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**NON-INVASIVE ANALYSIS OF
LEUKOTRIENES AS BIOMARKERS OF
AIRWAY INFLAMMATION: METHOD
DEVELOPMENT AND CLINICAL
APPLICATIONS**

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Institutet**

Stockholm 2008

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Published by Karolinska Institutet. Printed by Karolinska University Press
Box 200, SE-17177, Stockholm, Sweden

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ISBN 978-91-7409-143-4

ABSTRACT

Asthma and chronic obstructive pulmonary disease (COPD) are two common diseases characterized by chronic airway inflammation, but the inflammation in asthma is different from the one in COPD. In addition, asthma is a heterogeneous disease presenting with many different phenotypes (*e.g.* allergic asthma, aspirin-intolerant asthma), and different patterns of airway inflammation. A better characterization of the different phenotypes allows more accurate diagnosis and consequently tailor-made therapies. There is a need to develop new non-invasive methods for diagnosis of airway inflammation in asthma and COPD.

The use of exhaled breath condensate (EBC) has gained considerable interest as a new non-invasive technique for measuring markers of airway inflammation. The thesis examined the possibility that saliva could be a new, non-invasive means to measure markers of airway inflammation. The specific focus concerned the analysis of leukotrienes (LTs) in EBC and saliva. Leukotrienes generated by the 5-lipoxygenase (5-LO) pathway are potent inflammatory lipid mediators implicated in asthma, allergy and COPD. The cysteinyl-leukotrienes (CysLTs = LTC₄, LTD₄ and LTE₄) mediate central components of asthmatic airway inflammation, whereas leukotriene B₄ (LTB₄) is a very potent chemotactic and proinflammatory agent.

In **paper I**, LTB₄ and the saliva marker α -amylase were measured in EBC and sputum, collected before and after challenges that induced a strong neutrophilic inflammation in healthy subjects. For comparison, LTB₄ was measured in saliva from healthy subjects. **Results:** Only four out of 102 EBC samples had detectable LTB₄ (28–100 pg/mL). α -amylase activity was detected in the LTB₄-positive samples. In contrast, LTB₄ was detected in all examined sputum supernatants in the same study (median 1,190 pg/mL). The median LTB₄ level in saliva was 469 pg/mL. **Conclusion:** High levels of LTB₄ in saliva and the presence of LTB₄ in EBC only when α -amylase was detected, indicate that LTB₄ found in EBC is the result of saliva contamination.

In **paper II**, LT levels in urine, blood stimulated *ex vivo*, and saliva were compared, using the 5-LO inhibitor zileuton to assess the sensitivity of different sampling methods to detect inhibition of leukotriene formation. Healthy non-atopic subjects and atopic individuals (with or without asthma) were treated with zileuton. Blood, urine and saliva were collected before and after treatment. To investigate further the effects of zileuton, immunoexpression of 5-LO pathway enzymes was quantified in peripheral blood leukocytes by flow cytometry, and fraction of exhaled nitric oxide (FE_{NO}) was monitored. **Results:** LTB₄ and LTE₄ concentrations in saliva and blood stimulated *ex vivo*, were significantly decreased after zileuton treatment ($p < 0.05$), but urinary LTE₄ was not significantly altered. Flow cytometry showed high levels of 5-LO, FLAP and LTA₄ hydrolase expression in neutrophils and LTC₄ synthase in eosinophils, but zileuton had no acute effect on the expression of these enzymes. FE_{NO} was also unaffected. **Conclusion:** Leukotrienes in saliva are sensitive and convenient markers of altered 5-LO activity *in vivo*, as demonstrated by response to zileuton treatment.

In **paper III**, LT levels in saliva were compared with levels in sputum, blood stimulated *ex vivo*, and urine from subjects with aspirin-intolerant asthma (AIA) and aspirin-tolerant asthma (ATA), respectively. FE_{NO} and urinary 9 α ,11 β -prostaglandin F₂ (9 α ,11 β -PGF₂) were also measured. Samples were collected before and after aspirin provocation. **Results:** Subjects with AIA had higher basal levels of FE_{NO} and CysLTs in saliva, sputum, blood *ex vivo* and urine than subjects with ATA. There were no differences in basal levels of LTB₄ or 9 α ,11 β -PGF₂ between the groups. Levels of urinary LTE₄ and 9 α ,11 β -PGF₂ increased after aspirin provocation in AIA subjects, whereas LT levels in saliva and *ex vivo* stimulated blood were not increased post challenge. **Conclusion:** The findings support a global and specific exaggeration of CysLT production in AIA. Measurement of CysLTs in saliva has the potential to be a new and convenient non-invasive biomarker of AIA.

Overall conclusions: 1) Saliva is one likely source of LTB₄ in exhaled breath condensate; 2) Leukotrienes in saliva may be used as novel and sensitive biomarkers of altered *in vivo* 5-LO activity; 3) Cysteinyl-leukotrienes in saliva may be used as a new and non-invasive biomarker of aspirin-intolerant asthma.

Keywords: airway inflammation; aspirin-intolerant asthma; biomarker; exhaled breath condensate; FE_{NO}; induced sputum; leukotrienes; 5-lipoxygenase; non-invasive analysis; saliva

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-III)

- I. **Gaber F**, Acevedo F, Delin I, Sundblad BM, Palmberg L, Larsson K, Kumlin M, and Dahlén S-E.
Saliva is one likely source of leukotriene B₄ in exhaled breath condensate.
Eur Respir J. 2006 Dec;28(6):1229-35.
- II. **Gaber F***, James A*, Delin I, Wetterholm A, Sampson AP, Dahlén B, Dahlén S-E, and Kumlin M.
Assessment of in vivo 5-lipoxygenase activity by analysis of leukotriene B₄ in saliva: effects of treatment with zileuton.
J Allergy Clin Immunol. 2007 May;119(5):1267-8.
- III. **Gaber F**, Daham K, Higashi A, Higashi N, Gülich A, Delin I, James A, Skedinger M, Gyllfors P, Nord M, Dahlén S-E, Kumlin M, and Dahlén B
Increased levels of cysteinyl-leukotrienes in saliva, induced sputum, urine and blood from aspirin-intolerant asthmatics.
Thorax. 2008 Aug 29; [Epub ahead of print]

*equal contribution

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AIA	Aspirin-intolerant asthma
ATA	Aspirin-tolerant asthma
BAL	Bronchoalveolar lavage
BLT ₁	B Leukotriene receptor 1
BLT ₂	B Leukotriene receptor 2
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CysLT	Cysteinyl-leukotriene
CysLT ₁	Cysteinyl leukotriene receptor type 1
CysLT ₂	Cysteinyl leukotriene receptor type 2
EBC	Exhaled breath condensate
EIA	Enzyme immunoassay
FE _{NO}	Fraction of exhaled nitric oxide
FEV ₁	Forced Expiratory Volume in 1 Second
FLAP	Five-lipoxygenase activating protein
IL	Interleukin
L-ASA	Lysine-aspirin
5-LO	5-lipoxygenase
LT	Leukotriene
LTA ₄	Leukotriene A ₄
LTA ₄ H	Leukotriene A ₄ hydrolase
LTB ₄	Leukotriene B ₄
LTC ₄	Leukotriene C ₄
LTC ₄ S	Leukotriene C ₄ synthase
LTD ₄	Leukotriene D ₄
LTE ₄	Leukotriene E ₄
NSAIDs	Non-steroidal anti-inflammatory drugs
PG	Prostaglandin
PGD ₂	Prostaglandin D ₂
PGE ₂	Prostaglandin E ₂
9 α ,11 β -PGF ₂	9 α ,11 β -Prostaglandin F ₂
RP-HPLC	Reverse-phase high performance liquid chromatography
T _H 2	T helper 2
U	Units

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are two common airway inflammatory diseases.

Asthma affects around 300 million people worldwide, a figure that is expected to increase to 400 million by the year of 2025 (1, 2). The global prevalence of asthma varies between 1 and 18% (in Europe between 5 and 10%). Asthma is a chronic inflammatory airway disorder, which is also characterized by airway hyperresponsiveness (*i.e.* an excessive bronchoconstrictor response to inhaled triggers that does not have an effect on healthy airways) (1) and remodeling of the airways (*i.e.* structural changes of the airways) (3). The symptoms include recurring episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning, as described by the Global Initiative for Asthma (GINA) guidelines (1). The GINA guidelines further state that: “these episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment. The chronically inflamed airways become hyperresponsive to different risk factors, such as allergens, occupational or chemical irritants, tobacco smoke, respiratory (viral) infections, exercise, strong emotional expressions, and drugs (such as aspirin and beta blockers)” (1).

COPD is a major cause of morbidity and the fourth leading cause of mortality worldwide (4). Global epidemiologic studies have shown a high variability of COPD prevalence worldwide (5-22%, moderate COPD) (5). The Global Initiative for Obstructive Lung Disease (GOLD) guidelines define COPD as: “a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases” (4).

Hence, the guidelines recognize chronic airway inflammation as an important factor in asthma and COPD. Analysis of inflammatory markers help to identify different phenotypes of disease, and consequently a better characterization allow tailored therapies. Leukotrienes (LTs) are potent lipid mediators implicated in these diseases. There are two classes of leukotrienes: The cysteinyl-leukotrienes (CysLTs) and leukotriene B₄ (LTB₄) that play important roles in airway inflammation.

This thesis describes the non-invasive monitoring of airway inflammation, with particular focus on the analysis of leukotrienes as biomarkers in exhaled breath condensate and saliva. Exhaled Breath Condensate (EBC) has received great interest as a new non-invasive technique for measuring markers of airway inflammation (6). This thesis also investigated the possibility that measurement of leukotrienes in saliva could be a new, simple, convenient and non-invasive means to study airway inflammation.

GENERAL BACKGROUND

THE RESPIRATORY SYSTEM

Our bodies need energy (7-9). Oxidation of food substrates generates energy that drives our bodies. We maintain the vital process of oxidation by inhaling oxygen and exhaling the waste product carbon dioxide. Every day, about 10,000 liters of air enters the body through the nose or the mouth, passes the pharynx and larynx (upper airways) and continues further down through the trachea, bronchi (lower/proximal airways), bronchioles, alveolar ducts and finally reaches the alveolar sacs (lower/distal airways). The tracheobronchial tree consists of 23 generations of branches starting from the trachea, which divides into two bronchi that enter each lung (7, 8). Every generation of the tracheobronchial tree divides into two tubes (dichotomous branching) (7-9). The first 16 generations of the tracheobronchial tree are termed the conducting airways, since they conduct the air to the respiratory zone (generation 17 through 23), where the exchange between oxygen and carbon dioxide takes place (7, 8). The most distal part of the airways include around 300 million alveolar sacs (7, 8) (although a recent study reported the mean total number to be 480 million) (10). The combined surface area of the respiratory zone is enormous (around 100-150 m²) (11) and very thin, and this is optimal for the exchange of oxygen and carbon dioxide to take place between the alveoli and the pulmonary capillaries that line them (7-9).

The inner mucosal surface of the airways is to a large extent lined with ciliated epithelial cells (12). The upper airways (from the nasal cavity through the larynx) are also to some extent lined with squamous epithelium, which is the prevalent cell type lining the oral cavity. The airway epithelial cells are interspersed with secretory cells, *i.e.* mucus-secreting Goblet cells, and other cells (brush cells, basal cells, small granule cells). Mucus is also produced by glands that are found mainly in the submucosa (12). This mucoid epithelial fluid that lines the airway surface consists of two layers: an upper more viscous layer (traps foreign particles) and a lower more watery layer that enables the cilia to beat towards the mouth (13). The Goblet cells become less frequent distally and are eventually replaced by another type of secretory cell, the Clara cell (7, 12). A smooth muscle layer surrounds the mucosal layer. The trachea and bronchi are structurally supported by cartilage, whereas the bronchioles and more distal parts do not contain cartilage. The alveoli are composed of very large and thin epithelial cells of the squamous type (type I alveolar cells), and the more cuboidal surfactant-producing type II alveolar cells (11). The fluid that lines the alveoli is composed of surfactant phospholipids and an aqueous subphase (11).

In airway pathology, the numbers of Goblet cells may increase, and the mucus-secreting glands may hypertrophy, resulting in increased mucus formation (7, 12). The hypertrophy and hyperplasia of smooth muscle is an important pathophysiological component of airway remodeling in asthma (and to some extent in COPD) (14). These diseases are also characterized by chronic airway inflammation, which has been linked to bronchial hyperresponsiveness (15), and may also play a role in airway remodeling (3, 16). A pathological change of the airway structure and function in disease will eventually disturb the well-controlled process of ventilation.

The theme of this thesis is non-invasive monitoring of airway inflammation.

PULMONARY DEFENSE MECHANISMS

The healthy airways employ a number of defense mechanisms in order to keep invading foreign particles away (8, 17). Depending on their size and other physico-chemical properties, inhaled particles will be trapped by the mucus lining at different locations of the airways (18). The mucociliary escalator (cilia beating towards the mouth) transports mucus together with the trapped particles to the mouth where they may be swallowed (13). In airway pathology, mucociliary clearance may be altered by structural damage and inhibition or impairment of the cilia (8, 17). A cough or a sneeze initiated by receptors in the trachea and nose/nasopharynx, respectively, will transport the irritant out together with mucus (8). Cough may also serve as a back-up system for mucus clearance if the escalator does not function properly (13). Bronchoconstriction may prevent further penetration of the foreign particle into the airways (8). Pathologic bronchoconstriction is a feature of asthma (and to some extent, of COPD) (19). Particles that reach the alveoli may be phagocytosed by patrolling alveolar macrophages, which are derived from circulating monocytes (17). The alveolar macrophage may destroy the phagocytosed particles by its lysosomal contents or carry them to the mucociliary escalator or take them to the blood or lymph (7, 8). In addition to their role as defenders of the alveolus, alveolar macrophages are also important in the immune and inflammatory responses of the lung (17, 20, 21).

An inflammatory response is a protective mechanism that defends the body against foreign substances or invading microorganisms, and is involved in the healing of injury (22). The classic signs of acute inflammation are: *calor* and *rubor* (heat and redness; due to vasodilatation), *tumour* (swelling; due to plasma exudation and oedema), *dolor* (pain; due to sensitization and activation of sensory nerves), and *functio laesa* (loss of function; due to pain and swelling) (22, 23). The initial inflammatory response depends on the type of insult and on the site of invasion (17). Neutrophils are generally the first circulating cells to be recruited, followed by monocytes (which differentiate into macrophages in the tissue) and lymphocytes (22). These cells produce proinflammatory and chemotactic substances, which results in an influx of lymphocytes, neutrophils, monocytes, eosinophils, basophils, and mast cells to the site of inflammation. After a successful inflammatory response, resolution of inflammation normally occurs. Chronic inflammation develops when the antigen persists, or when there is an excessive and/or unresolved inflammatory response (22, 24).

CHRONIC AIRWAY INFLAMMATION

Asthma and COPD are both characterized by chronic inflammation of the respiratory tract, but the airway inflammation in asthma is different from the one occurring in COPD (16, 19). The airway inflammation in COPD is generally described as neutrophilic, with a predominance of neutrophils, macrophages, T helper 1 cells, and type 1 cytotoxic T cells (a subset of CD8⁺ T cells). Mediators produced by these cells include interferon- γ , tumor necrosis factor- α (TNF- α), interleukin (IL)-8, and LTB₄.

The pig house dust exposure is a useful human model to study neutrophilic inflammation (25), with potential relevance for COPD mechanisms (26, 27). The model involves a standardized exposure of healthy subjects in a pig house for three hours (25). This challenge results in a two- to three-fold increase in bronchial responsiveness to methacholine, and an associated intense neutrophilic airway inflammation. Studies have shown a 75-fold increase in the number of neutrophils in bronchoalveolar lavage (BAL) fluid (25), and a 16-fold increase in the number of neutrophils in sputum (28), as well as a two- to 5.5-fold increase in the levels of LTB₄ in nasal lavage (NAL) fluid following exposure in a pig house (29, 30).

In contrast, the airway inflammation in asthma is generally described as eosinophilic, with a predominance of eosinophils and mast cells (16, 19, 31). In addition, many other cells play an important role in asthma, including T lymphocytes, dendritic cells, macrophages, neutrophils, epithelial cells and smooth muscle cells. T helper 2 (T_H2) cells have a central role, especially in allergic asthma (32). Dendritic cells play an important role in the T_H2 regulation and antigen presentation to T_H2 (33, 34). Dendritic cells in the airway epithelium capture antigen (*i.e.* allergen in allergic asthma), process it while migrating to the draining lymph node, and present it to T helper cells. T helper 2 cells produce IL-4 and IL-13, which induce B cells to undergo class switching to immunoglobulin E (IgE) (35). Allergen cross-linking of two IgE-molecules, bound to the high-affinity receptor FcεRI on the surface of mast cells and basophils, will induce cell activation (36).

Mast cells originate from CD34⁺ pluripotent stem cells in the bone marrow, and enter the blood as mast cell progenitors (36). Upon entering the tissue, mast cell progenitors differentiate into mature mast cells primarily under the influence of stem cell factor (also termed Kit ligand or mast cell growth factor), which is expressed by endothelial cells and fibroblasts (37). Mast cells are widely distributed in vascularized tissue and can be found in the skin, connective tissue of various organs, and mucosal epithelial tissue of the respiratory, genitourinary, and gastrointestinal tracts (22). Mast cells also reside near blood vessels, lymphatic vessels and nerves (38). Asthmatic subjects were found to have markedly increased numbers of mast cells in bronchial smooth muscle, which positively correlated with bronchial hyperresponsiveness (39). Mast cells can be activated by a number of stimuli in addition to IgE cross-linking by allergen, resulting in a release of mediators (36). Mast cell derived mediators include several potent bronchoconstrictors such as histamine (preformed and granulae stored), leukotriene C₄ (LTC₄) and prostaglandin D₂ (PGD₂; *de novo* synthesized lipid mediators) (16). Mast cells also release proinflammatory cytokines, such as IL-4, 5 and 13.

Eosinophils are critically regulated by IL-5, which regulates eosinophil differentiation, priming, activation, and survival (40, 41). Eosinophils originate from CD34⁺ pluripotent stem cells in the bone marrow, and enter the blood as mature cells (40). After circulating in the blood for a few hours to a day, eosinophils enter the tissues, primarily the gastrointestinal, genitourinary, and respiratory tracts. Although capable of phagocytosis, eosinophils chiefly exert their function by releasing mediators that kill parasites and microorganisms, and they also release proinflammatory and chemotactic mediators (40, 41). Leukotriene C₄ is one of the key lipid mediators secreted by the eosinophils. Increased numbers of airway eosinophils (including increased infiltration of bronchial epithelium and submucosa) are a central feature of asthma (42). The number of eosinophils correlate with disease severity in asthma (42), and they may also play a role in airway remodeling (41). Increased eosinophil numbers are also observed in subjects with atopy (without asthma), although the increase is less marked as compared with asthmatic subjects (42).

The term atopy is described as a genetic predisposition to produce IgE antibodies in response to common environmental antigens (43). In comparison, the term allergy is defined as “a hypersensitivity reaction initiated by specific immunologic mechanisms”, which can be antibody-mediated (*e.g.* IgE) or cell-mediated. Immunoglobulin E-mediated allergic asthma is the most common form of asthma (43). Aspirin-intolerant asthma (AIA) is a type of *non*-allergic asthma, which will be described in further detail below.

It is becoming increasingly clear that asthma is a heterogeneous disease presenting with many different phenotypes, and different patterns of underlying airway inflammation (44). In addition, airway inflammation in asthma is overlapping with the inflammation seen in COPD (16). For example, subjects with severe asthma may have increased neutrophils as well as increased eosinophils. Given the fact that patients respond differently to anti-inflammatory therapy, a better understanding and characterization of the different phenotypes is important for tailor-made therapies. Bronchoscopy, the gold standard of collecting airway inflammatory cells and mediators, is expensive, invasive and involves some risk. Developing new non-invasive methods for diagnosis is therefore crucial.

The specific focus of this thesis concerns the non-invasive analysis of leukotrienes in airway inflammation. The leukotrienes are members of the eicosanoid family.

EICOSANOIDS

Eicosanoids are a large family of autocrine and paracrine lipid mediators derived from polyunsaturated fatty acids, *e.g.* arachidonic acid (AA; 20:4, ω 6) and eicosapentaenoic acid (EPA; 20:5, ω 3) (45). The eicosanoids name implies that they contain twenty carbon atoms (“eicosa-” is Greek for “twenty”). Mammals cannot introduce double bonds beyond C-9 in the fatty acid chain, *i.e.* the ω 6 and ω 3 fatty acids can not be endogenously synthesized and must be either supplied through the diet or synthesized from dietary precursors (46). Consequently, arachidonic acid and EPA are supplied through the diet or may be synthesized from dietary linoleic acid (18:2, ω 6), and α -linolenic acid (18:3, ω 3), respectively (45). Arachidonic acid is the most abundant precursor for the eicosanoids. It accounts for about 9-25% of the fatty acids in cellular phospholipids, in human adults living on a Western diet (47). In resting cells, the majority of AA is esterified at the *sn*-2 position to the glycerol backbone of phosphoglycerides (a major class of phospholipids, which form the membrane lipid bilayer) (48). Eicosanoids are not preformed, but rather *de novo* synthesized. Upon cell stimulation, AA is released from the lipid bilayer and may be metabolized to various eicosanoids by the lipoxygenase (LOX) pathway (generates leukotrienes (LTs), hydroxyeicosatetraenoic (HETEs) acids, and lipoxins), cyclooxygenase (COX) pathway (generates prostanoids) or the cytochrome P450 pathway (generates epoxy-eicosatrienoic acids). The eicosanoids are important mediators of tissue homeostasis and inflammation (49).

LEUKOTRIENES

The cysteinyl-leukotrienes (CysLTs, *i.e.* LTC₄, LTD₄ and LTE₄) mediate bronchoconstriction, mucus secretion and vasoconstriction which are central components of asthmatic airway inflammation, whereas LTB₄ is one of the most potent chemotactic and proinflammatory agents so far described (49, 50). Leukotriene C₄ is primarily produced by eosinophils, mast cells and basophils, but also by macrophages/monocytes (51). Leukotriene B₄ is predominantly produced by neutrophils, but also by macrophages/monocytes and dendritic cells. In addition, LTs are produced in transcellular reactions involving inflammatory cells and surrounding structural elements. The leukotrienes play important roles in both the innate and adaptive immune responses (51, 52).

Leukotriene B₄ is chemotactic for neutrophils, monocytes/macrophages, eosinophils, fibroblasts, dendritic cells and activated CD4⁺ and CD8⁺ T lymphocytes (53-56). In addition to its chemotactic properties, LTB₄ converts leukocytes from rolling on endothelium to firm adhesion, and promotes transendothelial migration (57, 58).

Leukotriene B₄ was shown to upregulate CD11b/CD18 β 2 integrin expression on neutrophils and monocytes (58). Leukotriene B₄-treated human endothelial cells were shown to promote transendothelial neutrophil migration (57, 58). Leukotriene B₄ activates leukocytes by enhancing macrophage and neutrophil phagocytosis (52). Leukotriene B₄ also augments phagocyte microbial killing by stimulating lysosomal enzyme release, and generation of defensins, reactive oxygen species (ROS), and nitric oxide (NO). In addition, LTB₄ stimulates production of cytokines and chemokines (*e.g.* TNF- α , IL-8 and monocyte chemoattractant protein-1), and also secretion of immunoglobulins. Thus, the role of LTB₄ in anti-microbial host defense is well established (52). Leukotriene B₄ exerts its effects by binding to two receptors, termed B leukotriene receptor 1 (BLT₁, high-affinity receptor) and B leukotriene receptor 2 (BLT₂, low-affinity receptor) (59). B leukotriene receptor 1, is primarily expressed on leukocytes, and mediates most of the LTB₄ mediated functions. B leukotriene receptor 2 is more ubiquitously expressed, and although its functional role is less clear, a recent study showed that LTB₄ mediates dendritic cell chemotaxis via BLT₂ (55).

The CysLTs were previously known as slow-reacting substance of anaphylaxis (SRS-A) (49, 51, 60, 61). They are potent bronchoconstrictors (49, 51, 60, 61), with LTC₄ and D₄ being more potent than E₄ (60, 61). In human subjects, the bronchoconstrictor effect of inhaled CysLTs were shown to be around 100 to 10,000-fold more potent than inhaled histamine (62, 63), and over 1000-fold more potent than inhaled methacholine (64). In addition, the CysLTs are potent vasoconstrictors (60, 61). Moreover, CysLTs mediate increased microvascular permeability (leading to leukocyte extravasation, plasma exudation and edema), and mucus hypersecretion (49, 60, 61). They may play a role in airway remodeling, *e.g.* by stimulating bronchial smooth muscle cell proliferation (both hyperplasia and hypertrophy), fibroblast proliferation, increased collagen deposition, and mucus gland hyperplasia (65). The CysLTs promote the recruitment of eosinophils, neutrophils, dendritic cells, and T lymphocytes (51, 60, 61). The CysLTs stimulate the proliferation and differentiation of bone marrow eosinophil hematopoietic progenitors, and also their subsequent migration into blood (51, 61). In addition, the CysLTs were shown to induce cell adhesion proteins and thereby promote leukocyte adhesion to vascular endothelial cells (49, 51, 61). The CysLTs were also shown to have some antimicrobial effects, although these actions are narrower than those of LTB₄ (52). Studies have demonstrated a capacity for CysLTs to induce ROS and NO formation. Furthermore, CysLTs promote leukocyte survival (51, 61). The CysLTs stimulate the production of T_H2 cytokines (*e.g.* IL-4, 5 and 13), which in turn stimulate the production of the CysLTs. Interleukin-4 upregulates LTC₄ synthase (see below) gene expression, whereas IL-4 and IL-13 upregulate the CysLT₁ receptor gene expression (51, 61).

There are at least two receptors for CysLTs, termed Cysteinyl leukotriene receptor type 1 and type 2 (CysLT₁ and CysLT₂), with a wide distribution (66). Cysteinyl leukotriene receptor type 1 is mainly expressed in the spleen, peripheral blood leukocytes (including eosinophils), and less strongly expressed in the lung (smooth muscle cells and interstitial macrophages), small intestine, pancreas and placenta. The CysLT₂ receptor is mainly expressed in the heart, adrenal medulla, placenta, peripheral blood leukocytes (including eosinophils), spleen and lymph nodes, and some expression throughout the CNS.

All LT receptors (CysLT₁, CysLT₂, BLT₁, and BLT₂) are seven-transmembrane G-protein coupled receptors (GPCRs) and the LTs either activate the Gq subtype

(resulting in an increased intracellular calcium concentration) and/or the Gi subtype (resulting in decreased intracellular cyclic adenosine monophosphate, cAMP) (59, 66). Current evidence suggests that the CysLTs are the LTs primarily involved in asthma, whereas LTB₄ has been implicated in other pulmonary diseases such as COPD (67) and cystic fibrosis (68). However, there are observations supporting a role also for LTB₄ in asthma and in particular bronchial hyperresponsiveness (60). In addition, both classes of LTs have been implicated in the pathophysiology of a number of extra-pulmonary diseases (51), most notably cardiovascular disease (69). This has created an interest in methods of measuring LT levels *in vivo*, both to characterize the involvement in different diseases and inflammatory reactions, and also to assess the *in vivo* activity of novel inhibitors of LT synthesis.

Antileukotriene drugs

The CysLT₁ receptor antagonists montelukast, zafirlukast and pranlukast are registered for the treatment of asthma and allergic rhinitis (51). These drugs bind competitively to the CysLT₁ receptor and thereby block the effects of the CysLTs. Zflo® (zileuton) is a registered drug in the U.S. for the prevention and chronic treatment of asthma. Zileuton inhibits LT biosynthesis by binding reversibly to the active site of 5-lipoxygenase, which catalyses the first committed step in the LT biosynthesis from arachidonic acid (51, 70).

Leukotriene biosynthesis

Leukotrienes are formed *de novo* within seconds to minutes upon cell stimulation. The biosynthetic pathway is outlined in figure 1. The reaction involves the release of arachidonic acid (AA, *all-cis*-5,8,11,14-eicosatetraenoic acid) from the phospholipid bilayer by phospholipase A₂ (PLA₂) enzymes (71), oxygenation into 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE), and subsequent dehydration into the unstable intermediate leukotriene A₄ (LTA₄, 5(S)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) by 5-lipoxygenase (5-LO) (72, 73). This epoxide is either hydrolyzed into leukotriene B₄ (LTB₄, (5(S),12(R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid) by LTA₄ hydrolase (LTA₄H) (74, 75) or conjugated to glutathione into leukotriene C₄ (LTC₄, 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) by LTC₄ synthase (LTC₄S) (76). Leukotriene C₄ is then enzymatically converted to leukotriene D₄ (LTD₄, 5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) by γ -glutamyl transpeptidase/leukotrienase (49, 50), and further to leukotriene E₄ (LTE₄, 5(S)-hydroxy-6(R)-S-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) by dipeptidase (50).

Among the large group of PLA₂ enzymes, the ubiquitously and constitutively expressed cytosolic PLA₂- α (cPLA₂- α) is believed to be the main enzyme releasing arachidonic acid from the phospholipid bilayer in eicosanoid biosynthesis (71). The activation of cPLA₂- α is regulated by Ca²⁺ and phosphorylation. Upon cell stimulation, resulting in an intracellular Ca²⁺ increase, cPLA₂- α translocates from the cytosol to the nuclear, endoplasmic reticulum (ER) and Golgi membranes, where it liberates AA bound to the *sn*-2 position of phospholipids.

The activation of 5-LO is regulated by Ca²⁺ and phosphorylation (72, 73). The enzyme contains a non-heme iron, which is important for its catalytic activity. Depending on the cell type, the subcellular localization of 5-LO in resting cells may be in the cytosol or in the nucleus associated with euchromatin. Upon cell stimulation, resulting in an intracellular Ca²⁺ increase, 5-LO translocates to the nuclear envelope, where it is

presented to AA. Cells expressing 5-LO are mainly of hematopoietic origin, *e.g.* granulocytes, monocytes/macrophages, mast cells, dendritic cells and B lymphocytes. However, 5-LO is not expressed in endothelial cells, T lymphocytes, platelets or erythrocytes.

Five-lipoxygenase activating protein (FLAP), which is thought to present AA to 5-LO, is a small integral membrane protein, and belongs to 'the membrane-associated proteins in eicosanoid and glutathione metabolism' (MAPEG) family. (72, 73)

Leukotriene A₄ hydrolase is a soluble zinc-containing enzyme, which catalyzes the conversion of LTA₄ to LTB₄ (74, 75). The enzyme is suicide inactivated by its substrate LTA₄. It is ubiquitously expressed, and the subcellular localization includes the cytosol and the nuclear compartment (77). Leukotriene A₄ hydrolase was found to be expressed by neutrophils, monocytes, lymphocytes and erythrocytes, whereas low or no levels were found in eosinophils, basophils and platelets (75). Cells containing LTA₄H but not 5-LO (*e.g.* erythrocytes, T cell lines, fibroblasts, endothelial cells, keratinocytes and airway epithelial cells), may be involved in transcellular metabolism, where LTA₄ is donated from a cell expressing 5-LO. Leukotriene B₄ is exported out of the cell by a yet unidentified carrier (49).

Leukotriene C₄ synthase is an integral membrane protein, located in the outer nuclear membrane and peripheral endoplasmic reticulum (78). Cells expressing LTC₄S are of hematopoietic origin, *i.e.* eosinophils, mast cells, basophils, monocytes/macrophages and platelets (76). Platelets lack however 5-LO and thus LTC₄ can only be formed in platelets from LTA₄ via transcellular metabolism (75). Leukotriene C₄ synthase is a member of the MAPEG family. Other members of the MAPEG family, namely MGST2 and MGST3 (microsomal glutathione-s-transferase 2 and 3) show similarity to LTC₄S and possess LTC₄S activity (75). Leukotriene C₄ is transported out of the cell by the multidrug resistance-associated protein, and is subsequently converted to LTD₄ (through cleavage of glutamic acid), and further to LTE₄ (through cleavage of glycine) (49). Leukotriene C₄, D₄ and E₄ are collectively termed the Cys-LTs since they all contain the amino acid cysteine.

Leukotriene metabolism

Metabolism of leukotrienes occurs via several pathways but commonly includes initial ω -oxidation and subsequent β -oxidation (79). The major inactivation pathway of LTB₄ occurs by enzymes belonging to the cytochrome P450 4F (CYP4F) subfamily. In neutrophils, LTB₄ is catalyzed by CYP4F3A to yield 20-OH LTB₄ (Figure 1). This less potent metabolite can bind to the BLT₁ receptor, and is further metabolized to the inactive metabolite 20-COOH LTB₄ (catalyzed by CYP4F3A in neutrophils, alternatively by alcohol dehydrogenase and aldehyde dehydrogenase in hepatocytes). Leukotriene C₄ is subjected to peptidases that generate the more stable metabolite of LTE₄ as described above. Approximately 5% of the total LTC₄ formed is metabolized and excreted into urine as intact LTE₄ (79). Leukotriene E₄ is also excreted via the hepatic route (80). Alternatively, LTE₄ may be ω -oxidized to yield 20-COOH LTE₄ (79). Subsequent chain-shortening by β -oxidation of 20-COOH LTB₄ and 20-COOH LTE₄ occurs in peroxisomes (20-COOH LTB₄ is also β -oxidized in mitochondria). The β -oxidation pathway of these unsaturated fatty acids additionally requires 2,4-dieneoyl-CoA reductase, which also has isomerase activity. Ultimately, products of ω - and β -oxidation may either be excreted or enter the citric acid cycle and eventually the electron transport chain (81).

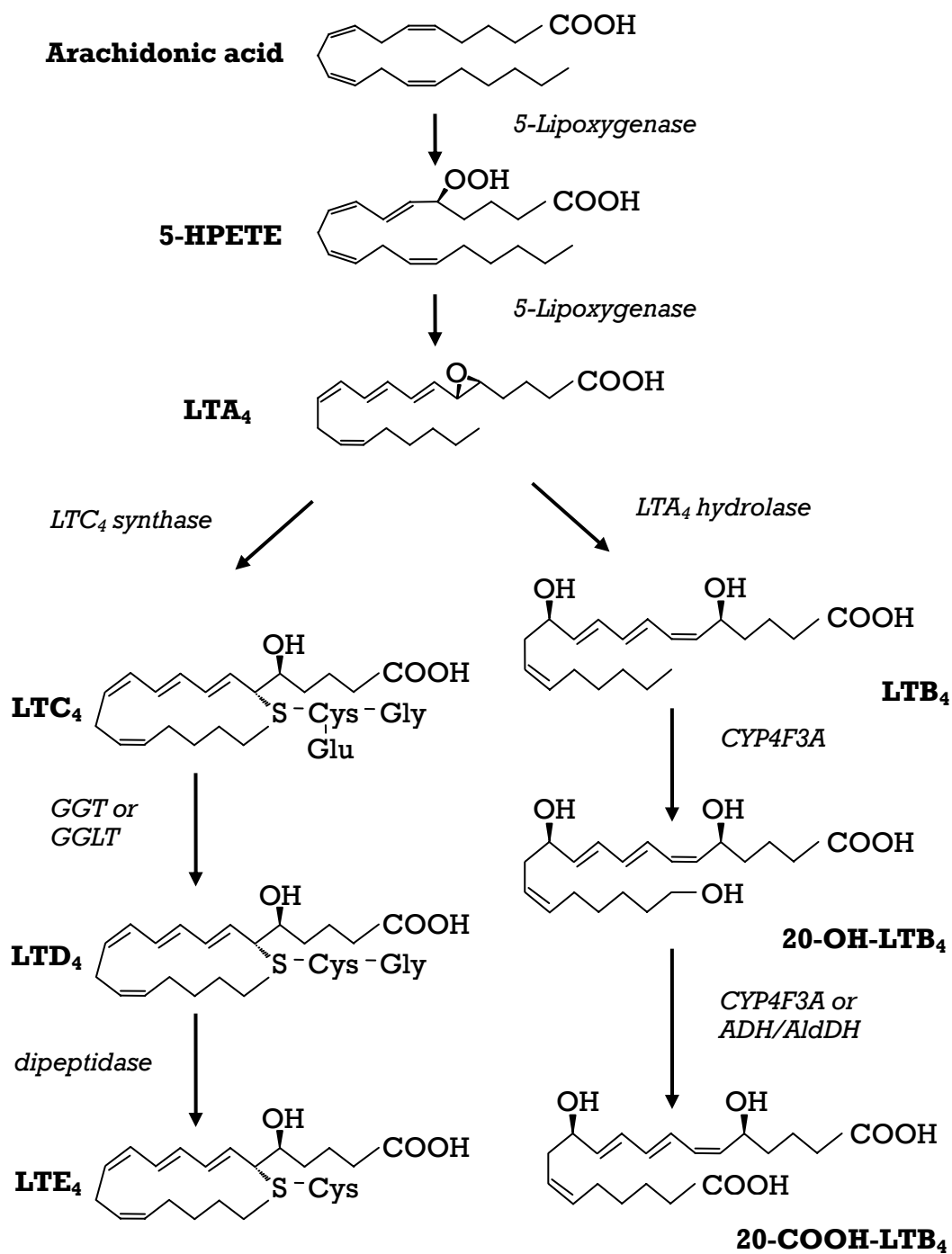


Figure 1. Leukotriene biosynthesis and LTB₄ metabolism.

GGT = γ -glutamyl transpeptidase; *GGLT* = γ -glutamyl leukotrienase; *CYP4F3* = cytochrome P450 F3; *ADH* = alcohol dehydrogenase; *AldDH* = aldehyde dehydrogenase

PROSTANOIDS

Another group of potent eicosanoids in inflammation are the prostanoids.

The cyclooxygenase pathway generates the prostanoids (*e.g.* prostaglandin (PG) D₂, E₂, F_{2α} and I₂, and thromboxane A₂) from AA (49). The prostanoids are produced by most cells in our bodies and are thus implicated in many physiological and pathophysiological processes, including inflammation, fever and pain, as well as bone resorption, ovulation, fertilization, platelet aggregation, angiogenesis and maintenance of renal function (82, 83). Prostaglandin endoperoxide H₂ synthase (PGHS; commonly designated as COX) is a heme-containing membrane-bound enzyme (with cyclooxygenase and peroxidase activities) that catalyzes the committed step in prostanoid synthesis. There are at least two isoforms of COX: COX-1, which is constitutively expressed in most cells and tissues (although it is inducible in some systems), and the inducible isoform, COX-2, which is induced by proinflammatory stimuli, hormones, growth factors and oncogenes (although constitutive expression of COX-2 has also been shown) (82, 83). The major steps in the biosynthesis of prostanoids include the liberation of AA from membrane phospholipids (by the action of phospholipase A₂), the conversion of AA to prostaglandin endoperoxide H₂ (PGH₂; by the action of PGHS), and the formation of specific prostanoids from PGH₂ (by the action of cell-specific unique synthases) (49).

The prostanoids can bind to specific GPCRs to mediate their functions (DP, EP, FP, IP and TP receptors), and the different receptors activate intracellular pathways that result in either increased intracellular Ca²⁺, or increased intracellular cAMP, or decreased intracellular cAMP (84).

Prostaglandin D₂ is the major prostanoid formed by activated mast cells (85). It is almost exclusively produced by mast cells, although it can be produced in 100-1000 lower amounts by other cells (*e.g.* platelets, macrophages and T lymphocytes) (86). Prostaglandin D₂ can bind to TP, DP₁ or CRTH2 (chemoattractant receptor-homologous molecule expressed on T_H2 cells) (86, 87). Activation of the TP receptor by PGD₂ results in contraction of the bronchial smooth muscle (88). The bronchoconstrictor effect of TP dominates over the effect of DP₁, which mediates bronchodilatation (89). The CRTH2 receptor induces chemotaxis of T_H2 cells, eosinophils, and basophils (87). Prostaglandin D₂ is converted by the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme 11-ketoreductase (in the human lung or liver) to 9α,11β-PGF₂ (86), which is a biologically active metabolite (90, 91) that can be detected in the urine (92, 93) as a marker of mast cell activation (86).

Prostaglandin E₂ can bind to four EP receptor subtypes: EP₁ mediates a Ca²⁺ increase; EP₂ and EP₄ mediate a cAMP increase; EP₃ consists of multiple isoforms, which can mediate a cAMP decrease or increase, or a Ca²⁺ increase (94). Among the effects of PGE₂ are bronchodilatation, inhibition of leukotriene synthesis, and inhibition of mast cell degranulation (95).

The biosynthesis of prostanoids can be inhibited by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs, *e.g.* ibuprofen) (96). The NSAIDs either bind irreversibly (*e.g.* aspirin) or reversibly to the active site of PGHS and thereby inhibit the cyclooxygenase activity. Aspirin-intolerant asthma is a type of asthma that is induced by NSAIDs and it is leukotriene-dependent (see below) (95).

ASPIRIN-INTOLERANT ASTHMA

Aspirin-intolerant asthma is a clinical syndrome where asthma and chronic nasal problems such as rhinosinusitis and recurrent polyps are associated with intolerance to aspirin and most other NSAIDs (97-99). This type of asthma should not be mistaken for allergy to aspirin (or any other NSAID), but is rather mediated by other mechanisms, where the inhibition of COX-1 precipitates the adverse reaction. Subjects with AIA were shown to have increased CysLTs at baseline, which increased further after aspirin provocation (100). In fact, hyperleukotrienuuria is a characteristic feature of AIA (98, 99), and antileukotriene drugs were shown to be effective in AIA patients (99). Also the PGD₂ metabolite, 9 α ,11 β -PGF₂, was increased after aspirin provocation (supporting mast cell activation), whereas most studies have not shown increased basal 9 α ,11 β -PGF₂ levels in AIA (98, 99, 101). Moreover, nasal tissue specimens and bronchial biopsies from AIA subjects contain increased numbers of eosinophils and mast cells (both cell types are major producers of LTC₄) as compared with subjects with aspirin-tolerant asthma (ATA) (98, 99). Levels of these markers in urine however overlap considerably between AIA and ATA subjects (97-99), and therefore the diagnosis of AIA can presently be definitely established only through aspirin provocations (98, 99). There are four types of aspirin provocation protocols: oral, bronchial, nasal and intravenous. These tests differ somewhat in terms of specificity and sensitivity. Studies on estimated prevalence of AIA among asthmatics show variable results (around 0.1-20%) (98) mainly because epidemiological tests have not included objective methods for diagnosis such as provocations. The absence of a simple *in vitro* test for AIA diagnosis is yet another limiting factor. In many countries, all asthmatics are advised against the use of NSAIDs since a potential intolerance reaction may in fact be life threatening, but as only a minority has this problem it means that many unnecessarily avoid effective drugs for fever, pain and inflammation. Therefore, there is a great need for improved and simpler methods of diagnosis.

Thus, the major aim of this thesis was to evaluate non-invasive methods for analyzing leukotrienes as biomarkers of airway inflammation, in e.g. aspirin-intolerant asthma.

CURRENT STRATEGIES FOR THE MEASUREMENT OF AIRWAY INFLAMMATION

Bronchoscopy

Bronchoscopy is the gold standard of assessing airway inflammation (102). The most common type of bronchoscope used today is a thin and flexible fiberoptic tube that is inserted through the mouth or nose and passed into the airways down to the level of subsegmental bronchi (*i.e.* the 4th or 5th generation) (103). Samples can be collected by methods such as washing, brushing and biopsy. The bronchoscope can even be used to sample the alveolar region by instillation of sterile saline through a channel of the bronchoscope. The fluid, containing cells and acellular components, is collected by suctioning through the bronchoscope. This sample is called bronchoalveolar lavage (BAL) fluid. A major drawback of this technique is the unknown dilution factor which causes great difficulties in interpreting the results, especially quantitation of the acellular components (104). Bronchoscopy is an invasive method that raises issues on safety. Special skills are required to perform this method and there is a high cost of procedure, which renders bronchoscopy less suitable for repeated testing.

Assessment of inflammatory cells in samples obtained by bronchoscopy has established the important effector cells in asthma (*e.g.* eosinophils and mast cells) (105, 106) and COPD (*e.g.* neutrophils, macrophages and CD8⁺ T lymphocytes) (107). There are a

number of studies that have shown increased leukotriene (both of CysLTs and LTB₄) levels in BAL fluid at baseline and after challenge in subjects with asthma (108). However, there is a large variation in the results, which makes standardization very difficult as was concluded by the European Respiratory Society (ERS) task force on BAL fluid (105).

Induced sputum

Induced sputum (IS) is a well validated, semi-invasive method to collect cells and mediators from the airways (102). Subjects inhale increasing doses of hypertonic saline and are asked to expectorate sputum into a container (109). The collected sputum sample is subsequently processed using a protocol where the cells are separated from the fluid phase (supernatant) (110). There are two methods of processing sputum (“plug picking” or “whole sputum”), and both methods have advantages and disadvantages (110). Since the inhaled hypertonic saline induces an inflammatory response and may even induce bronchoconstriction, it is recommended to wait at least 2 days before repeating the sputum induction in the same subject (109). Moreover, some (but not all) studies have shown that the duration of sputum induction can alter the cellular composition and therefore a standardized duration of sputum induction may be advisable. The IS method samples from more proximal airways in comparison with BAL fluid, and the cellular composition (and maybe even the fluid-phase mediator composition) is different between these two compartments (111). Bronchoalveolar lavage fluid tends to have relatively more macrophages and lymphocytes, while sputum tends to be relatively richer in neutrophils. However, the eosinophil count is similar in the two compartments. Induced sputum is more cost-effective than bronchoscopy and requires minimal training, although it is labor intensive (112).

Differential cell count (% cells) in IS has been shown to have useful clinical applications, with regard to diagnosis and monitoring airway disease, as well as predicting response to corticosteroid therapy (112). Eosinophil counts are generally increased in subjects with asthma, although IS studies have identified subjects with non-eosinophilic asthma, which was related to a poor response to corticosteroids. Subjects with COPD have generally increased sputum neutrophil counts, which was associated with reduced FEV₁ values (see methods section). In contrast, fluid-phase mediators are not as well validated mainly due to the unknown dilution factor of collected sputum, although studies have shown differences in sputum mediators. Basal levels of LTB₄ and CysLTs were significantly increased in IS from subjects with COPD and asthma as compared with healthy controls (113, 114). Moreover, basal CysLTs in IS from patients with AIA were significantly increased as compared with levels in ATA subjects (115, 116). Furthermore, asthmatics with exercise-induced bronchoconstriction (EIB) were shown to have significantly increased basal CysLT levels in IS, as compared with asthmatics without EIB (117). Sputum CysLT levels were significantly elevated after an exercise test in asthmatics with EIB (118). In patients with acute exacerbation of COPD, levels of sputum LTB₄ were significantly increased (119).

Exhaled Breath Condensate

Exhaled Breath Condensate (EBC) has gained considerable interest as a new non-invasive technique for measuring markers of airway inflammation (6, 102). The procedure normally involves tidal breathing for 5 to 15 minutes and passage of the expired air through a cold trap where the fluid phase is condensed. Ten minutes of breathing produces on average one milliliter of condensate liquid. No serious adverse events have been reported with this method and hence it can be used repeatedly and

even be applied on small children. Collection cost is relatively low, but the cost of mediator measurements depends on the molecule studied (102). However, this method is still unvalidated (6, 102). The condensate liquid consists mainly of exhaled water vapor (approximately 99.99%), and only a small fraction is respiratory droplets thought to originate from the airway lining fluid. Studies have assessed the numbers of aerosol particles to be 0.1-4 particles/cm³ (mean diameter: 0.3 µm) during normal tidal breathing, although these data do not include the smallest particles (6). Non-volatile molecules such as leukotrienes are hypothesized to be transported by these respiratory droplets from the airways and the amount of droplets may be related to the degree of airway turbulence (6, 102). A great number of mediators, markers and other molecules have been measured in EBC, such as eicosanoids (leukotrienes and prostaglandins), nitrogen oxides (*e.g.* nitrite/nitrate), adenosine, pH, hydrogen peroxide, ammonia and cytokines (*e.g.* IL-4, IL-8) (6). Several papers have reported elevated EBC levels of LTB₄ and CysLTs in asthma and increased LTB₄ in COPD. Following the original description of the method by Russian investigators in 1980 (120), there are as of date more than 400 papers published on the topic of EBC, and more than 300 of those are from the last five years. The method is thus very attractive but as pointed out in the recent report from an ERS Task Force (6), there are methodological issues that remain unresolved. In fact, there are only a few experimental studies that have addressed the question concerning the site of origin of exhaled markers.

Urine and Blood

There is evidence supporting the concept that asthma has a systemic component (2), and that LTE₄ in urine may be used as a surrogate marker of airway inflammation.

Previous studies have indicated that urinary LTE₄ is a useful index of whole-body CysLT production (80, 121). Cysteinyl-leukotrienes are released locally and those entering the systemic circulation are excreted into the bile and the urine. Systemically derived CysLTs may be recovered as intact LTE₄ in the urine, whereas urinary LTC₄ or LTD₄ are normally not detected. Measurements of urinary LTE₄ have been particularly useful in establishing CysLT involvement in bronchoconstriction induced by allergen or aspirin in susceptible subjects (80, 121), and more recently by mannitol in subjects with exercise-induced bronchoconstriction (122). Although subjects with severe and corticosteroid-dependent asthma have increased basal excretion of urinary LTE₄ (123), such measurements usually fail to detect significant differences in whole-body CysLT synthesis between healthy subjects and individuals with mild-to-moderate asthma (80, 121). Previous studies assessing the effects of the 5-LO inhibitor zileuton showed a 39% decrease from baseline in mean urinary LTE₄ levels after four weeks of zileuton treatment (600 mg QID) in patients with mild-to-moderate asthma, which was in contrast with a slight increase in urinary LTE₄ levels in the placebo group (124). Another study assessed the effects of zileuton on urinary LTE₄ levels after allergen challenge in subjects with asthma (125). The results showed a ~50% decrease in urinary LTE₄ levels after one oral dose (800 mg) of zileuton as compared with placebo. In contrast to LTE₄, measurements of urinary LTB₄ have not been possible since the majority of the LTB₄ produced is normally efficiently metabolized to water and carbon dioxide (60, 79).

Circulating levels of leukotrienes are very low (126) and with currently available methods, it is not possible to measure plasma concentrations of LTs reliably. The current standard for assessment of 5-LO inhibition is the method of analyzing *ex vivo* LT production in whole blood stimulated with calcium ionophore as an index of the *in vivo* capacity for LTB₄ formation (125). In previous treatment trials, immunoreactive

LTB₄ levels in *ex vivo* stimulated whole blood were profoundly inhibited by the 5-LO inhibitor zileuton (~90%), while urinary LTE₄ levels were inhibited to a much lesser extent (~50%). Ionophore-stimulated peripheral blood leukocytes from patients with asthma were previously shown to possess an increased production capacity of SRS-A (which were later known as the CysLTs) (127). A more recent study showed increased basal CysLT and LTB₄ levels from ionophore-stimulated peripheral blood leukocytes from patients with asthma as compared with controls (128). Moreover, the CysLT levels were significantly increased during asthma exacerbations.

Fraction of exhaled nitric oxide (F_{ENO})

Fraction of exhaled nitric oxide is a well validated non-invasive biomarker of airway inflammation (129). The F_{ENO} test is rapidly performed and no special skills are required. Although the F_{ENO} analyzer is expensive, the cost per test is low. Nitric oxide plays an important role in human airway biology, including modulating airway and vascular muscle tone, as well as mediating inflammatory responses (130). It may be produced by constitutive and inducible forms of nitric oxide synthases (NOS) that are expressed in a variety of cells including endothelial, epithelial and inflammatory cells. Subjects with asthma have increased inducible NOS expression, which is reflected as increased F_{ENO} (129). The degree of F_{ENO} is correlated with airway and blood eosinophilia, as well as with airway hyperresponsiveness, bronchodilator reversibility and atopy. Corticosteroids suppress inducible NOS and hence F_{ENO} levels. Fraction of exhaled nitric oxide is therefore regarded as a useful tool in the diagnosis and drug monitoring of asthma.

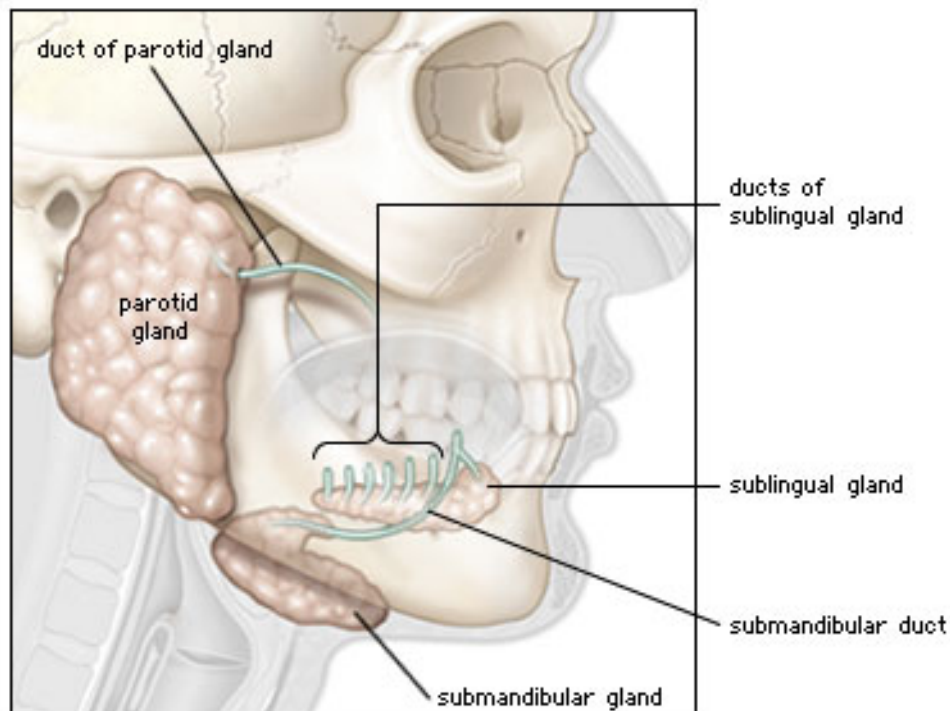
THE NEW OPPORTUNITY: SALIVA

This thesis investigated the possibility that measurement of leukotrienes in saliva could be a new, simple, convenient and non-invasive means to study endogenous leukotriene levels and to assess the effects of 5-LO inhibitors. Measurement of salivary leukotrienes have been attempted in some other diseases (131, 132), but there is only one report on salivary leukotrienes in asthma, which reported increased levels of salivary LTB₄ during acute asthma episodes in children (133). Leukotriene levels in saliva were first published in 1983 by Rigas and coworkers (134-137), and since then about twenty articles have been published on leukotriene measurements in whole saliva.

There is an increasing interest in the use of saliva as a diagnostic fluid (138-140). A number of studies have investigated the use of measurements in saliva, both in assessing local as well as systemic diseases. Saliva analysis is used in the diagnosis and monitoring of viral (*e.g.* HIV) and bacterial (*e.g.* *Helicobacter pylori*) infections, malignant diseases (*e.g.* squamous cell carcinoma, breast cancer), endocrine disorders (*e.g.* cortisol, estradiol), autoimmune diseases (*e.g.* Sjögren's syndrome), and neurological disorders (*e.g.* Alzheimer's disease) (138, 139). In addition, saliva is useful for the assessment of drug monitoring, particularly in matters of drug abuse (139).

A normal healthy adult produces around 500 to 1500 mL of saliva each day (134). Whole saliva is a mixed product of the three paired major salivary glands, the submandibular (produces approx. 65% of the total saliva secretion), parotid (approx. 23%), and sublingual glands (approx. 4%) as well as several other minor salivary glands found in the submucosa of the oral cavity (see Figure 2). In addition, whole saliva contains small amounts of other fluids and products of the mucosal surface, including mucosal transudate and gingival crevicular fluid, as well as desquamated

epithelial cells and other cells (138). The major functions of saliva are: 1. to maintain the integrity of the oral mucosal surface, to control the bacterial flora and protect the teeth, 2. to facilitate speech and swallowing, 3. to facilitate mastication and digestion of food substrates, and 4. to produce hormones and other biologically active substances (134). The grape-like structures of the glands are composed of secretory units of acinar cells that form an end-piece encircling a small central lumen that opens into a narrow duct. These ducts drain into somewhat larger ducts, which empty into still larger ducts and so forth. A single large duct ultimately drains into the oral cavity. The glands differ in their cellular composition: the parotid glands have mainly serous acinar cells (produce α -amylase), the sublingual have mainly mucous acinar cells (produce mucins) and the submandibular a mixture of the two. Mucins (glycoproteins) lubricate food to optimize mastication and swallowing. Salivary α -amylase digests starch in food (141). Saliva is formed in two steps: first an isotonic primary secretion is formed in the end-pieces, and as saliva flows down the ducts, it becomes gradually hypotonic (134). Salivary secretion is dependent on blood flow and nerve stimulation (parasympathetic and sympathetic autonomic nerves collaborate to evoke secretion). The glands have a high blood flow that is proportional to the rate of saliva formation. The content of saliva is approximately 99% water and 1% proteins and salts (138). The protein content of saliva is complex and a recent study identified approximately 1200 different proteins in saliva derived from ductal secretions (142). Proteins and other components of saliva may either be produced locally by the glands or transferred from the blood by different processes (passive diffusion, active transport, ultrafiltration through pores, or by pinocytosis) (134). A change in the composition of the salivary components may correlate with both local and systemic disease processes (138, 142).



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Figure 2. The salivary glands

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AIMS

The primary aims of this thesis were:

To explore the use of non-invasive biomarkers to monitor airway inflammation, with focus on the analysis of leukotrienes in exhaled breath condensate and saliva.

To evaluate the use of such methods on patients with suspected overproduction of leukotrienes.

The secondary aim was:

To compare measurements of leukotrienes in exhaled breath condensate and saliva with previously applied methods of leukotriene analysis, *e.g.* analysis in induced sputum, blood *ex vivo*, and urine.

METHODS

Table I summarizes the methods used in this thesis, with reference to the methods sections in the respective paper. Methods not presented in the included papers are described in detail below.

Table I.

Method and study design	Paper
Subjects and study design	I, II, III
Saliva sampling and processing	I, II, III
Blood collection and <i>ex vivo</i> blood stimulation	II, III
Sputum induction and processing	I, III
Collection of exhaled breath condensate	I
FE _{NO} analysis	III
Enzyme immunoassay (EIA)	I, II, III
Alpha-amylase assay	I
Flow cytometry	II
Statistical analyses	I, II, III

Measurement of lung function

In **paper III**, lung function was expressed as FEV₁ or FEV₁ (% predicted). Lung volume was measured with a spirometer (7). The forced expiratory volume in one second is the FEV₁. The predicted value is a reference value of FEV₁ that takes into account age, sex and height. The FEV₁ (% predicted) is calculated as (observed FEV₁/predicted FEV₁) %.

Pig-house dust exposure

In **paper I**, 34 subjects were exposed to pig-house dust in a pig confinement building while weighing pigs for three hours. This exposure is previously shown to elicit an acute neutrophilic inflammation in the airways and an associated two- to three-fold increase in bronchial responsiveness to methacholine (25).

Bronchoprovocations

In **paper I** and **paper III**, subjects were subjected to bronchoprovocations with lipopolysaccharide (LPS), lysine-aspirin (L-ASA), or allergen. Detailed descriptions of LPS and L-ASA provocation, respectively, can be found in (28) and (143). In brief, bronchoprovocations with LPS, L-ASA, and allergen, respectively, were performed by inhalation of the agent dissolved in diluent, using a dosimeter-controlled jet nebuliser. Lung function measured as FEV₁ was continuously monitored. In **paper I**, 34 subjects were provoked with a LPS solution corresponding to 53.4 µg which was inhaled by six breaths, and FEV₁ was measured before and after challenge. Lipopolysaccharide (bacterial endotoxin) is a component of pig house dust and bronchial challenge with LPS resulted in a nearly 10-fold increase in the number of neutrophils in sputum from healthy subjects (28). In **paper III**, 21 subjects were provoked with L-ASA which was inhaled at 30 minutes intervals starting with inhalation of sterile saline (diluent), followed by 1 µmol, 3 µmol, 10 µmol, 30 µmol, 100 µmol, 180 µmol, 300 µmol up to the cumulative dose of 600 µmol of L-ASA. The provocation was ended when there was a fall in FEV₁ ≥ 20% as compared with post saline baseline value in FEV₁ or when the maximum cumulative dose of L-ASA was reached without any adverse symptoms. In **paper III**, eight subjects were provoked with allergen, which was inhaled at 20

minutes intervals starting with inhalation of diluent, followed by step-wise half-log increments of the cumulative dose of allergen (7-7100 Standardized Quality (SQ)-units, cumulated dose). The provocation was ended when there was a fall in $FEV_1 \geq 20\%$ as compared with post diluent baseline value in FEV_1 or when the maximum cumulative dose of allergen was reached. The cumulative provocative dose causing 20% decrease in FEV_1 (PD_{20}) was calculated by linear interpolation of the last 2 points of the dose-response curve.

Purification of saliva and RP-HPLC analysis of salivary LTB_4

In **paper II**, saliva was subjected to qualitative LTB_4 analysis on reverse-phase high performance liquid chromatography (RP-HPLC). Saliva (10 mL) was collected from a healthy subject, diluted with an equal volume of methanol, and supplemented with PGB_2 (51.6 pmol) as an internal standard. The sample was left at 4°C for 1 h followed by centrifugation (1500xg, 15 min). Water (15 mL) was added, and the sample was subsequently acidified to $pH \approx 4$ with acetic acid and subjected to solid phase extraction (SPE) on 100 mg Isolute® C_{18} silicic acid columns (International Sorbent Technology Ltd, Mid-Glamorgan, UK). The SPE column was washed with water (1 mL), methanol/water (1:4, 1 mL) and leukotrienes were finally eluted with pure methanol (200 μ l) and water (200 μ l). Water (100 μ l) was added to 100 μ l of the eluate and 200 μ l was injected onto a RP-HPLC column (Nova-Pak C_{18} , 3.9 x 150 mm, Waters, Milford, MA, USA), eluted with mobile phase (30/30/40/0.01, methanol/acetonitrile/water/acetic acid) at a flow rate of 1 mL/min. Retention time for LTB_4 was determined by detection of ultraviolet absorbance at 270 nm of authentic standard. One minute HPLC-fractions were collected, evaporated under a stream of nitrogen (N_2), and assayed for LTB_4 by enzyme immunoassay.

Measurement of FE_{NO}

In **paper II** and **paper III**, baseline FE_{NO} measurements were included as phenotyping of the subjects. Also in **paper II**, the possible effect of the 5-LO inhibitor zileuton on FE_{NO} levels was assessed. All FE_{NO} measurements were performed using a NIOX® nitric oxide monitoring system (Aerocrine NO-system type EBA:1, Aerocrine AB, Sweden), which is based on detecting NO in exhaled air by chemiluminescence (for review see (144)). Nitric oxide in the sample reacts with ozone, and the photons released in this reaction are detected by a photomultiplier tube, producing a voltage that is linearly proportional to the sampled NO. The subject was instructed to inhale NO-free air to total lung capacity. After maximal inhalation, exhalation was immediately followed at a constant flow rate of 50 mL/s against a resistance, which facilitated velum closure in order to exclude contamination by nasal NO. Each exhalation lasted 10 seconds and the resultant FE_{NO} was defined as a 3-second NO plateau at the end of the exhalation. The mean of the three standardized single-breath measurements was used. Results are expressed as parts per billion (ppb). The subjects were instructed to avoid intake of caffeine-containing beverages, vegetables or to exercise 4 hours before FE_{NO} measurements. In **paper II**, a water mouthwash preceded the measurement procedure to decrease contamination from the oral cavity, and the results are presented in this thesis as previously unpublished data. In **paper III**, the subjects washed their mouth with water followed by 10% bicarbonate solution before the measurement procedure to decrease contamination from the oral cavity (145).

Analysis of LTB_4 , LTE_4 , and $9\alpha,11\beta$ -PGF₂ by competitive enzyme immunoassay

Analyses of eicosanoids (CysLTs, LTB_4 and $9\alpha,11\beta$ -PGF₂) were performed in serially diluted aliquots of the respective samples by enzyme immunoassays (EIA, Cayman Chemical Co., Ann Arbor, MI, USA) as briefly described below. The assay is based on

the principle of competition between the eicosanoid in the sample and an added tracer molecule, which is conjugated to the enzyme acetylcholine esterase. A 96-well plate was coated with a coating antibody (mouse monoclonal antirabbit IgG), which binds to the primary antibody (rabbit polyclonal antiserum). The primary antibody is specific for the respective eicosanoid, with the following cross-reactivities: The $9\alpha,11\beta$ -PGF₂ antibody had a cross-reactivity with $9\alpha,11\beta$ -PGF₂ (100%), 2,3-dinor- $9\alpha,11\beta$ -PGF₂ (10%), and PGD₂, PGF_{2 α} and 8-epi-PGF_{2 α} (all <0.01%); the LTB₄ antibody had a cross-reactivity with LTB₄ (100%), 20-OH-LTB₄ (15%) and 20-COOH-LTB₄ (0.14%); the CysLT antibody had a cross-reactivity with LTC₄ (100%), LTD₄ (100%) and LTE₄ (67%). Leukotriene E₄ was used as tracer for the CysLT assay. Leukotriene B₄ and $9\alpha,11\beta$ -PGF₂ were used as tracers for their respective assays. Primary antibody, acetylcholine esterase-conjugated tracer and serially diluted samples were added to the coated 96-well plate and left to incubate overnight at darkness. After washing the plate, Ellman's Reagent was added, which contains the substrate for acetylcholinesterase. The reaction yields a product (5-thio-2-nitrobenzoic acid) that has a strong absorbance at 412 nm. The concentration of the analyte was determined by comparing the results to a standard curve. The assay detection limits for LTB₄, LTE₄ and $9\alpha,11\beta$ -PGF₂ were 3.9, 7.8 and 7.8 pg/mL, respectively.

Results of CysLTs analyses are somewhat differently presented in **paper II** (as LTE₄ equivalents), and in **paper III** (as CysLTs), respectively. It is therefore of importance to note that the same assay protocol was used in both studies. However, urinary results are at all times presented as LTE₄, because previous studies have established that only LTE₄ is excreted into the urine (80).

Determination of creatinine concentration in urine

The final concentrations of LTE₄ and $9\alpha,11\beta$ -PGF₂ in urine are presented as ng per mmol of creatinine in order to correct for dilution due to diuresis variations. The reaction of creatinine with added alkaline picrate to urine produced a red color, the intensity of which was determined spectrophotometrically at 490 nm. The following addition of acid destroyed the color and the difference in absorbance before and after acidification was proportional to the creatinine content in urine. The sample creatinine concentration expressed as mmol per liter was determined by the use of calibrated standards of known concentrations.

RESULTS AND DISCUSSION

ANALYSIS OF LTB₄ IN EXHALED BREATH CONDENSATE: POSSIBLE SALIVA CONTAMINATION (paper I)

In **paper I**, EIA was used to measure LTB₄ levels in samples of EBC, collected in a study where a pronounced neutrophilic inflammation was induced (pig house dust exposure and LPS provocation (28)). A silicon-coated glass condenser was used in order to minimize possible binding of the leukotrienes to the coating during EBC collection (146, 147). Alpha-amylase activity in EBC was monitored to control for possible saliva contribution.

Levels of LTB₄ in EBC were low or below the limit of detection

Out of totally 102 EBC samples tested, only four samples were positive for immunoreactive LTB₄ (median 46.5, range 28-100 pg/mL). The LTB₄ assay had a detection limit of 3.9 pg/mL. Alpha-amylase activity was detected in these four samples. In an additional sample, low α -amylase activity was detected but LTB₄ was below the limit of detection. The α -amylase activity assay had a detection limit of 0.078 U/mL, and the samples that were positive for α -amylase activity (n=5) ranged between 1.2 and 28.0 U/mL (median 3.2 U/mL). Moreover, there was a strong correlation between immunoreactive LTB₄ levels and α -amylase activity ($r_s = 1.00$; $p = 0.017$; $n = 5$). To further study the possible salivary contribution to the LTB₄ levels, increasing amounts of saliva were added to samples of EBC. These spiking experiments showed that saliva addition of ≥ 0.5 –1% gives rise to increased α -amylase and LTB₄ in EBC samples. In this study, it was thus concluded that the LTB₄ presence in the EBC was most likely due to saliva contamination (**paper I**).

In addition, stability tests showed that levels of immunoreactive LTB₄ in EBC were stable for 24 h at RT and at least 3 months at -20°C (**paper I**).

There are to date around 100 publications reporting levels of leukotrienes and other eicosanoids in EBC (for review see (6, 148-154)). Altered eicosanoid levels in EBC have been shown in diseases such as asthma (155-157), chronic obstructive pulmonary disease (158, 159), cystic fibrosis (160, 161), acute lung injury/adult respiratory distress syndrome (162), primary ciliary dyskinesia (163), and interstitial lung diseases (164). Our negative finding has therefore received some attention (165-171). However, in accordance with our results, others have also found low or no levels of eicosanoids in EBC (166, 172-178).

Nevertheless, the question remains about the source of LTB₄ found in EBC. Although a number of studies have measured amylase activity in order to control for possible saliva contamination, the majority (approx. 80%) of the papers presenting eicosanoid levels in EBC to date have not tested for possible salivary contribution.

A current hypothesis states that respiratory droplets containing nonvolatile mediators (e.g. LTB₄) are released from the airway surface and transported out with the exhaled air (179, 180). A change in the composition of the epithelial lining fluid would therefore be reflected in the EBC (180). Effros *et al* have tried to estimate the dilution

of the respiratory droplets in the condensate, and found it to be on average 2443:1 (n=20) (179). This conclusion was made on the assumption that the epithelial lining fluid is isotonic relative to plasma. By measuring the concentration of cations in the EBC samples, the authors calculated the dilution of the respiratory droplets by dividing an assumed concentration of the cations in plasma with the concentration of cations in EBC. In addition, a very low amylase activity was detected in 75% of the collected samples, which was less than 0.01% of the amylase activity in the corresponding saliva samples (179). The authors therefore concluded that only a few of the total droplets in EBC were derived from the mouth and that most of the droplets came from elsewhere in the respiratory tract. However, it is interesting to note that a majority of the EBC samples actually were positive for the saliva marker. Leukotrienes or any other eicosanoids were not measured in Effros' study. In this context, it may also be important to consider that amylase activity in saliva has been found to be highly variable (181) and this reduces the value of amylase as a quantitative measurement of saliva content. Also, as was concluded by Effros *et al*, the respiratory droplet formation varies due to differences in the dilution by the water of vaporization (179). In other words, differences in mediator levels might reflect an increased or decreased droplet formation rather than true differences of that particular mediator in the epithelial lining fluid. Hence, in a more recent study by the same authors, the dilution of the respiratory droplets was shown to be highly variable, *i.e.* between 50000:1 to 1000:1 (average 20000:1) (182). Another group concluded that the non-volatile compound nitrite found in EBC mainly originated from the oropharyngeal tract (183). Thus, it may not be advisable to draw any general conclusions on the EBC composition due to large variations between different samples of EBC. Regular testing for the possible saliva/oropharyngeal tract contribution to EBC may therefore be warranted.

Other studies have tried to eliminate the problem of saliva contamination by collecting EBC samples from mechanically ventilated patients. Eicosanoids were measured in samples of EBC, collected from intubated (162, 184) and tracheotomized (185) patients by placing the condenser in line with the expiratory limb of the ventilator circuit. The authors concluded that the detected levels of 8-iso-PGF_{2α} (162), LTB₄ (184), and TXB₂ (185), respectively, reflected the inflammatory state of the (lower) airways, indicating that these samples were not affected by oral contamination (6). However, Nandapalan *et al* have shown around 100-1000 percent increase of the saliva marker amylase in tracheobronchial secretions, the third day after patients were tracheotomized, suggesting a possible saliva aspiration in patients with tracheostomy or endotracheal intubation (186).

Is α-amylase a relevant marker of saliva presence? Although the highest levels of α-amylase have been found in saliva and pancreatic juice (141), amylase has been detected in lung tissue (187) and may be raised in certain pulmonary disorders. Increased amylase activity (>0.2 IU/ mL) was recorded in pleural effusions from patients with *e.g.* lung cancer and tuberculosis (188). Tumor tissue from lung adenocarcinoma and adenosquamous carcinoma was shown to produce α-amylase of the salivary type (range 1-28 U/g) (189). One study found 0.33 to 145.3 U/mL (median: 2.25 U/mL) of amylase activity in bronchial secretions obtained by transtracheal aspiration from patients with severe and moderate chest infections (190). However, the authors concluded that the highest levels of amylase activity found in the bronchial secretions, were a result of saliva aspiration (190). In comparison, α-amylase activity in saliva from 17 healthy subjects in our study ranged between 147 and 807 U/mL (**paper I**).

Levels of immunoreactive LTB₄ in induced sputum of healthy subjects exposed to pig house dust

In **paper I**, it was shown that the levels of immunoreactive LTB₄ in the supernatant of induced sputum from healthy subjects (n=11) were much higher than the corresponding LTB₄ levels found in EBC. Previous studies that have analyzed LTB₄ in sputum present a wide range of results among different studies. For example, one study found the levels of LTB₄ in EBC (87 pg/mL) to be higher than in the LTB₄ levels in the corresponding sputum samples (2.6 pg/mL) from patients with COPD (113). Another study showed high levels of LTB₄ (around 1800-3500 pg/mL) in induced sputum from subjects with COPD (191). In **paper I**, there was an increase in the levels of sputum LTB₄ post exposure to pig house dust as compared to pre exposure, but this difference did not reach statistical significance (Figure 3; pre 793 (484-2176) vs. post 1904 (872-2825) pg/mL (median (IQR)). However, sputum neutrophils (%) increased significantly (3 to 90-fold) post exposure in these subjects (28). A major drawback of measuring fluid-phase mediators in induced sputum is the unknown dilution factor of collected sputum. However, presenting cellular data as a percentage (of total cell number) eliminates this problem. In comparison to the sputum results, our group previously reported a significant increase in LTB₄ levels in nasal lavage fluid from healthy subjects exposed in the pig house dust model (30), which was attenuated by pre-treatment with the 5-LO inhibitor zileuton (29). It may also be of interest to note that levels of the inflammatory marker FE_{NO} increased significantly post pig house dust exposure in healthy subjects (28).

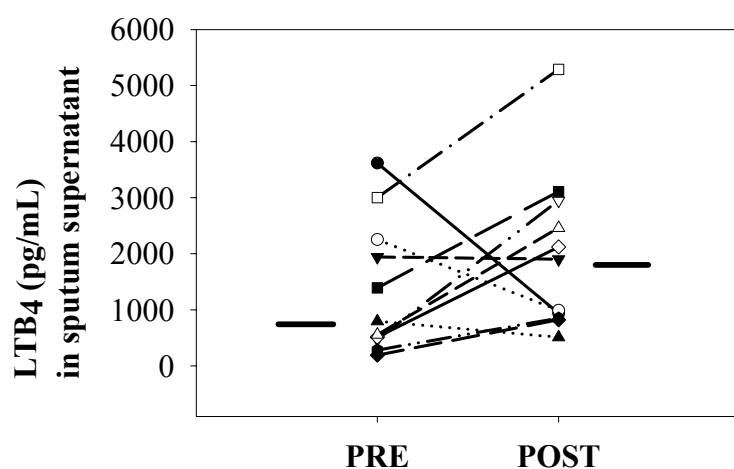


Figure 3. Immunoreactive LTB₄ levels in sputum supernatant from healthy subjects exposed to pig house dust, while weighing pigs in a piggery for three hours. Sputum was collected before (PRE) and seven hours after exposure (POST). Horizontal lines represent the medians. (p=0.24, n=11).

ASSESSMENT OF *IN VIVO* 5-LIPOXYGENASE ACTIVITY BY ANALYSIS OF LEUKOTRIENE B₄ IN SALIVA: EFFECTS OF TREATMENT WITH ZILEUTON (paper II and unpublished data)

In **paper II**, the effects of treatment with the 5-LO inhibitor zileuton on leukotriene levels (as measured by EIA) in urine and ionophore-stimulated whole blood were for the first time compared with those in saliva. Healthy non-atopic subjects (n=14) and atopic individuals (n=12) with or without asthma were treated with the clinically used dose of zileuton (600 mg QID, p.o., for 3 days). Blood, urine and saliva were collected at baseline, after the first zileuton dose, and after 3 days of zileuton treatment. Eight of the healthy subjects participated in a double-blind cross-over study in which they were randomized to receive either zileuton or placebo, with a minimum of a one-week washout period (due to technical problems, complete data sets were not obtained from one subject in the placebo-treated arm).

Levels of LTB₄ in ionophore-stimulated whole blood were significantly decreased after zileuton treatment

Baseline levels of LTB₄ in ionophore-stimulated whole blood or degree of inhibition by zileuton did not differ between the healthy (n=14) and the atopic subjects (n=12; p>0.05, data not shown). The results are therefore presented for the combined group (n=26). Zileuton effectively inhibited levels of LTB₄ in ionophore-stimulated whole blood after the first dose in the combined group (p<0.001, n=26, Figure 4A) and also after three days of treatment (p<0.001, Figure 4A). The median percentage inhibition in the combined group was 98% (95-99 (IQR)) after one dose, as well as after 3 days of treatment (n=26). In the cross-over study (Figure 4B, n=7), *ex vivo* formation of immunoreactive LTB₄ levels was not affected by placebo, whereas zileuton significantly attenuated LTB₄, as in the open study.

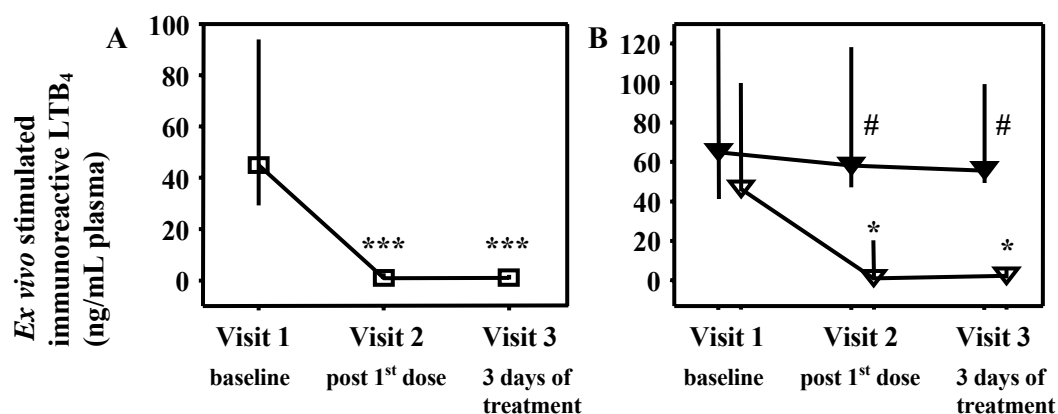


Figure 4. Leukotriene B₄ in blood *ex vivo*. Values are medians; vertical lines: 25th–75th percentiles. **(A)** Open study: Zileuton attenuated LTB₄; ***p<0.001 (n=26) as compared to baseline. **(B)** Cross-over study: LTB₄ was attenuated by zileuton (open triangles) but not by placebo (filled triangles); *p<0.05 as compared to baseline. There was a significant difference between the two study arms at visits 2 and 3; #p<0.05

Identification of LTB₄ in saliva

The authenticity of salivary LTB₄ immunoreactivity was confirmed by separation on RP-HPLC prior to EIA. One saliva sample was purified on RP-HPLC and fractions were assayed by EIA for LTB₄ immunoreactivity (Figure 5). The results indicate that the majority of the immunoreactivity eluted at the retention time of LTB₄ with a smaller proportion at the retention time of the metabolite 20-OH-LTB₄. The EIA cross-reacts to 15% with the ω -hydroxylated metabolite of LTB₄ and the results showed that both compounds were present in the sample.

The RP-HPLC method is a highly specific method to identify leukotrienes. However, it is expensive, labor intensive, and requires pre-purification by solid-phase extraction that may cause variable recovery or even significant loss of analyte. On the other hand, EIA does not require pre-purification, it is relatively inexpensive, rapid, simple to perform, and highly sensitive. However, EIA is not as specific as the RP-HPLC method, since it is difficult to eliminate cross-reactivity with structurally related substances. The LTB₄ antibody used in **paper II** cross-reacted with 20-OH-LTB₄ (by 15%) and 20-COOH-LTB₄ (by 0.14%), which are ω -oxidation metabolites of LTB₄. In other words, the antibody detected LTB₄ as well as substances that were originally derived from LTB₄. Thus, the EIA measurements may in fact gain in sensitivity.

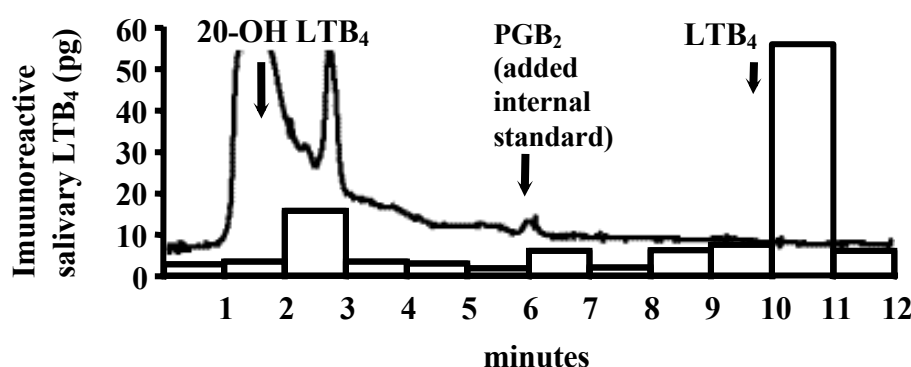


Figure 5. Saliva was subjected to RP-HPLC and one-minute fractions were analyzed by EIA. Solid line depicts ultraviolet absorbance at 270 nm and bars depict LTB₄ immunoreactivity. The arrows indicate retention times of authentic compounds. The majority of the immunoreactivity eluted at the retention time of LTB₄. The antibody used in the EIA cross-reacted with LTB₄ (100%) and 20-OH-LTB₄ (15%).

Levels of LTB₄ in saliva were significantly decreased after zileuton treatment

Zileuton effectively attenuated immunoreactive LTB₄ levels in saliva after the first dose (visit 2) in healthy ($p < 0.001$, $n = 14$) and in atopic subjects ($p < 0.001$, $n = 12$, Figure 6A), and also after three days (visit 3) in both the healthy ($p < 0.001$) and atopic subjects ($p < 0.001$). The median percentage inhibition in the healthy group was 71% (57-76) at visit 2 and 68% (57-82) at visit 3 ($n = 14$). The median percentage inhibition in the atopic group was 84% (76-90) at visit 2 and 86% (73-91) at visit 3 ($n = 12$). In the cross-over study in a sub-group of healthy subjects, immunoreactive LTB₄ levels were not affected by placebo, but were significantly attenuated by zileuton (Figure 6B, $n = 7$).

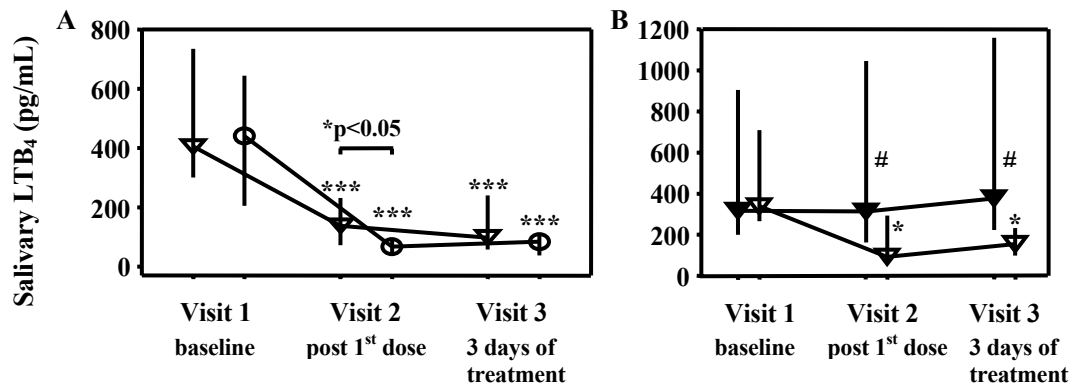


Figure 6. Salivary LTB₄ before and after zileuton treatment. Values are medians with 25th–75th percentile range. **(A)** Open study: Zileuton attenuated LTB₄ in healthy (triangles; n=14) and atopic subjects (circles; n=12); ***p<0.001 compared to baseline. **(B)** Cross-over study: LTB₄ was inhibited by zileuton (open triangles) but not placebo (filled triangles); *p<0.05 compared to baseline. There was a significant difference between placebo and zileuton (visits 2 and 3); #p<0.05

Levels of LTE₄ in urine were not significantly altered by zileuton treatment

Urinary LTE₄ levels did not differ between the healthy and the atopic subjects (p>0.25 at all study points). Zileuton did not alter urinary levels of immunoreactive LTE₄ after the first dose [22.9 (16.1-32.2) vs. 22.1 (17.7-32.4) ng/mmol creatinine, p=0.264, n=26] or after three days of treatment [21.5 (16.3-25.3) ng/mmol creatinine, p=0.477, n=26]. Urinary LTE₄ levels also did not differ between the placebo-treated arm and the zileuton-treated arm in the cross-over study (p=0.688, data not shown).

In contrast with the current short-term study, in which zileuton treatment for 3 days did not affect urinary LTE₄, there are several studies in subjects with asthma where 2-6 weeks of zileuton treatment inhibited urinary LTE₄, ranging between 36% and 86% reductions from baseline (124, 192-194). Our study raised the possibility that direct measurements of LT levels in saliva may be a more sensitive marker of altered *in vivo* 5-LO activity as compared with urine.

The effects of zileuton on LT levels *in vivo* were previously studied by Hui and coworkers (125); they showed, in agreement with our results, that zileuton produced significant inhibitions in *ex vivo* LTB₄ generation in whole blood. However, minor reductions in urinary LTE₄ were positively correlated with zileuton-induced reductions in the early airway response (EAR) to inhaled allergen. In contrast, *ex vivo* LTB₄ levels generated in whole blood correlated better with plasma concentrations of zileuton (125).

Levels of LTE₄ in saliva and in whole blood *ex vivo* were significantly decreased after zileuton treatment

To determine whether the failure of zileuton to reduce LTE₄ levels in urine also applied to CysLT levels elsewhere, LTE₄ was measured in the saliva and in the plasma (after *ex vivo* ionophore-stimulation of whole blood) from six of the atopic subjects. Three days of zileuton treatment significantly attenuated LTE₄ levels both in ionophore-stimulated whole blood [inhibition 98 % (98-99), p<0.05] and in saliva [inhibition 59 % (53-70), p<0.05]. The degree of inhibition of salivary LTE₄ was not

significantly different ($p>0.5$) from the extent of inhibition of salivary LTB₄ in the same subjects.

Fraction of exhaled nitric oxide was unaffected by zileuton treatment

Measurements of F_{ENO} were performed in parallel with leukotriene measurements, in order to assess the effect of 5-LO inhibition on this non-invasive marker of airway inflammation (**own unpublished data**). Atopic subjects had higher levels of F_{ENO} at baseline when compared to the healthy group ($p<0.05$, Table II), and this difference was maintained at visits 2 and 3. F_{ENO} was moderately attenuated by zileuton at visit 2 in the healthy group ($p<0.05$), but there was no significant effect of zileuton at visit 3, and zileuton did not influence F_{ENO} in the atopic group at either visit 2 or visit 3. Two atopic subjects taking inhaled corticosteroids were excluded from the statistical analysis of F_{ENO}. In the cross-over study, F_{ENO} values were moderately attenuated at visit 2 in the placebo group, but there were no significant difference between the groups at baseline, and zileuton had no effect on F_{ENO} at visit 2 or visit 3 (Table III).

Table II. Fraction of exhaled nitric oxide (F_{ENO}) in healthy subjects and atopic subjects. Values are medians with 25th-75th percentiles.

	F _{ENO} (ppb)		
	Baseline	after one dose of zileuton	after 3 days of zileuton
Healthy subjects (n=14)	13.4 (10.4-16.8)	12.2 (10.7-14.5) p=0.020*	11.8 (10.6-14.9) p=0.058*
Atopic subjects (n=10)	27.0§ (22.3-39.7)	25.9§ (19.3-37.7) p=0.426*	23.2§ (11.2-42.2) p=0.492*

* Within-group comparison, compared with baseline value

§ Between-group comparison, $p<0.05$

Table III. Seven healthy subjects participated in a cross-over study. Values are medians with 25th-75th percentiles.

	F _{ENO} (ppb)		
	Baseline	after one dose of treatment	after 3 days of treatment
placebo group	15.3 (9.7-18.1)	12.4* (9.4-17.1)	12.3 (10.8-15.1)
zileuton group	12.9 (10.1-16.5)	12.4 (9.3-14.3)	11.8 (9.9-14.6)

* Within-group comparison, compared with baseline value $p<0.05$

We could not demonstrate an inhibition of F_{ENO} after this short-term treatment with zileuton in healthy or atopic subjects, which is in accordance with a previous study where zileuton pretreatment of healthy subjects did not affect pig house dust mediated rise in F_{ENO} levels (29). In asthmatic subjects, treatment with anti-leukotriene drugs (in this case CysLT₁ receptor antagonists) resulted in decreased exhaled nitric oxide (195, 196), although other authors have reported no effect on F_{ENO} with the leukotriene receptor antagonists (197).

In vitro studies have demonstrated interactions between nitric oxide and leukotrienes. Leukotrienes (both CysLTs and LTB₄) were shown to induce NO production in human neutrophils (52). Conversely, NO suppresses LT production in rat alveolar macrophages and human mast cells (52). Nitric oxide-induced inhibition of 5-LO activity in rat alveolar macrophages was shown to be mediated via soluble guanylyl cyclase and protein kinase G dependent-mechanisms (198). Interestingly, in addition to its 5-LO inhibiting function, zileuton was shown to inhibit the LT suppressing effect of nitric oxide in rat alveolar macrophages (199).

The results in **paper II** showed that the basal levels of F_{ENO} were significantly increased in the atopic group. This is in accordance with previous studies of F_{ENO} comparing healthy with atopic subjects (200). Fraction of exhaled nitric oxide have been positively correlated with the number of airway eosinophils (201) and subjects with atopy were shown to have increased eosinophils in the airways (42). Therefore, the increased basal F_{ENO} levels in the atopic group in **paper II** may imply that these subjects have increased eosinophils in their airways.

Zileuton had no acute effect on the expression of LT pathway enzymes in blood leukocytes

To investigate further the effects of zileuton, immunoexpression of 5-LO pathway enzymes in peripheral blood leukocytes was analyzed by flow cytometry. Evidence that the 5-LO pathway product LTB₄ can itself modulate the transcription of 5-LO pathway enzymes in animal models and in human leukocytes *in vitro* (202-205) led us to investigate whether inhibiting LTB₄ biosynthesis *in vivo* with zileuton would affect intracellular immunoexpression of 5-LO, FLAP, LTA₄H and LTC₄S in peripheral blood leukocytes. Basal expression of 5-LO, FLAP, LTA₄H and LTC₄S was readily detectable by flow cytometry in peripheral blood leukocytes, although levels varied between the cell types examined. As expected, neutrophils prominently expressed the enzymes required for LTB₄ biosynthesis (5-LO, FLAP, LTA₄H), and eosinophils expressed enzymes required for CysLT biosynthesis (5-LO, FLAP, LTC₄S). In contrast to previously reported observations in a mouse model (202), our crossover study with zileuton for three days in normal subjects did not show changes in expression of 5-LO pathway enzymes in peripheral blood leukocytes. The findings in **paper II** (online repository) do not preclude the possibility that longer-term treatment with zileuton may result in compensatory changes in the expression of the LT pathway enzymes.

INCREASED LEVELS OF CYSTEINYL-LEUKOTRIENES IN SALIVA, INDUCED SPUTUM, URINE AND BLOOD FROM ASPIRIN-INTOLERANT ASTHMATICS (paper III and unpublished data)

In this study, 11 subjects with AIA and ten with ATA were provoked with inhaled lysine-aspirin (L-ASA). Samples of saliva, induced sputum, blood and urine were collected before, during and/or after the provocation and FE_{NO} was measured (see Figure 7 for study design). Cysteinyl-LTs, LTB_4 , and $9\alpha,11\beta$ -PGF₂ were measured with EIA. Cell differentials were counted in induced sputum.

↑ Baseline visit (visit 1):		↑ Provocation visit, L-ASA (visit 2):			
	3–10 days	baseline	during	End of	after EP
FE_{NO}		FE_{NO}	provocation	provocation (EP)	saliva up to 2-3h
saliva		saliva	saliva and	$FEV_1 \geq 20\%$ or	urine up to 3h
urine		urine	urine every	max L-ASA dose	
FEV_1		blood	hour	blood	
induced sputum		FEV_1			

Figure 7. Study design, paper III. FE_{NO} = fraction of exhaled nitric oxide; FEV_1 = forced expiratory volume in one second; L-ASA = lysine-aspirin

Inflammatory markers at baseline vs. after provocation

Subjects with aspirin-intolerant asthma had increased basal levels of CysLTs and FE_{NO}

The results showed that subjects with AIA had increased basal FE_{NO} levels, and increased basal levels of immunoreactive CysLTs in saliva, sputum supernatant, blood *ex vivo*, as well as increased basal LTE_4 levels in urine. There was a trend for increased sputum eosinophils (%) at baseline in subjects with AIA, although it did not reach statistical significance. However, basal levels of LTB_4 in saliva, sputum supernatant and blood *ex vivo*, or basal levels of $9\alpha,11\beta$ -PGF₂ in urine were not significantly different between the AIA and ATA group. In conclusion, these results support the concept that there is a global deviation in leukotriene metabolism in AIA, with selective overproduction of CysLTs.

The increase in CysLT and FE_{NO} levels lends circumstantial support to the theory of eosinophil involvement in AIA. The eosinophil is a main producer of LTC_4 (51) and previous studies have shown that tissue eosinophils (from bronchial biopsy specimens) were increased 4-fold in AIA as compared with ATA subjects (99) . Although induced sputum reflects the cellular composition of the airway lumen, it does not represent an identical situation to the local inflammatory process in the mucosa (206). Speculatively, it may be that tissue eosinophils were significantly increased in the AIA subjects of **paper III**. In addition, levels of FE_{NO} have previously been shown to be positively correlated with sputum eosinophils in a larger study group (201).

Overproduction of LTE_4 in the urine is one of the major characteristic features of aspirin-intolerant asthma (for review see (95)). Previous studies have shown increased levels of LTE_4 in the urine from patients with AIA (100, 207), as well as an association between hyperleukotrienuria and clinical features that are similar to those of AIA (208). Previous studies have also indicated increased CysLTs levels in induced sputum (115,

116) and in exhaled breath condensate (209, 210) of patients with AIA. However, this is the first time increased levels of CysLTs have been shown in saliva. This is also the first time an increased basal CysLT producing capacity of ionophore stimulated whole blood in AIA as compared with ATA has been shown.

However, measurements of CysLT levels in blood and sputum from subjects with AIA show variable results. In contrast to our data, Sanak *et al* did not detect increased basal LTC₄ levels in whole blood stimulated *ex vivo* from subjects with AIA (211). In accordance with our results, Obase *et al* showed increased basal sputum CysLT levels in patients with AIA (115, 116). However, Higashi *et al* did not detect increased CysLTs concentrations in sputum from AIA at baseline, although basal LTE₄ levels in the urine were increased (212). In light of these variable results, it cannot be ignored that the different aspirin provocation protocols (oral, inhaled, intravenous, and nasal) used in different studies may contribute to conflicting results. In this context, it may also be important to point out that the subjects' AIA status was confirmed in **paper III** with an aspirin bronchoprovocation test at the time of the study, which was not the case in all other reports.

Antczak *et al* showed increased basal CysLT levels in exhaled breath condensate from patients with AIA as compared with ATA (209). Cysteinyl-leukotriene levels in EBC from steroid-naïve subjects were 152.3±30.4 pg/mL for AIA, and 36.6±7.1 pg/mL for ATA subjects, respectively (p<0.05). In a study by Sanak *et al*, exhaled CysLTs were similar between AIA and ATA patients at baseline (9.3±10.3 vs. 13.8±17.1 pg/mL, AIA vs. ATA) (210). However, after aspirin provocation, the exhaled CysLT levels were increased in the AIA group only (34.0±33.5 pg/mL, p<0.001) (210). In contrast, we did not detect CysLT levels in any of the EBC samples collected from patients with AIA or other forms of asthma or in healthy subjects (n=36, **own unpublished data**). Moreover, activity of the saliva marker α-amylase was low or below the detection limit (0.3 U/L) in all EBC samples in our study. In the studies performed by Antczak *et al* and Sanak *et al*, there was no information given regarding assessment of possible saliva contamination in the EBC samples (209, 210).

We also showed for the first time increased basal F_{ENO} levels in the AIA group. Our results are in contrast with Rolla *et al*, who did not observe a significant difference in baseline F_{ENO} levels between AIA and ATA patients (213). However, Rolla *et al* detected a F_{ENO} increase 2-4 hours after L-ASA bronchoprovocation in the AIA group, which is suggestive of eosinophil recruitment (213).

Levels of leukotrienes and 9α,11β-PGF₂ before vs. after provocation

There was a significant increase in urinary LTE₄ and 9α,11β-PGF₂ levels post L-ASA provocation as compared with baseline in the AIA group (**paper III**), confirming previous reports and supporting the theory of mast cell activation in AIA (101, 207).

Blood was collected for *ex vivo* ionophore stimulation at baseline and immediately after the end of the L-ASA provocation. However, leukotriene levels in plasma from whole blood stimulated *ex vivo* were unaltered post provocation in the AIA group. Since there was no blood collected for differential counting, it is difficult to conclude whether the increased basal CysLT levels in ionophore-stimulated whole blood reflects an up-regulation of the LTC₄ synthetic pathway or increased numbers of LTC₄ producing cells (e.g. eosinophils) in subjects with AIA. In absence of blood count results, it is also difficult to provide a good explanation for the post-provocation results of leukotrienes

in blood stimulated *ex vivo*. Effects of a possible change in blood eosinophil number post provocation may have been missed since blood was collected immediately after end of provocation. In comparison, when sputum CysLT levels were expressed as per million eosinophils, levels were not greater in the AIA group. This suggests that the increased basal levels of CysLTs in sputum are an effect of increased eosinophil numbers rather than overactivation of each eosinophil.

Somewhat unexpectedly, levels of CysLTs and LTB₄ were decreased in saliva after L-ASA provocation. In order to further study this, saliva and urine collected before and after allergen bronchoprovocation from eight subjects with atopic asthma were analyzed. The results confirmed a decrease of leukotriene levels in saliva, and an expected increase of LTE₄ and 9 α ,11 β -PGF₂ in urine after allergen challenge (101, 214). Further studies on the origin and kinetics of salivary leukotrienes are needed in order to understand the decrease in salivary leukotrienes post provocation. In contrast to urinary data that are corrected for creatinine, data from salivary measurements are presented in pg/mL. This may at least partly explain the discrepancy between salivary and urinary results.

Association between parameters of lung function and inflammatory markers

In addition to assessing differences between asthmatic groups, a relationship between the inflammatory markers and lung function was also evaluated (**own unpublished data**). Spearman rank correlation test was used to calculate correlations on baseline values.

Results showed that lung function expressed as FEV₁ (% predicted) correlated negatively with urinary 9 α ,11 β -PGF₂ (Figure 8). Interestingly, a significant correlation between clinical severity of asthma and intraepithelial mast cells in bronchial brush biopsies from corticosteroid-treated asthmatic subjects has been shown (215).

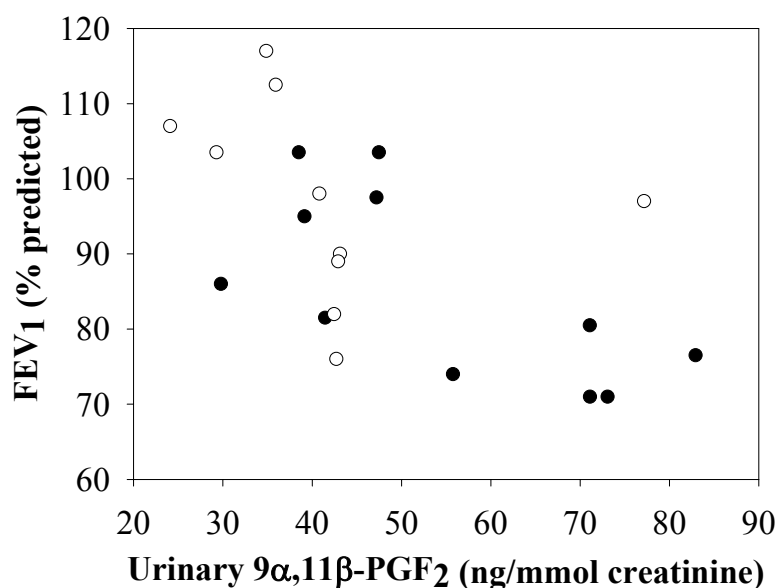


Figure 8. There was a negative correlation between FEV₁ (% predicted) and 9 α ,11 β -PGF₂ levels in urine ($r_s = -0.65$, $p = 0.001$, $n = 21$). Filled circles represent subjects with AIA, whereas open circles represent subjects with ATA.

The lung function (FEV₁ % predicted) also correlated with levels of CysLTs in *ex vivo* stimulated whole blood (Figure 9). This is the first time a correlation between lung function and levels of CysLTs in *ex vivo* stimulated whole blood has been shown in patients with asthma. Both eosinophils and mast cells are main producers of LTC₄, which is metabolized to LTD₄ and subsequently to the more stable form LTE₄ (60).

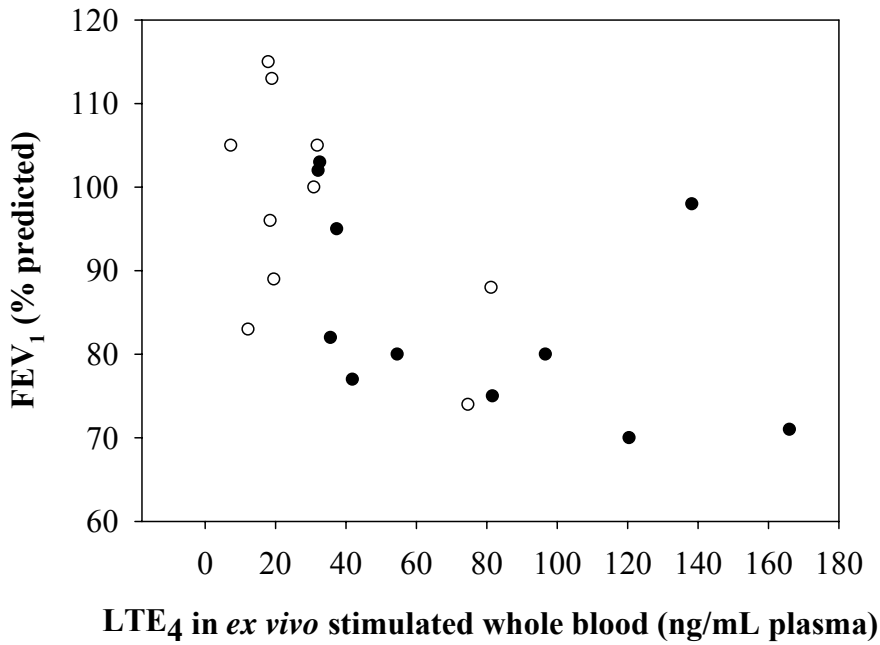


Figure 9. There was a negative correlation between FEV₁ (% predicted) and CysLT levels (expressed as LTE₄ equivalents) in *ex vivo* stimulated whole blood ($r_s = -0.67$, $p < 0.001$, $n = 21$). Filled circles represent subjects with AIA, whereas open circles represent subjects with ATA.

Only in the AIA group did FEV₁ (% predicted) correlate with percentage eosinophils in induced sputum ($r_s = -0.85$, $p < 0.001$, $n = 11$). Sputum eosinophils have previously been found to weakly correlate with lung function measured as FEV₁ (216, 217).

GENERAL DISCUSSION:

The potential implication of salivary leukotrienes as biomarker of disease

This thesis investigated two methods for the non-invasive measurement of leukotrienes: EBC and saliva. In **paper I**, LTB₄ could not be detected in nearly 100 samples of EBC. It was concluded that the LTB₄ levels found in EBC were a result of saliva contamination, as assessed by the concomitant detection of the saliva marker α -amylase activity in the LTB₄ positive samples. This was further investigated by measuring CysLTs as well as α -amylase activity in EBC from subjects with asthma and healthy controls (n=36, **own unpublished data**). The results showed that there were no or very low basal levels of CysLTs or α -amylase activity in EBC. In **paper II**, the potential of LT measurements in saliva was investigated. Salivary LTs were shown to be attenuated by the 5-LO inhibitor zileuton. It was therefore concluded that LTs in saliva may be used as novel and sensitive biomarker of altered *in vivo* 5-LO activity. In **paper III**, increased basal CysLTs in saliva as a potential biomarker of aspirin-intolerant asthma was demonstrated for the first time.

Following the first report by Rigas *et al* in the early 1980s (137), only one brief study has presented LTB₄ levels in saliva from patients with asthma (133) (not published in English). In that study, levels of LTB₄, prostaglandin F_{2 α} and thromboxane B₂ in blood and saliva were increased, and levels of PGE₂ and prostacyclin were reduced, during an asthma attack in children. The study presented in **paper II** is the first to present data on the effect of anti-leukotriene treatment on salivary leukotrienes in both healthy and atopic subjects. The study presented in **paper III** is the first to present data on salivary leukotrienes from subjects with aspirin-intolerant asthma. The measurement of salivary leukotrienes has been attempted in clinical conditions other than airway inflammation. One study found lower levels of immunoreactive salivary LTB₄ in patients with migraine as compared to controls, and the salivary levels correlated with LTB₄ levels in nasal lavage fluid (131). Another study demonstrated enhanced levels of salivary LTB₄ in patients with squamous cell carcinoma of the head and neck (132). Others have detected higher levels of CysLTs in gingival crevicular fluid (which is a component of whole saliva) from subjects with atherosclerosis (218). These studies support the hypothesis that salivary LT measurements may reflect systemic levels and have a wide applicability to diseases in which CysLTs and LTB₄ may be involved, for demonstration of involvement and for assessment of the efficacy of LT synthesis inhibitors. Furthermore, in the absence of an appropriate urinary marker for whole-body LTB₄ synthesis, our data on salivary LTB₄ responses to short-term zileuton therapy (**paper II**) may have particular relevance to functional trials of novel LT synthesis inhibitors in inflammatory conditions in which LTB₄ is more closely implicated than CysLTs.

The leukotriene content of saliva should also be recognized as a possible contributing source of leukotrienes when collecting other biological samples via the mouth, *e.g.* EBC (see **paper I**) and induced sputum. In **paper III**, possible salivary contamination of sputum samples was estimated to be similar in the two groups as sputum squamous cell counts were not different between the two groups.

Salivary measurements of leukotriene levels appear to introduce significant advantages with regard to their non-invasive nature and in providing direct assessment of *in vivo* leukotriene concentrations. Collection of saliva is inexpensive. It can be frozen for

storage immediately on collection, and an advantage in the clinical setting is that this requires no processing at the time of collection. Children in particular would benefit from not being required to undergo venepuncture. Saliva is already routinely used in other contexts to measure levels of cortisol and IgA (for review see (138, 219)). In fact, using saliva for different types of diagnostic testing have been described by more than 20,000 publications (220). The diagnostic value of saliva has been assessed for the detection of a number of systemic diseases, such as infectious diseases, cancer and Alzheimer's disease (138, 220, 221).

However, a number of issues must be addressed before salivary LTs can be applied for diagnostic use, including optimizing methods of collecting and processing saliva before analysis, as well as more studies on kinetics, diurnal variability and stability are needed. In the early studies by Rigas *et al*, it was shown that the arachidonic acid metabolites are independent of salivary flow rate (136), and that they have a circadian rhythm, with the highest levels at 5 a.m. (135). Therefore, saliva samples in **paper II** and baseline saliva samples in **paper III** were always collected between 8 a.m. and 10 a.m. to reduce the influence of diurnal variation. Furthermore, there is intra- and intervariability of LTs in saliva to take into account. The analysis is also hampered by the fact that the dilution factor is largely unknown, and there is no established reference marker of dilution at present. This is an important area for future research. The source of LTs in saliva remains to be conclusively established. In addition to overflow from the circulation, it is conceivable that local production by salivary glands contributes. Green *et al* showed a lipoxygenase-generated production capacity of cells taken from the oral cavity (222), but it remains to be determined whether the leukotrienes are mainly secreted from the salivary glands, or if other cells in the oral cavity contribute to leukotrienes found in whole saliva.

With respect to the ease and non-invasiveness by which saliva is collected, in combination with the vast amount of mediators that can be measured in saliva (142, 220), the future applications and opportunities in saliva diagnostics are promising. High-throughput approaches may allow us to study several biomarkers in saliva simultaneously in inflammatory diseases such as asthma. Future studies of leukotrienes in saliva may involve a more comprehensive analysis on the leukotrienes and factors that interact with them, *i.e.* the lipidomics approach (223), in aspirin-intolerant asthma or other diseases with increased production of leukotrienes (51).

C ONCLUSIONS

- Saliva is one likely source of leukotrienes in exhaled breath condensate.
- Measurements of leukotrienes in exhaled breath condensate should include a sensitive test for the possible saliva contribution. This precaution should also extend to all measurements in EBC of compounds that are secreted in relatively high quantities in saliva.
- Leukotrienes in saliva may be used as novel and sensitive biomarkers of *in vivo* 5-lipoxygenase activity.
- Cysteinyl-LTs in saliva may be used as a new and non-invasive biomarker of aspirin-intolerant asthma.
- Measurement of basal LTs in saliva generally gave the same mechanistic information as measurements in blood *ex vivo*, sputum, and urine.
- Salivary leukotrienes may be explored in other diseases associated with increased leukotriene production.
- Further work is needed to optimize measurements of leukotrienes in saliva, especially with regard to identifying a suitable marker of dilution.

POPULÄRVETENSKAPLIG SAMMANFATTNING (Swedish)

Icke-invasiv analys av leukotriener som markörer för luftvägsinflammation

Astma och kroniskt obstruktiv lungsjukdom (KOL) är två vanliga sjukdomar som drabbar luftvägarna. Personer med astma eller KOL har en kronisk inflammation i luftvägarna. Det betyder att inflammationen alltid finns där, även vid symptomfria perioder. Inflammation är kroppens försvar mot infektioner och andra främmande ämnen, t.ex. ämnen i tobaksrök eller allergiframkallande substanser. Inflammationen har också som uppgift att reparera en skada på vävnaden. När en kroppsdel skadas eller blir angripen, så svarar vårt immunförsvar med att skicka dit flera olika typer av vita blodkroppar (inflammatoriska celler) som i sin tur bildar ett flertal inflammatoriska substanser. Leukotriener (LT) är substanser som visat sig spela en viktig roll i den inflammatoriska processen. De tre cysteinylinnehållande leukotrienerna (CysLT = LTC₄, LTD₄ och LTE₄) utsöndras från eosinofiler och mastceller, medan neutrofiler bildar LTB₄. De inflammatoriska cellerna och substanserna angriper det kroppsfrämmande ämnet och reparerar den skadade vävnaden. Detta är vad som menas med en akut inflammation och är vanligtvis en mycket väl kontrollerad process. Inflammationen övergår till att bli kronisk när det kroppsfrämmande ämnet kvarstår eller när det uppstår en obalans i systemet som medför att inflammationen inte läker ut. Den kroniska inflammationen hos astmatiker skiljer sig från den hos KOL-patienter: astma domineras av eosinofiler och mastceller, medan KOL domineras av neutrofiler. Dessutom finns det olika varianter av astma och KOL. Den underliggande kroniska inflammationen ser olika ut hos dessa olika varianter och det är mycket viktigt att förstå vilken typ av underliggande inflammation patienten har för att kunna ge korrekt behandling.

Bronkoskopi är standardmetoden för att ta prover för mätning av inflammationen i luftvägarna. Bronkoskopet är ett långt böjligt instrument som förs ned i luftrören (=bronkerna) via näsborren eller svalget efter lätt bedövning. Bronkoskopi är en invasiv procedur som kräver specialtränade läkare och assistenter samt inte lämpar sig för alla patienter. Mindre invasiva eller helt icke-invasiva metoder har därför utvecklats för att studera luftvägsinflammationen, t.ex. inducerat sputum och utandningskondensat. Inducerat sputum är ett prov som fås genom att patienten inandas en salthaltig lösning vilket retar till slembildning och hosta. Patienten hostar följaktligen upp provet. Utandningskondensat (*eng.* exhaled breath condensate = EBC) samlas helt icke-invasivt. Patienten andas ut genom ett munstycke som är kopplat till nedkyllt rör, vilket resulterar i att den utandade luften kondenserar och därmed bildar en vätska som samlas upp i en kopp i änden av röret. I denna vätska mäts sedan halten av exempelvis leukotriener, som används som ett mått på luftvägsinflammationen. Teorin bakom EBC-metoden är att leukotrienerna färdas från luftvägarna ut med utandningsluften. Höga halter av leukotriener i EBC kan vara ett tecken på en pågående luftvägsinflammation.

Avhandlingsarbetet syftade till att mäta leukotriener i olika kroppsvätskor. Förutom inducerat sputum och EBC har vi samlat blod, urin och även salivprov för att ta reda på om vi får samma resultat i olika kroppsvätskor. Salivprovet samlas helt icke-invasivt (Patienten uppmanas att spotta i en kopp). Fördelen gentemot ett urinprov är att man kan mäta fler ämnen i saliv, t.ex. så går det bara att mäta endast en av de ovannämnda leukotrienerna i urin, nämligen LTE₄. Både EBC och saliv samlas alltså helt icke-invasivt, men ingen av dessa metoder har validerats för mätningar av leukotriener.

Huvudsyftet med detta avhandlingsarbete var att utvärdera mätningar av leukotriener i EBC och saliv, samt att tillämpa dessa metoder i olika sjukdomsmodeller som tidigare uppvisat höga halter av leukotriener. Det sekundära syftet var att jämföra leukotrienmätningar i EBC och saliv med leukotrienmätningar i inducerat sputum, blod och urin.

Studien omfattade även mätningar av halten av utandad kvävemonoxid. Mätning av FE_{NO} (eng. "fraction of exhaled nitric oxide") är en väl validerad icke-invasiv analysmetod för att bestämma halten av utandad kvävemonoxid. Flera tidigare studier har visat att FE_{NO} är förhöjd vid inflammation i luftvägarna och denna metod har dessutom visat sig vara användbar vid diagnos och uppföljning av luftvägsallergi och astma.

I delarbete I studerades LTB_4 i EBC från försökspersoner exponerade för damm i svinhus. Man har tidigare visat att friska försökspersoner får kraftigt förhöjt antal neutrofiler i luftvägarna efter att ha andats in svinhusdammet i tre timmar. Neutrofiler utsöndrar LTB_4 och det var därför lämpligt att mäta denna leukotrien i svinhusmodellen. En markör för saliv (amylas) mättes för att kontrollera eventuell salivtillblandning. Amylas är ett ämne som vanligtvis återfinns i höga halter i saliven, vilket gör den till en lämplig markör för att påvisa förekomsten av saliv. Då EBC-metoden innebär att man andas ut genom ett munstycke, så kan saliv följa med utandningsluften och därmed kontaminera provet. Saliv innehåller leukotriener och även en liten mängd saliv kan kontaminera EBC-provet. Därför är det viktigt att kontrollera detta och vi utvecklade därför en känslig metod för mätning av salivkontaminering (dvs. amylas). Totalt samlades 102 EBC-prover. Endast fyra av dessa prover innehöll LTB_4 och samtliga av dessa fyra prover innehöll även salivmarkören amylas. Slutsatsen blev att de uppmätta LTB_4 -halterna i EBC var ett resultat av salivkontaminering.

I denna studie samlades även inducerat sputum före och efter svinhusexponeringen. Mätningar av LTB_4 i sputum visade att halterna blev förhöjda efter vistelsen i svinhuset, men det blev inte statistiskt signifikant. Däremot var neutrofilerna (som utsöndrar LTB_4) 16 gånger förhöjda i de sputumprov som samlades efter svinhusvistelsen, vilket tyder på att leukotrienmätningar i sputum inte är lika känsliga som mätningar av neutrofiler i sputum.

I delarbete II jämfördes leukotrienhalter i saliv med leukotrienhalter i blod och urin. Både friska och luftvägsallergiker (med eller utan mild astma) deltog i studien. Försökspersonerna intog ett astmaläkemedel (zileuton) som verkar genom att sänka halten av leukotriener. Prover samlades före respektive efter intag av zileuton och halten av FE_{NO} mättes också. Blodprovet förbehandlades med ett ämne som tvingar blodcellerna att maximalt producera och utsöndra leukotriener. Förbehandling av blodprovet är nödvändigt då leukotrien-nivåerna i blodet är för låga för att kunna detekteras med de mätmetoder som vanligtvis används. Däremot var det inte nödvändigt att förbehandla saliv- eller urinprovet då dessa kroppsvätskor innehåller mätbara halter av leukotriener. Resultaten visade att leukotrien-nivåerna i saliv och blod sjönk efter tre dagars behandling med zileuton. Däremot påverkades inte LTE_4 -nivåerna i urin av tre dagars behandling, vilket tyder på att mätningar i urin inte är lika känsliga som mätningar i saliv eller blod. Slutsatsen var att leukotriener i saliv kan användas som ett känsligt och icke-invasivt mått på en förändrad leukotrienproduktion.

FE_{NO}-nivåerna var förhöjda hos försökspersoner med luftvägsallergi i denna studie. FE_{NO} påverkades dock inte av zileuton.

I **delarbete III** mättes leukotriener i saliv, inducerat sputum, blod och urin, samt FE_{NO} hos patienter med aspirinkänslig astma. Patienter med denna form av astma kan få mycket svåra och ibland livshotande astma-attacker genom intag av acetylsalicylsyra och andra liknande läkemedel (t.ex. ibuprofen). Det är sedan tidigare känt att dessa patienter har förhöjda halter av LTE₄ i urin. Denna studie visade att LTE₄ halterna var förhöjda i samtliga kroppsvätskor hos dessa patienter i jämförelse med astmatiker som tål aspirin. Av speciellt intresse var att LTE₄ halterna i saliv var förhöjda hos aspirinkänsliga astmatiker och detta har inte studerats tidigare. Dessutom visades det för första gången att FE_{NO} var förhöjt hos dessa patienter.

Vi gav också patienterna en liten dos acetylsalicylsyra för att studera hur leukotrien-nivåerna påverkas. En liten dos som ges under kontrollerade former orsakar en liten men övergående försämring av lungfunktionen hos dessa patienter. Detta kallas för aspirinprovokation och är idag det enda sättet att slutgiltigt fastställa diagnos för aspirinkänslig astma. Som väntat orsakade aspirinprovokationen en ökad utsöndring av LTE₄ i urinen hos de aspirinkänsliga patienterna. Dock skedde ingen ökning av leukotrienerna i blod eller saliv, snarare sågs en minskning i saliven. Fortsatta studier av leukotriener i saliv krävs för att förklara resultaten.

Sammanfattningsvis:

- ▶ Studier av leukotriener i utandningskondensat (EBC) bör inkludera en känslig metod som detekterar eventuell salivkontaminering.
- ▶ Leukotrienmätningar i saliv kan användas som ett känsligt och icke-invasivt mått på förändrad leukotrienproduktion.
- ▶ Aspirinkänsliga astmatiker hade förhöjda nivåer av LTE₄ i saliv, samt i inducerat sputum, blod och urin.
- ▶ Salivprover kan vara en framtida metod för diagnos av inflammatoriska sjukdomar.
- ▶ FE_{NO}-nivåerna var förhöjda hos patienter med aspirinkänslig astma.

ACKNOWLEDGEMENTS

First,

I would like to thank all the patients and volunteers who have selflessly and generously participated in these studies.

The work in this thesis was made possible with the help and support of a number of people:

Associate Professor Maria Kumlin (main supervisor): for supervising this thesis project, for generously sharing your tremendous knowledge on eicosanoids with me; and especially for thoroughly and critically reading my thesis summary, despite having another full-time job to think about at the same time.

Professor Sven-Erik Dahlén (co-supervisor and head of EAAF): for supervising and sponsoring this thesis project, for providing with excellent work facilities, for valuable comments on the thesis summary, and especially for being positive in helping me apply to and present our data at various international congresses.

Professor Kjell Larsson (mentor) and Associate Professor Lena Palmberg who supervised the project that provided the basis for paper I. For kindly answering all my questions, especially those regarding lung physiology, statistics, and pig house dust exposure. I think I have asked you guys about everything there is to ask and still you have time for my questions. Also, for critically reading my manuscripts and for being utterly friendly and supportive!

Dr. Fernando Acevedo and Ingrid Delin, my main lab-mates: for all the help inside and outside the lab, for good laughs, for your friendship and for being there for me. I am indebted to Fernando for always having time for me and my questions (whatever they may be), and also for critically reading my manuscripts. I am also indebted to Ingrid for excellent help with all the analyses. Thanks for inviting me to collaborate on the EBC-project! It has been a real pleasure working with you guys.

My sincere thanks to all co-authors and co-workers who in any way contributed to my projects: Dr. Fernando Acevedo, Prof. Kjell Alving, Heléne Blomqvist, Karin C Carlberg, Dr. Kameran Daham, Margitha Dahl, Ass. Prof. Barbro Dahlén, Katarina Damm, Ingrid Delin, Gunnel de Forest, Marianne Eduards, Dr. Alexandra Ek, Inger Ericsson, Ass. Prof. Per Gerde, Agneta Gulich, Dr. Pär Gyllfors, Elisabeth Henriksson, Dr. Ai Higashi, Dr. Noritaka Higashi, Dr. Anna James, Ann-Sofie Lantz, Prof. Kjell Larsson, Dr. Helena Marteus, Ass. Prof. Magnus Nord, Björn Nordlund, Marianne Olsson, Ass. Prof. Lena Palmberg, Karin Sahlander, Dr. Anthony P Sampson, Dr. Nurdan Sandalci, Ida von Scheele, Dr. Maria Skedinger, Dr. Karin Strandberg, Dr. Britt-Marie Sundblad, Ass. Prof. Anders Wetterholm, and Nancy Zonneveld

I would especially like to acknowledge Dr. Anna James, who I collaborated with on several projects, and Drs. Ai and Noritaka Higashi, who I could not have done without on the aspirin-intolerant asthma project. I wish to thank Agneta Gulich and Ann-Sofie Lantz for taking your time to answer my clinically related questions; and also Björn Nordlund for helping with the blood sampling. It was a pleasure working amongst such friendly and talented people!

Associate Professor Per Gerde, Dr. Helena Marteus and Professor Kjell Alving are gratefully acknowledged for helpful discussions relating to the EBC-project.

All the past and present members of the EAAF group for fun and interesting discussions, and for a pleasant time both within and outside the lab: Anita, Anna, Anna-Karin, Annika, Claudia, Ewa, Fredrik, Ingrid D, Ingrid M, Jesper, Josephine, Katarina, Lena L, Linda, Magdalena, Maria B, Marianne H-A, Marianne O, Margareta, Margot (I loved your lactose-free cakes!), Marieke x2, Mikael, Nancy, Pär, Per, Roelinde, Sophia, Yvonne and Åke.

All the past and present members of the ALI group and the rest of IMM/division of Physiology: for creating a friendly and stimulating work environment.

I would especially like to mention Karin Strandberg and Magdalena for being really cool “office mates”; Claudia for friendly company in the lab as well as outside of work; Ewa, Lena L, and Pär for your support; Marianne O for all the help with the practical matters and especially for constantly being in a good and friendly mood; Yvonne and Ulla for taking care of the practical stuff; Brittis and Åsa for your kind help with the blood sampling and lung function measurements; Karin Sahlander and Anna-Karin for fun activities after-work; Camilla Kallin for enjoyable discussions when sometimes sharing train on our long way home to Bålsta; Linda and Mathias for the fun trip to Fjällen; Anne-Sophie K and Linda E for friendly company in the lab. And of course: all the Dutch students (Nancy, Marieke x2, Deline) who among other things showed me how I really should use my skates. ☺

I would also like to mention Anders W, Ulrike, Marija and Cecilia R at MBB for interesting and fun discussions regarding leukotrienes and many other things.

My gratitude goes to Fredrik Rosengren and Sten Thorold for the IT-support!

A part of my PhD studies was conducted at the school bench and I would like to thank all the teachers and course leaders at KI for the excellent PG-courses! The two teachers at my own department, Associate Professor Anne Renström and Professor Emeritus Bengt Björkstén are gratefully acknowledged for teaching me what I need to know about allergy in a highly pedagogical and enthusiastic manner! And what would the JC:s be like without Associate Professor Mikael Adner and his little guinea pigs? Thank you for teaching “Flurra” something about intracellular mechanisms!

This thesis work would not have been possible without the friendship and support from my family and all my friends (both within and outside KI, fosko-friends) who give me the encouragement to go on, and for reminding me that there is a life outside of work. Here, none mentioned, none forgotten.

Last,

I consider myself extremely lucky to have a loving family who are always there for me!

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