (a) PGDM, (c) PGEM, (e) PGIM and (g) TXM during 2 untreated baseline days and on 3 consecutive days after daily intake of placebo (open square) or celecoxib (filled square). (b, d, f, h) Levels of the respective metabolite expressed as percent of baseline (dotted line in figures indicate baseline level).



**Figure 15.** Baseline urinary concentrations of (a) PGDM and (b) PGEM in healthy volunteers and in three groups of asthmatic subjects. Open diamonds indicate individual levels and horizontal lines indicate median value for each group

#### 4.4 Paper IV

##### **Selective COX-2 inhibition did not change allergen-induced airway obstruction or airway inflammation in subjects with mild atopic asthma. Short-term use of COX-2 inhibitors is safe in asthmatics (figures 16 and 17).**

Almost two weeks of treatment with the selective COX-2 inhibitor etoricoxib was well tolerated with respect to a number of physiological outcomes in this study. Etoricoxib, a new and highly selective COX-2 inhibitor, was used for the first time in an allergen challenge study in a cross-over manner. The atopic asthmatic subjects did not display any differences neither in the pre-challenge baseline lung function nor in the systolic or diastolic blood pressure between the treatment and control study period.

Following treatment with etoricoxib, the airway sensitivity to cumulatively increased doses of inhaled allergen was not affected with geometric mean PD<sub>20</sub>FEV<sub>1</sub> being 234 (range 31.7-5244) and 200 (range 12.2-3198) SQ units after drug and control, respectively. Neither was the immediate peak fall in FEV<sub>1</sub> nor the decrease after 30 minutes different between the study arms. Methacholine responsiveness, expressed as PD<sub>20</sub>FEV<sub>1</sub>, was not affected by etoricoxib, the geometric mean being 229 (range 29-4655) and 222 (range 56-2018) µg, after drug and control respectively

The total number of cells and the percentage of eosinophils in induced sputum increased six hours after allergen challenge during both sessions supporting the allergen-induced cellular inflammatory response. The increases after inhaled allergen in total sputum cells and the percentage of eosinophils displayed no differences between the two study periods. Neither were there differences in sputum cell numbers or differential cell counts between baseline samples and sputum samples collected after etoricoxib or after the control period. Furthermore, treatment with etoricoxib did not give rise to any significant changes in F<sub>E</sub>NO measurements within or between the two periods.

Given this profile of essentially no changes after etoricoxib treatment of the primary physiological endpoints, it was a particular strength of the study that the biochemical effectiveness of the treatment was established with appropriate methods, i.e. blood assays of COX activities and measurements of urinary metabolites.

First, the effectiveness of COX-2 selectivity was confirmed by biochemical assays where measurements of thromboxane generation in clotted blood determined the activity of COX-1 and LPS-induced formation of PGE<sub>2</sub> in leukocytes that of COX-2, respectively. After *in vivo* treatment with etoricoxib, the baseline PGE<sub>2</sub> levels were consistently inhibited and the findings supported good adherence to the treatment by the study subjects. In contrast, during the untreated period, there were no significant changes of the PGE<sub>2</sub> levels in the blood. The COX-1 activity was assessed as the level of serum TXB<sub>2</sub> after one hour of *ex vivo* clotting. There were no differences in TXB<sub>2</sub> levels between samples collected after etoricoxib or after no treatment. In the experiments using blood from control sessions, it was documented that addition of etoricoxib *ex vivo* had no effect on TXB<sub>2</sub> levels.

Second, the measurement of metabolites also validated the selective effects of the active treatment with a major inhibition in urinary excretion of the main PGE<sub>2</sub> metabolite PGEM and unaffected excretion of the corresponding major tetranor metabolite of PGD<sub>2</sub>, PGDM.

During the control arm of the study, there was increased excretion of tetranor-PGD<sub>2</sub> and 2,3-dinor TXB<sub>2</sub> in urine during the hour following the peak drop in FEV<sub>1</sub>, indicating increased biosynthesis of PGD<sub>2</sub> and TXA<sub>2</sub>. Excretion of PGD<sub>2</sub> metabolite in urine increased the most and was still higher than baseline at 2 hours post peak fall in FEV<sub>1</sub>, whereas excretion of 2,3-dinor TXB<sub>2</sub> had returned to basal levels during the second hour after the challenge. However, there was no change following the challenge in the urinary excretion of the PGE<sub>2</sub> and PGI<sub>2</sub> metabolites tetranor-PGE<sub>2</sub> and 2,3-dinor-6-keto-PGF1 $\alpha$ , respectively.

Excretion of tetranor-PGEM in urine at baseline was however significantly higher in males than females, whereas no gender differences were observed with respect to the other metabolites. Inhibition of COX-2 led to a profound reduction in the basal excretion of PGE<sub>2</sub> metabolite in urine and the levels remained depressed in the samples collected after the challenge. Etoricoxib also caused about a 50% decrease in the basal urinary excretion of the PGI<sub>2</sub> metabolite, whereas the basal and the allergen-induced levels of urinary TXA<sub>2</sub>/PGD<sub>2</sub> metabolites were unaffected by celecoxib.

A consistent and significant increase in urinary excretion of LTE<sub>4</sub> was observed following the allergen challenge. However, no difference in the urinary levels was found between the two study arms.

In this first assessment of the impact of COX-2 inhibition on airway homeostasis in atopic asthmatic patients following allergen challenge, etoricoxib had no effect on baseline lung function, airway responsiveness to methacholine, sensitivity to allergen or the magnitude of the fall in FEV<sub>1</sub> following the PD<sub>20</sub> allergen dose. As surrogate markers of asthmatic airway inflammation(231,237), the F<sub>E</sub>NO values and increased sputum eosinophils following the inhaled allergen challenge did not demonstrate any differences between the two study arms.

These findings provide evidence that using COX-2 inhibitors are safe in asthmatics and even during mild asthmatic exacerbations. The results are also in line with those from paper III where we found that COX-2 contributed substantially to whole body PGE<sub>2</sub> biosynthesis and that the increased basal biosynthesis of PGD<sub>2</sub> was exclusively catalysed by COX-1(238).

Substantial evidence from previous observations points towards a good tolerability of COX-2 inhibitors in AIA(34). The fact that the airway responses at

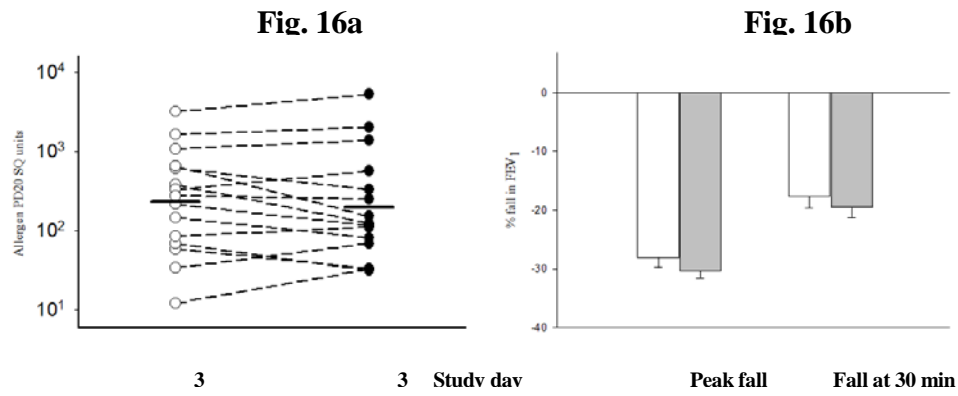
baseline and after inhaled allergen were not affected by COX-2 inhibition, suggests that COX-1 is the only enzyme of importance for PGD<sub>2</sub> formation in humans.

The atopic asthmatics in this study demonstrated signs of on-going airway inflammation including increased F<sub>E</sub>NO, sputum eosinophils and AHR to methacholine suggesting minimal involvement of COX-2 pathway in PGD<sub>2</sub> biosynthesis even under conditions of airway inflammation. This is consistent with the results of a bronchial biopsy study in seasonal allergic asthmatics, that was performed during pollen season and demonstrated involvement of 5-LO pathway without any increase in the expression of COX-2 or PGD-synthase(239). Despite the profound COX-2 related inhibition whole-body biosynthesis of PGE<sub>2</sub>, the PGE<sub>2</sub> that controls the airway dynamics was not affected which is in line with *in vitro* observations demonstrating that COX-1 is responsible for PGE<sub>2</sub> biosynthesis in human airway epithelium(232). Furthermore, the results are in agreement with the replicated studies of bronchoconstriction in AIA being triggered by non-selective NSAIDs, and not by COX-2 inhibitors(34,35,59). Early studies have suggested that NSAIDs can affect different components of the response to allergen-challenge in subjects with asthma(240,241). More recent studies have shown that non-selective COX-inhibitors have no effect on the early or late reaction to allergen challenge(229,242). However, the effects of selective COX-2 inhibitors on the allergen-induced airway responses have not been investigated previously. In addition, proper urinary excretion of prostanoid metabolites urges a pre-treatment time of at least 3-5 days(243).

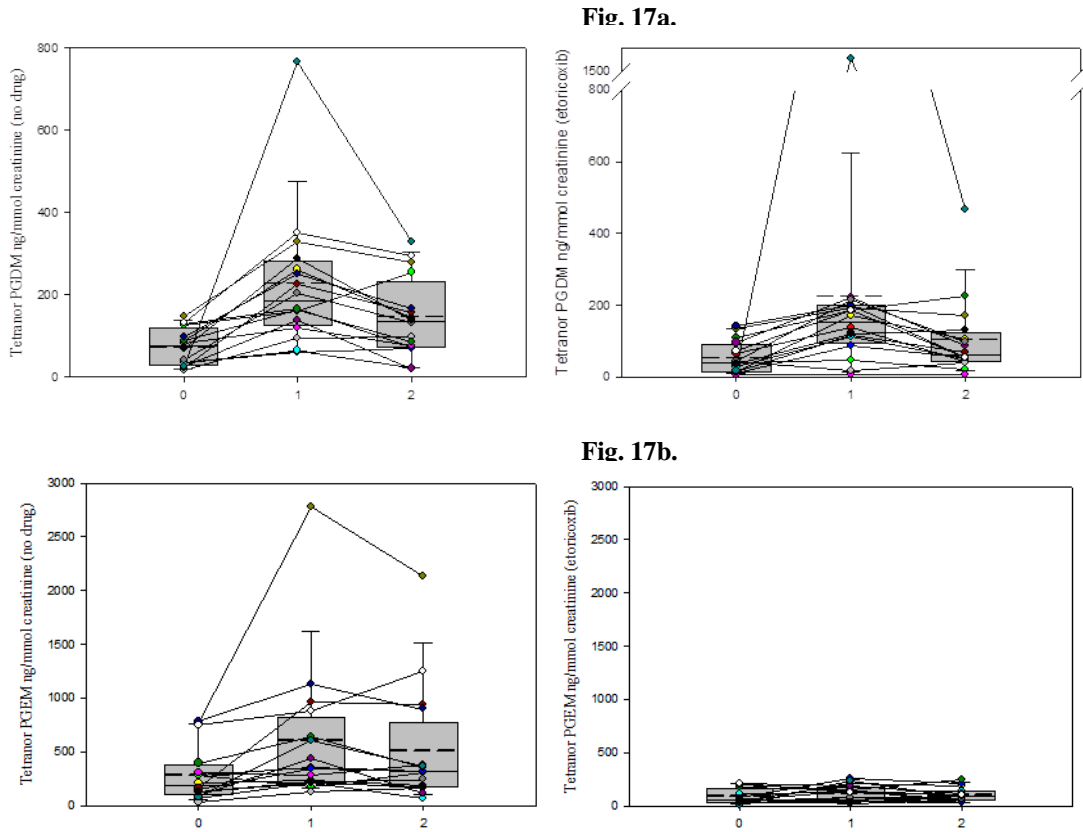
It is worth consideration that, in common mice models, selective COX-2 inhibition has been reported to enhance airway responsiveness but also to inhibit airway inflammation. Administration of a selective COX-1 inhibitor during ovalbumin (OVA) challenge in mice resulted in enhanced AHR without affecting the airway inflammatory response, while selective COX-2 inhibition caused a reduction in the inflammatory cells without affecting AHR after OVA challenge(244). Peebles and colleagues(245,246) found that inhibition of both COX isoenzymes, COX-1 and 2, resulted in enhanced AHR as well as decreased PGE<sub>2</sub> bearing in mind that both PGD<sub>2</sub> and PGE<sub>2</sub> are potent relaxants of airway smooth muscle in mice(106,247,248). In view of the findings in our study, the relevance of these mice models to human asthma may be low, at least with respect to defining the role of COX-products.

In the control session, the allergen-induced increases in the biosynthesis of LTE<sub>4</sub> and TXB<sub>2</sub> confirm the results from previous works(40,93,128,229,249). PGI<sub>2</sub> seems to have a regulatory role in allergen-induced airway inflammation in humans(144,250). However, the urinary excretion of PGI<sub>2</sub> metabolites was not affected during the untreated period.

In conclusion, selective COX-2 inhibition in the allergen-challenge setting does not appear to exert any negative effects on the lung function or the inflammatory responses in subjects with mild atopic asthma. This provides evidence that short-term use of COX-2 inhibitors may be acceptable in this group of asthmatics. However, in more severe asthma or during heightened asthmatic airway responses, the effects may differ from those demonstrated in mild asthmatic subjects with stable disease. PGD<sub>2</sub> appears to be generated predominantly by COX-1 when the allergic airway inflammation is aggravated and the data also support that the bronchoprotective PGE<sub>2</sub>, mechanistically, is derived from COX-1.



**Figure 16.** Effects of etoricoxib on: **a.** airway sensitivity to allergen **b.** FEV<sub>1</sub> fall (peak and 30 min after allergen)



**Figure 17.** Effects of etoricoxib on metabolites in urine **a.** tetranor-PGD<sub>2</sub> **b.** tetranor-PGE<sub>2</sub>, 0 = Baseline, 1 and 2 = one hour and two hours after allergen challenge, respectively.

## 5. General discussion and conclusions

The heterogeneous nature of asthma, with its different patterns of airway inflammation, prompts a more specific characterization of different phenotypes. Studying the role eicosanoids play in the asthmatic airway inflammation, under constitutive and triggered conditions, may offer the possibility of finding new diagnostic phenotype-specific biomarkers.

In ASA/NSAID-intolerant and allergic phenotypes the asthmatic inflammation is characterized by involvement of mast cells and eosinophils. The clinical characteristics of AIA, with its salient eosinophilic inflammation and overproduction of CysLTs, make this clear-cut syndrome an intriguing experimental model.

Measurement of CysLTs in different body matrices was one of the approaches used in this thesis to help explore a new test of capacity for *in vivo* CysLT release that would serve as a clinically applicable sensitive biomarker of AIA. A particular strength of the study in paper I was that AIA diagnosis was verified at the time of the study to avoid the known fluctuation in the clinical picture.

In aspirin-sensitive asthmatics, the higher basal levels of LTE<sub>4</sub> in the three body matrices (induced sputum, *ex vivo* stimulated blood and saliva), compared with those in aspirin-tolerant asthmatics, provides further support to the selective overproduction of CysLTs which has been suggested to be due to increased expression of LTC<sub>4</sub> synthase in eosinophils in this distinct phenotype. However, when sputum CysLT levels were expressed per million eosinophils, the levels were not greater in the AIA group suggesting that the increased baseline levels of CysLTs in AIA may be due to increased numbers of eosinophils rather than an overactivation of each eosinophil(37,251). This study has for the first time shown higher baseline levels of salivary CysLTs in aspirin-sensitive asthmatics than subjects with aspirin-tolerant asthma. However, there was no increase in salivary CysLTs after either bronchial challenges suggesting that salivary levels of CysLTs are unlikely to reflect direct overflow from the circulation. Measurement of CysLTs in saliva appears to introduce an advantage over that in other body matrices with regard to the non-invasive nature of saliva and that it offers a direct assessment of *in vivo* concentrations of LTs. However, it has to be addressed that methods of collecting and processing saliva have to be optimized. The influence of diurnal variation and the lack of a dilution factor are issues that also should be considered. Furthermore, it is important to determine the sources of leukotrienes in saliva, e.g. factors related to the cells in the oral cavity. The observation that basal CysLT levels are raised in *ex vivo* stimulated whole blood from subjects with AIA is also new. However, the method is time-consuming with the need of an immediate incubation of the cells. Measurement of CysLTs in asthmatic subjects is important from the diagnostic and therapeutic point of view as the biosynthesis of leukotrienes is not affected by treatment with corticosteroids despite their widespread anti-inflammatory action(252,253)(132).

In an exploratory experiment, highly purified eosinophils in subjects with AIA revealed the capacity to release the 15-LO product, EXC<sub>4</sub>, which was higher when the cellular *ex vivo* responsiveness to COX inhibition was tested through incubation with ASA.

The similarity between the aspirin-sensitive and severe asthmatics, with regard to the effect of ASA on release of arachidonic acid-induced 15-LO products (EXC<sub>4</sub> and 15-HETE) and ionophore-induced 5-LO product (LTC<sub>4</sub>), indicates that aspirin

hypersensitivity is unlikely to be involved in the underlying mechanism. The most likely explanation is therefore that the COX inhibition abolished PGE<sub>2</sub> known to exert an important negative regulatory role on mediator release in inflammatory cells in general. In addition to the known eosinophilia in AIA and SA groups, another contributing factor to the remarkable degree of 15-LO activity would be increased numbers of hyperactive hypodense eosinophils. These findings indicate that the capacity of eosinophils to produce 15-LO products could be used as a biomarker for asthma severity. The possible involvement of 15-HETE in aspirin-sensitive and severe asthma suggests that therapies targeting the 15-LO pathway might be of value in treatment of these two asthma phenotypes. However, further exploration of the biological role of lipid mediators synthesized via 15-LO are required. Some metabolites such as lipoxin A<sub>4</sub> have even been suggested to be protective factors(254) which would prompt for agonist intervention.

In this thesis, pharmacological interventions using selective COX-2 inhibitors were performed to evaluate the role of COX-1 and COX-2 iso-enzymes in the biosynthesis of the bronchoconstrictive/proinflammatory PGD<sub>2</sub> and the bronchoprotective PGE<sub>2</sub> in asthmatic subjects under basal and triggered conditions.

The basal biosynthesis of PGD<sub>2</sub> was maintained during three days of treatment with celecoxib indicating that formation of this pro-inflammatory prostaglandin is catalyzed by COX-1. In contrast, COX-2-inhibition caused a profound inhibition of biosynthesis of PGE<sub>2</sub> indicating that whole body biosynthesis of PGE<sub>2</sub> is predominantly COX-2 dependent. Thus, COX-1 was shown to be the quantitatively dominant pathway for *in vivo* biosynthesis of PGD<sub>2</sub>, and this would presumably apply to the mast cell that is thought to be the major source of PGD<sub>2</sub> in humans. Subjects with asthma were shown to have higher basal biosynthesis of PGD<sub>2</sub> without any difference between the asthmatic phenotypes.

The unaltered biosynthesis of PGD<sub>2</sub> after celecoxib indicates that the tolerance of COX-2 inhibitors in AIA is not explained by inhibition of PGD<sub>2</sub>. The paradoxical increased release of PGD<sub>2</sub> during the intolerance reaction(87) is thought to be attributed to mast cell activation occurring as a result of removal of protective PGE<sub>2</sub>.

Close to two weeks of treatment with the selective COX-2 inhibitor etoricoxib was well tolerated with respect to a number of physiological outcomes in this study. The traditional method of measuring PD<sub>20</sub> to allergen was applied because this a reliable index of sensitivity to allergen. Etoricoxib is a new and highly selective COX-2 inhibitor and this strategy was considered suitable for the first time use in an allergen challenge setting.

This first investigation of the impact of COX-2 inhibition on airway homeostasis in atopic asthmatic patients following allergen challenge demonstrated no significant effects on the baseline lung function, AHR to methacholine, sensitivity to allergen or the magnitude of FEV<sub>1</sub> fall for the PD<sub>20</sub> allergen dose. Furthermore, the surrogate marker of airway inflammation, exhaled nitric oxide, and the allergen-induced increase in sputum eosinophils displayed no significant differences in comparison with the untreated study arm. These results support the notion that short-term use of a coxib is safe in asthmatics, even during a mild asthma attack.

A particular strength of the study was that the blood assays for COX-1 and COX-2 inhibition confirmed that the study participants had complied with the treatment and that only COX-2 activity, measured as LPS-induced PGE<sub>2</sub> formation, was inhibited. In addition, there was a profound inhibition of PGEM excretion in urine, whereas urinary



PGDM was unaffected. These results demonstrate that COX-1 by far is the most important enzyme for PGD<sub>2</sub> formation in humans. Our data also suggest that the PGE<sub>2</sub> which controls airway dynamics is not affected by systemic COX-2 inhibition, despite the pronounced involvement of COX-2 in whole-body PGE<sub>2</sub> formation. This finding is consistent with *in vitro* observations demonstrating that COX-1 is responsible for PGE<sub>2</sub> biosynthesis in human airway epithelium(232). These results warrant future studies with selective receptor antagonists or selective inhibitors of distal class-specific isomerases (e.g. PGD-synthases or PGE-synthases) to define the role of individual prostaglandins in allergen evoked reactions.

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