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QUANTIFICATION OF INFLAMMATORY MEDIATORS
TO EXPLORE MOLECULAR MECHANISMS AND SUB-PHENOTYPES
OF ASTHMA

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QUANTIFICATION OF INFLAMMATORY MEDIATORS
TO EXPLORE MOLECULAR MECHANISMS AND SUB-PHENOTYPES
OF ASTHMA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedicated to patients suffering from asthma

ABSTRACT

This thesis summarizes a series of studies using liquid chromatography coupled to mass spectrometry methodologies to quantify metabolites of fatty acids (*i.e.*, oxylipins) and histamine in different samples from experimental models and clinical studies with the overall aim to define mechanisms and identify biomarkers for improved sub-phenotyping of asthma.

Asthma is characterized by variable airflow obstruction, hyperresponsiveness and chronic inflammation in the airways. The substantial overlap among clinical descriptors has resulted in difficulties to establish diagnosis and predict response to treatment. Instead, a shift in focus towards identifying specific cellular and molecular mechanisms has emerged, aiming to define new treatable traits based on specific cellular and molecular pathways (defined as endotypes). Important pathobiological components involve the release of potent inflammatory mediators, such as histamine, prostaglandins (PGs) and leukotrienes (LTs), that cause bronchoconstriction and airway inflammation.

A rapid hydrophilic interaction chromatography method failed to quantify the major histamine metabolite 1,4-methyl-5-imidazoleacetic acid (*tele*-MIAA) due to ion suppression from inorganic salts present in urine. Ion-pairing chromatography was therefore employed and the resulting increase in precision enabled the detection of higher baseline levels of *tele*-MIAA in females compared to males (3.0 vs. 2.1 $\mu\text{mol}/\text{mmol}$ creatinine, respectively) (**Paper I**). In addition, levels of *tele*-MIAA reached up to 30 $\mu\text{mol}/\text{mmol}$ creatinine in spot urine samples from mastocytosis patients.

Three liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods quantified 130 oxylipins and were able to define kinetic release and enzymatic contribution of mast cell-derived mediators to smooth muscle contraction using isolated and intact airways from humans and guinea pigs *in vitro*. PGD_2 levels were elevated 24-hour post anti-IgE stimulation of human bronchus, suggesting a prolonged mast cell activation (**Paper II**). Furthermore, exposure to house dust mite (HDM) induced strong release of lipoxygenase-derived LTB_4 , 5,15-DiHETE, 15-HETE and 15-HEDE along with eosinophilic infiltration in a C57BL/6 murine model of asthma. Interestingly, high levels of cysteinyl-leukotrienes (CysLTs) remained unchanged suggesting a different role of CysLTs in mice (**Paper III**).

Urinary profiles of 11 eicosanoid metabolites in 100 healthy control subjects and 497 asthmatics defined normal baseline levels and revealed increased concentration of PGs, LTE_4 and isoprostanes with asthma severity. Consensus clustering of 497 asthmatics identified a five-cluster model with distinct clinical characteristics, which included two new phenotypes, U_1 and U_5 , with low levels of thromboxanes and PGs respectively (**Paper IV**). At the 12 to 18-month longitudinal time point for the 302 subjects with severe asthma, z-scored eicosanoid concentrations retained the five-cluster profile, despite technical and intra-subject variability.

In conclusion, the developed bioanalytical methods were applied to define levels of histamine and eicosanoid metabolites in urine from healthy subjects. In addition, release of multiple oxylipins following mast cell-mediated bronchoconstriction and HDM-induced airway inflammation in model systems were explored to relate functions to levels of lipid mediators. For the first time, grouping of asthmatics according to profiles of eicosanoid metabolites in urine was performed and demonstrated sufficient resolution to identify five sub-phenotypes of asthma possessing distinct clinical characteristics. The presented approaches, for both *in vitro* and *in vivo* respiratory research, offer an opportunity to progress the development of new treatment options and suggests a panel of PGs, LTE_4 and isoprostanes to be further validated as diagnostic markers in patients with asthma.

LIST OF SCIENTIFIC PAPERS

- I. Kolmert J, Forngren B, Lindberg J, Öhd J, Åberg K-M, Nilsson G, Moritz T and Nordström A
A quantitative LC/MS method targeting urinary 1-methyl-4-imidazoleacetic acid for safety monitoring of the global histamine turnover in clinical studies
Analytical and Bioanalytical Chemistry, 2014, February 406(6):1751-62
- II. Kolmert J*, Fauland A*, Fuchs D, Säfholm J, Gómez C, Adner M, Dahlén S-E and Wheelock C-E
Lipid mediator quantification in isolated human and guinea pig airways – an expanded approach for respiratory research
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- III. Kolmert J*, Piñeiro-Hermida S*, Hamberg M, Gregory J-A, López I-P, Fauland A, Wheelock C-E, Dahlén S-E, Pichel J-G and Adner M
Prominent release of lipoxygenase generated mediators in a murine house dust mite-induced asthma model
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- IV. Kolmert J, Lefaudeux D, Sjödin M, Gómez C, DeMeulder B, Auffray C, Balgoma D, Sousa A, Chung F, Dahlén B, Knowles R, Sterk P-J, Djukanović R, Dahlén S-E, Wheelock C-E, on behalf of the U-BIOPRED Study Group
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During my time at the Karolinska Institutet I have also been involved in published studies not included in the thesis.

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Enhanced oxidative stress in smoking and ex-smoking severe asthma in the U-BIOPRED cohort.

PlosOne 2018, under revision

Lazarinis N, Bood J, Gomez C, **Kolmert J**, Lantz AS, Gyllfors P, Davis A, Wheelock C-E, Dahlén S-E and Dahlén B

Leukotriene E₄ induces airflow obstruction and mast cell activation via the CysLT₁ receptor.

Journal of Allergy and Clinical Immunology, 2018, in press

Naz S, **Kolmert J**, Yang M, Rhenike S-N, Kamleh M-A, Snowden S, Heyder T, Levanen B, Erle D-J, Sköld C-M, Wheelock Å-M and Wheelock C-E

Metabolomics identifies sex-associated metabotypes of oxidative stress and autotaxin-lysoPA axis in COPD.

European Respiratory Journal, 2017, June 22;(49)6

Gülen T, Möller Westerberg C, Lyberg K, Ekoff M, **Kolmert J**, Bood J, Öhd J, James A, Dahlén S-E, Nilsson G and Dahlén B

Assessment of *in vivo* mast cell reactivity in patients with systemic mastocytosis.

Clinical and Experimental Allergy, 2017, July 47(7):909-917

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LIST OF ABBREVIATIONS

AHR	airway hyperresponsiveness
AA	arachidonic acid
COX	cyclooxygenase
CRTH2	Chemoattractant receptor-homologous molecule express on type 2 cells, also named DP2
CYP450	Cytochrome P450
CysLT	cysteinyl-leukotriene
FEV ₁ (%)	Forced exhaled volume in 1 second
F _E NO	Forced exhaled nitric oxide
HILIC	Hydrophilic interaction liquid chromatography
HDM	house dust mite
IL	interleukin
LC-MS	liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LOX	lipoxygenase
LT	leukotrienes
OCS	oral corticosteroid
OVA	ovalbumin
PAF	platelet activation factor
PG	prostaglandins
PUFA	polyunsaturated fatty acids
PBS	phosphate buffered saline
RNS	reactive nitrogen species
ROS	reactive oxygen species
sEH	soluble epoxide hydrolase
SPE	solid phase extraction

1 INTRODUCTION

Asthma is a chronic inflammatory disease with symptoms of wheeze, cough and variable airway obstruction (Fireman, 2003). A substantial proportion of asthmatic patients have severe asthma with insufficient control of their symptoms and experience worsening events (exacerbations), and consequently have an impaired quality of life. The need for better treatment options for this group of patients is evident (Bell and Busse, 2013). Despite the fact that some mechanisms in asthma were described early in the 20th century by William Osler (1849-1919) the pathogenesis remains unclear. In more specific terms - there is currently an incomplete understanding of the molecular mechanisms driving the disease. Consequently, there is an unmet need for new biomarkers that better reflect molecular and inflammatory mechanisms, and which could also distinguish sub-groups of severe asthmatics.

During airway inflammation, there is a large release of inflammatory mediators. The mast cell is one important immune cell in asthma (Bradding and Arthur, 2016; Metcalfe et al., 2016), known for its release of potent inflammatory and bronchoconstrictive mediators histamine, cysteinyl-leukotrienes (CysLTs) and prostaglandins (PGs). As the released mediators bind their specific receptors (H₁, CysLT₁ and TP) located on the smooth muscle, they directly induce constriction of the airways. Following chronic inflammation, the airway function can also change and the tissue undergoes cellular remodelling. Herein, oxylipins refer to oxygenated polyunsaturated fatty acid (PUFA) metabolites, of which eicosanoids have been the most studied to date. The research field of lipidomics is expanding and a large number of additional oxygenated lipids have recently been identified as downstream products of PUFA metabolism in biological systems (Buczynski et al., 2009; Dumlao et al., 2011). The role in the pathogenesis of asthma of a few mediators with pro- or anti-inflammatory properties have been studied in more detail, but biochemical function and physiological relevance is largely unknown for the majority of oxygenated lipid molecules that can be detected today. It therefore remains to be determined if released oxylipins have a mediator function or not. Likewise, the baseline excretion levels of many metabolites in healthy human urine have not been established.

The focus of this thesis has been to develop advanced bioanalytical methods, using liquid chromatography coupled to mass spectrometry (LC-MS), that can quantify mediators released by mast cells and other inflammatory cells. The developed LC-MS methods have been characterized to accurately determine the urinary marker of released histamine (*tele*-MIAA) and arachidonic acid-derived eicosanoids. In addition, a comprehensive panel of 130 oxylipins was developed to enable assessment of biochemical pathways of cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 (CYP450) and soluble epoxide hydrolase (sEH) enzymes with the corresponding physiological responses of airway smooth muscle contractions and airway inflammation.

To demonstrate the utility of these methods, urine samples from clinical studies including healthy controls and asthmatic subjects have been evaluated. The large cohorts of subjects enabled levels of the major urinary histamine metabolite and eicosanoids to be characterized. Furthermore, the comprehensive oxylipin profiling platform was applied to an *in vivo* asthma model in mice to study airway inflammation, and to an *in vitro* model of mast cell mediated bronchoconstriction where changes in oxylipin levels was compared with alteration of smooth muscle force following pharmacological treatment.

It is hypothesized that developing quantitative methods for oxylipin profiling and urinary *tele*-MIAA renders it possible to study molecular processes related to the mast cell, and perhaps other cells, involved in airway inflammation and bronchoconstriction.

2 AIM OF THESIS

A series of studies were conducted to develop and apply bioanalytical methods that determine the levels of the major urinary histamine and eicosanoid metabolites, as well as a broad range of oxylipins in multiple samples from different experimental models and in clinical studies. The overall aim of the collected efforts was to define mechanisms and identify biomarkers for improved sub-phenotyping of asthma.

In more detail, the methodological aims of this thesis were:

- To develop a simple and repeatable method for quantification of systemic histamine turnover in the clinical setting together with use of a sensitive method for comprehensive quantification of the major eicosanoid metabolites in human urine
- To set up and demonstrate a translational approach to study oxylipin release from isolated and intact human and guinea pig airways following mast cell-mediated smooth muscle contractions *in vitro*

The secondary aims were:

- To describe the oxylipin release profile in bronchoalveolar lavage fluid following house dust mite (HDM)-induced airway inflammation in a mice model of asthma and from isolated and intact human and guinea pig tissue preparations
- Establish baseline excretion levels in human urine of metabolites of histamine and eicosanoids
- Define associations of eicosanoid metabolite levels in urine with clinical traits in subjects with asthma
- Use urinary eicosanoid metabolite profiles to sub-phenotype subjects with asthma in the clinical observational European U-BIOPRED multicenter study

3 BACKGROUND

3.1 THE NEED FOR BETTER DIAGNOSIS AND TREATMENT IN ASTHMA

Asthma is commonly described as a heterogeneous and variable respiratory disease involving both an acute and a chronic inflammatory response (Papi et al., 2017). The biological mechanisms initiating, regulating and terminating (resolving) the airway inflammation are not well understood, but are dependent on the interaction between cellular and molecular mechanisms. Participating immune cells constitute fundamental parts of both the innate and adaptive immune system and exert their coordinated action via signals (mediators). Individuals suffering from asthma have variable or persistent airway obstruction that give the symptoms of breathlessness, re-occurring wheeze, cough with increased mucus production and impaired quality of life.

The aim of current asthma treatment is to: 1) alleviate inflammation and 2) reduce smooth muscle constriction. This can be accomplished by the use of inhaled or oral steroids and inhaled bronchodilators, such as short or long acting β 2-agonists. For the majority of individuals with asthma, this treatment strategy stabilizes the symptoms; however, about 5-10% of asthmatics present frequent exacerbations and lack of control of their symptoms despite a high dose of oral corticosteroids. Add-on therapy, such as the use of anti-leukotrienes or anti-IgE, may alleviate symptoms for some individuals with asthma (Chung et al., 2014), but a substantial proportion of patients with severe asthma lack effective treatment. Frequent hospital and emergency visits, causing a reduced ability to work, are associated with increased societal costs and a reduced quality of life. Consequently, individuals with severe, or “difficult-to-treat”, asthma are in great need for improved diagnosis and treatment. However, to address this requirement, there is a need to redefine the subgroups of asthmatic patients.

The heterogeneous disease asthma is often considered to be an umbrella term comprised of several related airway diseases. It is most likely caused by multiple factors. Viral infections are believed to be the most common cause for worsening of symptoms and exacerbations (Busse et al., 2010; Rosenthal et al., 2010). Viruses are recognized by the toll-like receptors (TLRs) on antigen presenting cells initiating one type of inflammatory response. A genetic susceptibility component is suggested to be an inducing factor at all ages (Moffatt et al., 2010). Environmental factors and psychosocial factors, such as stress, are all potential triggers of airway obstruction. Allergens are a common environmental trigger (Lemanske and Busse, 2010). To add further complexity, a loss of certain microbiome species has been associated to negatively influence the immunology of asthma (Heederik and von Mutius, 2012). Indeed, asthma is caused by multiple factors and the causes of airway inflammation in one particular patient may differ from another. As the underlying mechanisms are unclear, one main aim of asthma research is to identify the roots of asthma.

Asthma is classically ascribed as being allergic, with elevated T-helper cells of type 2. These cells release type 2 cytokines (IL-4, 5 and 13) and recruit eosinophils to the site of inflammation. In particular, among severe asthmatics, about 50% have > 2% eosinophils in collected sputum samples despite high doses of steroids (Wenzel, 2012; Woodruff et al., 2009). However, some patients have non-type 2 asthma, where instead, neutrophils predominantly infiltrate the lung under the control of type 17 T-helper cells. This type of asthma is often associated with a late age of onset (Miranda et al., 2004).

3.1.1 New ways to change the clinical definition of asthma

Our understanding of asthma pathobiology is incomplete. The clinical practice to stratify asthmatics into mild-to-moderate, or severe, may be a too simplistic categorization. This severity based stratification has been proposed to limit asthma management and treatment, and could be one cause of poor clinical outcomes during drug development (Pavord et al., 2018).

To address the need for improved diagnosis and treatment of asthma, increased research focus has the last decade been directed towards identifying novel sub-phenotypes of asthma (Wenzel, 2012). In an early study by Haldar et al. 2008, four different asthma phenotypes were described by a clustering approach using clinical variables and sputum eosinophils from two cohorts. Later, the Severe Asthma Research Program (SARP) identified five asthma phenotypes by another clustering approach, using a reduced set of physiological and clinical variables (Moore et al., 2010; Wu et al., 2014). At best, new clinical phenotypes may help to explain clinical differences among patients, but identifying specific underlying molecular mechanisms would increase the potential to suggest new treatment targets (Wenzel, 2016). A research area of increasing interest is therefore to uncover functional and pathological mechanisms, referred to as an endotype, or specific mechanisms that respond to treatment (Anderson, 2008).

Based upon the concepts mentioned above, the Unbiased BIOMarker Prediction for Respiratory Disease outcomes (U-BIOPRED) study was designed with the primary aim of performing unbiased molecular data clustering approaches to identify new phenotypes and endotypes. To accomplish this, 607 subjects were enrolled, undergoing clinical evaluation and collection of multiple biofluid samples for multiple omics-platforms to acquire the most comprehensive molecular data set to date. The basic premise of the study was to challenge this simplistic description in which asthma has been subdivided by age of onset, type 2 and non-type 2 (Wenzel, 2012), and to instead identify detailed molecular descriptors involved in asthma.

Clinical cluster analysis of the U-BIOPRED data used a bootstrapping methodology based on consensus clustering, which is a different approach to clustering than was used in the SARP study and by Haldar et al. Four clinical clusters were identified using only nine included clinical variables (Lefaudeux et al., 2016), partly overlapping with previously published clinical clusters. Further investigations of the U-BIOPRED cohort have explored changes in blood and sputum mRNA transcript, protein profiles as well as in bronchial biopsies and brushings. Findings from these studies highlighted molecular signatures related to altered epithelial barriers, responsiveness to steroids, type 2 inflammation and activation in the IL-6 trans signalling pathway (Bigler et al., 2017; Jevnikar et al., 2018; Kuo et al., 2017; Takahashi et al., 2018). As the U-BIOPRED project is still in its data analysis phase, additional results from multiple omics data integration analysis remain to be reported.

Given the above-mentioned clinical efforts, experimental research models are complementary by the use of intervention with specific mechanisms. In this way, important features such as airway inflammation and bronchoconstriction can be modulated to further increase our understanding.

3.1.2 *In vivo* and *in vitro* models of asthma

The murine asthma models enable allergen-induced airway inflammation and airway hyperresponsiveness to be studied while systematically investigating cellular and molecular changes in biological samples from, for example, bronchoalveolar lavage fluid (BALF), lung

tissue and biofluids. To study cellular and molecular mechanisms involved in mediating allergic airway inflammation, both BALB/c and C57BL/6 mice strains have been used to document the involvement of eosinophils, mast cells and neutrophils in this condition. Despite the many opportunities with these models, mice lack the branching structure of the lung as well as the basic signalling pathways that exist in humans. The serotonin-to-acetylcholine pathway is the primary mechanism for mediating bronchoconstriction in the mice, which distinctly differs from humans where histamine, PGs and LTs are the primary mediators (Säfholm et al., 2015). Despite these differences, mice models offer many opportunities to characterize specific mechanisms such as comparison of different allergens, e.g., alternaria, house dust mite (HDM) and ovalbumin (OVA) (Fuchs and Braun, 2008).

In *in vitro* experiments, the role of the human epithelial layer in mediating type 2 inflammation has been studied. The cytokines IL-4, IL-5 and IL-13 are central to the type 2 mechanism and are frequently upregulated in allergic asthma. Woodruff and colleagues demonstrated in patients with mild-to-moderate asthma that using isolated bronchial epithelial cells, two subgroups (type 2 high and low) of patients could be identified following IL-13 stimulation of cultured cells (Woodruff et al., 2007). The type 2 high group was further associated with elevated IL-5 and IL-13 cytokines in their biopsies, AHR, elevated serum IgE and eosinophilia, were responsive to inhaled steroids. The cellular and molecular evidence for the type 2 mechanism is important, as steroid treatment has proven effective for this group of patients and therefore constitutes a fundamental treatment option in asthma.

The events following allergen exposure result in bronchoconstriction and airway inflammation. One experimental set up to study the constriction of the airway smooth muscle is by use of the organ bath methodology, which enables functional responses to be monitored following pharmacological treatment using receptor agonists/antagonists (Säfholm et al., 2015; Yu et al., 2018). The results from intervening with specific molecular pathways in one species can be compared with the same intervention in another species, thereby addressing important translational aspects. The guinea pig is the particular species where the lung anatomy resembles that of humans (Canning and Chou, 2008).

3.1.3 Airway physiology

The upper respiratory system includes the nose and nasal cavity and connects to the lower regions where the proximal and distal portions are located. The proximal portion consist of the trachea and the main bronchi, while the distal portion hosts the bronchioles and alveoli. In human and guinea pigs, the lungs have a clear branching structure where each bronchi repeatedly subdivides into two smaller bronchi, **Figure 1**. During the transport of inhaled air from the main bronchi down the bronchial tree to the alveoli, the site where the gas exchange occurs, about 23-27 divisions can be observed (Macklem, 1998).

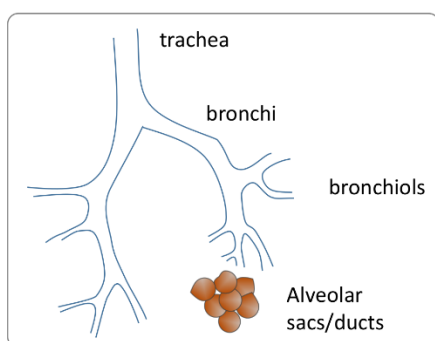


Figure 1. The branching structure of the human lung ends with the bronchioles and the alveolar sacs where the gas-exchange occurs.

The distal airways have an inner diameter of ≤ 2 mm and start at division 8, also referred to as the small airways. During obstruction of asthmatic airways, the smooth muscle constricts to narrow the bronchi, which can lead to almost complete loss of airflow. In particular, among subjects with severe asthma, bronchial wall thickening, edema and increased secretion of mucus is frequently observed, leading to reduced efficiency in the O₂/CO₂ gas exchange and a reduction in blood oxygenation. The airway smooth muscle surrounding the bronchi is under involuntary control and has attained additional interest in asthma research because of its role also to produce many signaling molecules, such as cytokines, chemokines and growth factors (Black and Johnson, 2002; Knox et al., 2000).

The epithelial layer is the inner lining of the airways where inhaled air, particles and allergens come into contact with the host and therefore constitute an important first defense barrier. Furthermore, invading viruses and bacteria are also at this epithelial barrier further prevented to penetrate the tissue. Another important function of the epithelial layer is the production of pro-inflammatory cytokines IL-13, IL-25, IL-33, TSLP and eotaxin (Holgate, 2011). These cytokines can recruit eosinophils to amplify inflammatory conditions. In this tissue, oxylipins derived from COX, LOX and CYP450 enzyme activity are actively produced as well as nitric oxide (NO).

Innate immune cells such as basophils, eosinophils and mast cells are potent effector cells and are actively involved in airway inflammation, bronchoconstriction and associated with type 2 asthma (Lambrecht and Hammad, 2015). Mast cells are derived from the bone marrow and found resident in the skin and within the respiratory tract. Furthermore, eosinophils and neutrophils can be recruited to the airways to further enhance inflammation. Recently, a lower number of BALF natural killer (NK) cells was observed in severe asthmatics (Duvall et al., 2017). The recently identified innate lymphoid cells type II (ILC2) cells have shown to substantially contribute to the pathobiology of asthma, but interestingly, can be regulated by PGs (Maric et al., 2018).

3.2 MEDIATORS OF AIRWAY INFLAMMATION AND BRONCHOCONSTRICTION

Maintaining normal physiological conditions requires communication between multiple cells and organs. This communication is mediated (signalled) by cell-to-cell contacts, released proteins and small molecules. During inflammation, these signaling events can be strongly enhanced during cellular activation and follow a kinetic profile that can be rapid or slowly changing. The net response can be short, or long acting, and the occurrence can be local (autocrine) or peripheral (paracrine).

Important inflammatory signaling pathways are under strict regulation at both the cellular and the molecular level. Mast cells are important effector cells in asthma that can be activated by either binding of IgE to the high affinity receptor Fc ϵ R, binding of cytokines, neuropeptides and adenosine, or by changes in osmolarity in the surrounding milieu, **Figure 2**.

Once the activated mast cell degranulates, preformed mediators, such as histamine, tryptase, heparin and certain cytokines (*e.g.*, TNF α), are released locally. In addition, *de-novo* synthesis of cytokines and lipid mediators, such as PGs and LTs, lead to their release. Histamine, PGs and LTs are well known for their bronchoconstrictive effects and therefore the H₁, TP and CysLT₁ receptors constitute both established and new targets for drug intervention. However, the role of the majority of ω 3, ω 6 and ω 9 derived oxylipins are still not well understood. These lipid species have therefore also been addressed in this thesis.

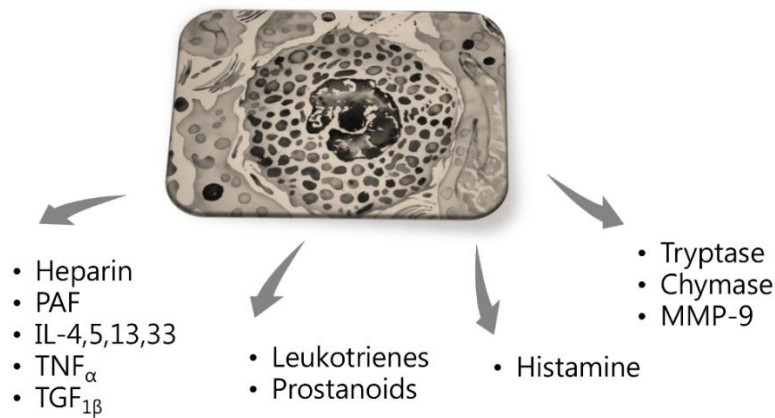


Figure 2. The mast cell contains potent inflammatory mediators, such as histamine and proteases, which are stored in preformed granula. Upon cellular stimulation, degranulation releases these constituents to the extracellular environment. Furthermore, activation of cytosolic phospholipase A₂ liberates arachidonic acid from the cellular membrane to *de novo* synthesize prostaglandins, leukotrienes and other eicosanoids.

3.2.1 Histamine

Mast cells and basophils contain granules with high amounts of preformed histamine. Histamine binds the H₁ receptor located on the smooth muscle and in the vasculature to induce constriction and increased vascular permeability, respectively. Once histamine is in the blood stream, it is rapidly metabolized and transported via the kidneys for excretion. Histamine N-methyl-transferase (HMT), diaminoxidase (DAO) and aldehyde dehydrogenase (ALDH), are the enzymes that sequentially metabolise histamine into 1-methyl-4-imidazoleacetic acid (commonly referred to as *tele*-MIAA), **Figure 3**. About 75-80% of infused histamine is excreted as *tele*-MIAA in the urine of humans (Granerus et al., 1999b).

In order to accurately determine the release and turnover of histamine, the structural analogue *pi*-MIAA needs to be separated from *tele*-MIAA. The two molecules differ by the methyl-group positioned at the 4th or the 5th carbon position and therefore require careful consideration in the choice of separation method, **Figure 1, Paper I**. Hydrophilic interaction liquid chromatography (HILIC) is an attractive option for separation of polar analytes because they are better retained than with reversed phase. In reversed phase chromatography, low molecular weight, and polar analytes, elute early and any un-retained sample constituent will elute at the same time. In the presence of inorganic salt, which is abundant in biological samples, co-elution of inorganic salt can result in marked ion suppression in the electrospray source.

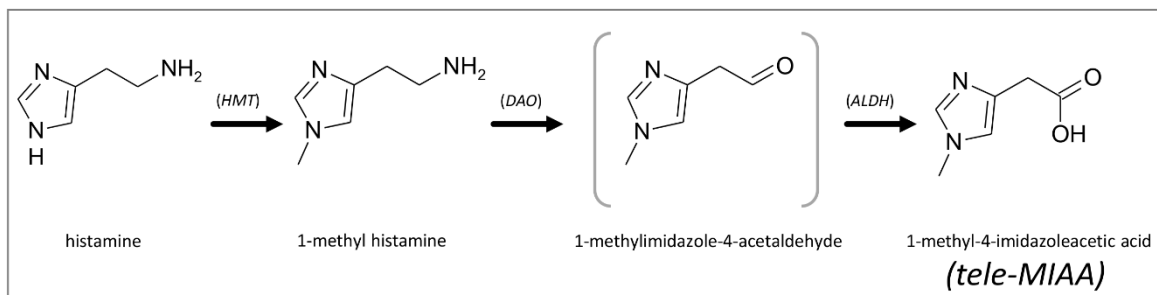


Figure 3: Histamine metabolism via the enzymes HMT, DAO and ALDH produces the stable end-point metabolite *tele*-MIAA, which is present in urine.

3.2.2 Arachidonic acid and its main metabolites

Lipids have an essential role in maintaining physiological functions and are constituents of every cell membrane. Oxidized lipids also participate in physiological and pathophysiological signaling processes and regulate cellular functions such as proliferation, migration, apoptosis and metabolism. Arachidonic acid (AA: 5,8,11,14-eicosatetraenoic acid) is a 20 carbon ω 6 PUFA and is the most well studied lipid species. Its downstream metabolites are known to play a key role in mediating inflammatory signals, such as pain, vasodilation/contraction or recruitment of inflammatory cells (Dennis and Norris, 2015), **Figure 4**. AA is present in all cells as conjugated via an ester bond at the *sn*2 position of membrane phospholipids.

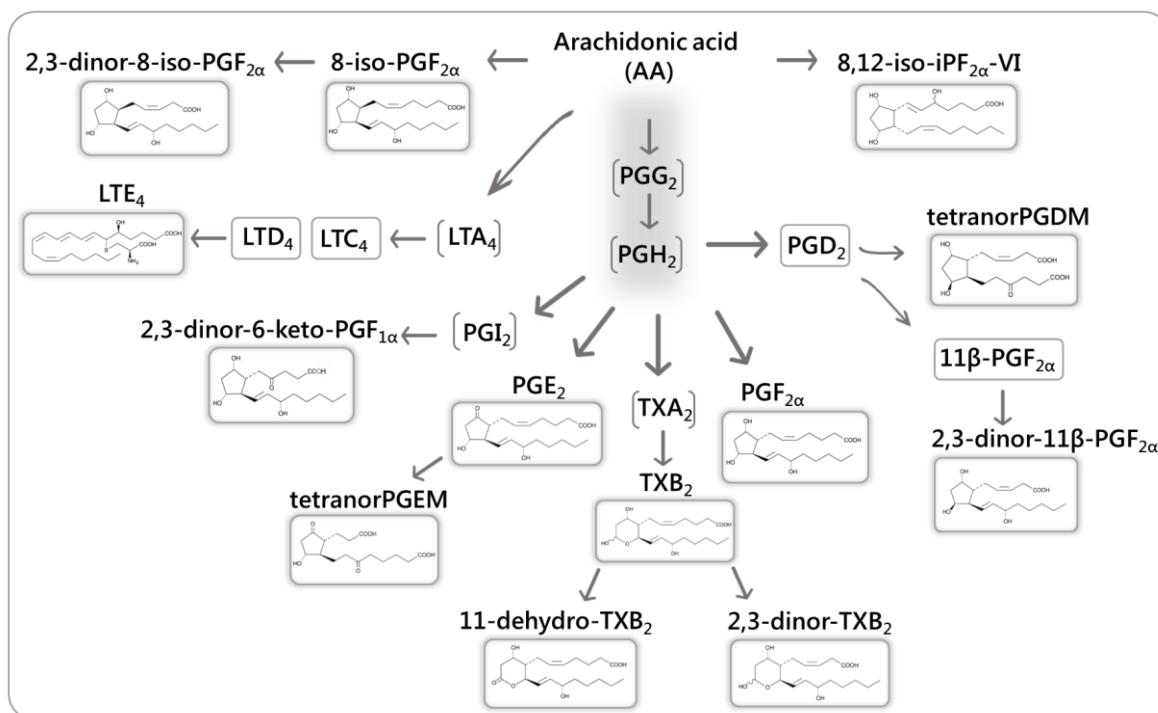


Figure 4. Selected eicosanoids and eicosanoid metabolites excreted in urine. Eicosanoids for which the chemical structure is shown are quantified in **Paper IV**.

The 85kDa cytosolic phospholipase A₂ (cPLA₂) enzyme liberates AA following cellular activation (Leslie, 2004). cPLA₂ is found in the endoplasmic reticulum (ER) and the nuclear membrane of the cell. Metabolism of AA follows two major pathways, cyclooxygenase (COX) and lipoxygenase (LOX). This produces prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs) and lipoxins (LXs) collectively known as eicosanoids (Dahlén et al., 1986). They have several fundamental and important biochemical functions, such as regulating renal function, blood pressure, inflammation and host defense. After release of AA from the intracellular membrane, COX enzymes can catalyze the formation of PGH₂ (the main precursor for synthesis of PGs and TXs) through a two-step reaction. First, abstraction of hydrogen from AA carbon 13 followed by introduction of oxygen at carbon 11 produces a peroxy fatty acid, which by further intramolecular rearrangement adds a second oxygen into the molecule producing the cyclic endoperoxide PGG₂. Further enzymatic reduction generates PGH₂, which is the substrate for the various PG and TX synthases (Smith et al., 2000).

3.2.3 Prostaglandins (PGs) and Thromboxanes (TXs)

Mast cells almost exclusively express hematopoietic prostaglandin D synthase and convert PGH₂ into PGD₂. PGD₂ is therefore considered a marker of mast cell activity (Lewis et al., 1982). With a relatively short half-life, PGD₂ is metabolized locally along two pathways. The 11-keto-reductase product, 11 β -PGF_{2 α} , is less biologically active and can almost exclusively be detected in blood. However, shortening of the carbon chain length by β -oxidation metabolism occurs on the way passing the liver and kidney forming 2,3-dinor-11 β -PGF_{2 α} which is referred to as the earlier PGD₂ metabolite in urine (Granström et al., 1982; Roberts and Sweetman, 1985). The second route of PGD₂ metabolism goes via carbon 15-dehydrogenase, delta 13-reductase and both β - and ω -oxidation to form tetranorPGDM, which is the most abundant PGD₂ metabolite in urine (Song et al., 2008).

Since the identification of prostaglandin E₂ (PGE₂) and F_{2 α} (PGF_{2 α}) in human seminal plasma in 1963, PGE₂ has been attributed both pro- and anti-inflammatory properties, partly being tissue dependent (Samuelsson, 1963). PGE₂ is also produced from PGH₂ but there are three alternative synthases that may catalyze the reaction leading to PGE₂, microsomal PGE synthase -1 and -2 (mPGES-1, mPGES-2) and cytosolic PGE synthase (cPGES). PGE₂ is metabolized to tetranorPGEM and excreted in the urine together with primary PGE₂ and several intermediate metabolites (Hamberg and Samuelsson, 1971), however, substantial contribution to urinary levels of primary PGE₂ can be attributed to its formation in the kidney and prostate. Similarly, the primary PGF-synthase product PGF_{2 α} is detected in urine, but is known to originate, to some extent, also from the kidney (Frölich et al., 1975).

Inactivation of PGs occurs via oxidation of the secondary alcohol group at C-15 and reduction of the double bond at the 13th carbon position. These reactions are catalyzed by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and Δ^{13} -reductase. The metabolites formed by these reactions, the 15-keto and the 15-keto-13,14-dihydro compounds, have been considered a key step in the biological inactivation of prostaglandins since they have much lower biologic activities than the parent prostaglandins (Diczfalusy and Alexson, 1990).

Other important PGH₂ metabolites include products of prostacyclin synthase (PGI₂) and thromboxane synthase (TXA₂) activity (Hamberg et al., 1975; Moncada et al., 1977). PGI₂ and TXA₂ have opposing physiological functions in the cardiovascular system, where PGI₂ induces relaxation of smooth muscles and TXA₂ contraction and platelet aggregation. Similar effects occur in the bronchial smooth muscle. Metabolites of PGI₂ include 6-keto-PGF_{1 α} and the 2,3-dinor-6-keto-PGF_{1 α} , the former found in plasma and the latter excreted in urine (Brash et al., 1983). As the half-life of the instable TXA₂ is seconds, the inactive product TXB₂ is rapidly formed and detectable in circulation, but is further converted to 11-dehydro-TXB₂ and 2,3-dinor-TXB₂ excreted in urine (Patrono et al., 1986).

3.2.4 Leukotrienes (LTs)

In contrast to COX metabolism of AA, five lipoxygenase activating protein (FLAP) binds AA in the cellular nuclear membrane. AA is transferred to co-localized 5-LOX enzyme, which produces 5-hydroxyeicosatetraenoic acid (5-HETE) and the unstable product leukotriene A₄ (LTA₄) (Smith, 1989). Depending on cell type, LTA₄ hydrolase generates LTB₄ by the addition of water (Haeggström et al., 2007), as in neutrophils, while the presence of LTC₄-synthase, as in mast cells, eosinophils and basophils, enables the incorporation of a glutathionyl group producing the first cysteinyl-leukotriene LTC₄ (Welsch et al., 1994). Following extracellular extraction, LTC₄ is rapidly metabolized by γ -glutamyl transpeptidase, which cleaves off γ -glutamyl to create LTD₄. LTD₄ is the most potent bronchoconstrictor among the CysLTs. It is about 1000 times more potent than histamine in

mediating bronchoconstriction (Dahlén et al., 1980). Finally, LTE₄ is formed by dipeptidase activity. Because approximately 20% of locally formed LTE₄ is rapidly excreted in the urine LTE₄ is frequently used as a marker of CysLT production (Kumlin et al., 1992; Maltby et al., 1990).

3.2.5 Isoprostanes

In contrast to enzymatically produced oxylipin species, isoprostanes constitute a class of non-enzymatically produced oxylipins resulting from increased oxidative stress, they are however believed to contribute to the pathobiology of asthma (Milne et al., 2011; Wood et al., 2003).

Isoprostanes are formed by free radical induced peroxidation such as by O₂⁻, H₂O₂, •OH, ozone (O₃), commonly termed ROS. Similarly, intracellular NO originates from a family of three nitric oxide synthases (NOS) and together with reactive nitrogen species (RNS) contribute to the production of oxidized lipids, *i.e.*, isoprostanes (Janssen, 2001). Both RNS and ROS have in experimental studies been suggested to mediate important biological functions in maintaining homeostasis, and during inflammation by regulating apoptosis, or killing invading pathogens (Fadeel et al., 1998; Valko et al., 2007). However, due to the lack of specific inhibitors or antagonists, it remains to define the function of isoprostanes in humans.

3.2.6 Oxylipins generated from other PUFAs

During the last decades, considerable attention has been paid to the role of metabolism of PUFAs with different carbon chain lengths, *i.e.*, C₁₈-C₂₂, in inflammatory and infectious diseases (Serhan, 2017). Similar to AA, several other PUFAs are stored in the cellular membrane and may be metabolized by the incorporation of oxygen at one or more of the unsaturated double bonds. Taking all PUFA substrates evaluated in this thesis into account, **Figure 5**, this results in a diverse set of oxygenated lipid metabolite species. Acknowledging that membrane phospholipids constitute precursors for oxylipin production, any change in cellular membrane composition, *i.e.*, of esterified phospholipids conjugated with PUFAs, may have an impact on many pathobiological states (Spector and Yorek, 1985). The main PUFA substrates covered in **Papers II-IV** are highlighted in **Figure 5** below.

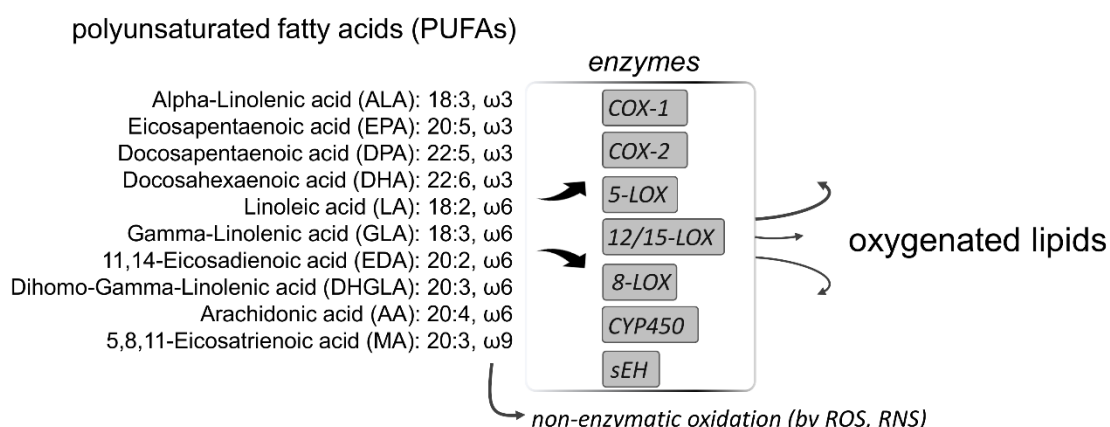


Figure 5: Omega-3, -6 and -9 PUFA substrates can undergo enzymatic and non-enzymatic metabolism to generate a plethora of oxygenated lipid species. Abbreviations are: soluble epoxide hydrolase (sEH), cytochrome P450 (CYP450), lipoxygenase (LOX), cyclooxygenase (COX), reactive oxygen species (ROS) and reactive nitrogen species (RNS). For more expanded details regarding biosynthetic production of oxylipins see **Figure 1, Paper II**.

The roles of various AA derived oxylipins from the COX enzymatic pathway are most well studied. However, as an expanding number of oxidized PUFA metabolites are identified, there is an increased demand to characterize their physiological function. In both **Paper II** and **III (Figure 1)**, the detailed pathway figures describes the oxylipins quantified in this thesis and the enzymes responsible for their synthesis. One approach to define the role of individual oxylipins is by using *in vivo* and *in vitro* models where multiple lipid species can be quantified and the relative change in concentration of individual lipid species described.

3.2.7 Enzymes involved in oxylipin production

During inflammation, the production of specific oxidized lipids and eicosanoids is a direct consequence of activated structural and inflammatory cells. These cells sometimes share the same enzymes, but expression of enzymes may also be distinctly different, which will dictate the set of oxylipins that will be produced in a particular cell, or in the particular inflammatory milieu.

Of the two cyclooxygenase enzymes, the COX-1 isoform is constitutively expressed, while COX-2 is upregulated during inflammation (Samuelsson et al., 2007). As the COX enzymes are largely involved in the production of pro-inflammatory oxylipins, they have been frequently used as targets for anti-inflammatory treatment, such as by the non-steroidal anti-inflammatory drugs (NSAIDs) aspirin, indomethacin and ibuprofen. Selective inhibition of COX-2 has been clinically tested to circumvent the unwanted gastro-intestinal side effects of blocking COX-1, which thereby removes the protective function of PGE₂. However, due to increased cardiac adverse events, selective COX-2 inhibitors have not been as successful as anticipated (Mukherjee et al., 2001).

The family of lipoxygenase enzymes (5, 8 and 12/15-LOX) are in principle involved in two basic mechanisms, lipid peroxidation and redox-status regulation (Kuhn et al., 2015). These enzymes catalyze deoxygenation of the cis-double bonds on PUFAs, which results in the introduction of a hydroxyl group. In general, LOX enzymes selectively produce oxidized lipids as the *S*-enantiomers. Four human 12-LOX enzymes have been described, in platelets (*S*-isomer), leukocyte (*S*-isomer) and epidermis (*S*- and *R*-isomers). Furthermore, 5-LOX produces 5(*S*)-HETE. 15-LOX-1 in humans is mainly expressed in eosinophils, monocytes and reticulocytes (Haeggström and Funk, 2011) producing 15(*S*)-HETE, which is the most abundant eicosanoid in the human lung (Dahlén et al., 1983; Hamberg et al., 1980; Kumlin et al., 1990). A human 15-LOX-2 enzyme also exist and share a high degree of sequence identity with mice 8-LOX.

As previously mentioned, 5-LOX is localized in the nuclear membrane and under strict control by Ca²⁺ and ATP (Rådmark et al., 2007) and requires the FLAP protein for delivery of arachidonic acid to 5-LOX (Mancini et al., 1993). Pro-inflammatory 5-LOX products include LTB₄, which is a strong chemoattractant for neutrophils, and CysLTs, which mediate bronchoconstriction (Dahlén et al., 1980; Ford-Hutchinson et al., 1980). As a direct consequence, montelukast is used for anti-leukotriene treatment in asthma where it antagonizes the CysLT₁ receptor to reduce bronchoconstriction and airway inflammation (Noonan et al., 1998). Another approach to block LT formation is to inhibit FLAP activity by the use of MK-886, preventing formation of LTA₄. On the other hand, blocking FLAP also reduces the ability of 5-LOX to produce the anti-inflammatory mediator lipoxin A₄ (LXA₄) and B₄ (LXB₄). Formation of LXA₄ also depend on 15-LOX interaction. LXA₄ has demonstrated several pro-resolving effects in the lung by promoting phagocytosis of apoptotic polymorphonuclear cells (PMNs) and blocking eosinophil trafficking and both lower levels of LXA₄ and its receptor in the lungs of severe asthmatic patients has been reported (Haworth and Levy, 2008). The 8-LOX has to date not been described in humans.

A diverse set of LOX products are quantified in **Paper II** and **III** and are commonly abbreviated by their PUFA origin; such as HODEs (linoleic acid), HODTrEs (α - and γ -linolenic acid), HDoHEs (docosaheptaenoic acid), HETrEs (dihomo-gamma-linolenic acid), HETEes (arachidonic acid), HEDEs (11,14-eicosadienoic acid) and HEPEs (eicosapentaenoic acid). Their roles in regulating inflammatory events are poorly understood, and therefore, a specific aim of this thesis was to assess the presence and abundance of multiple lipoxygenase products during airway inflammation in mice and in response to mast cell mediated bronchoconstriction in guinea pig and isolated human airways.

The large family of CYP450 enzymes form epoxy-fatty acid compounds from the primary substrates LA, AA, EPA and DHA. The epoxy-fatty acid products are involved in important physiological functions regulating inflammation and nociception and are generally ascribed protective and beneficial properties (Wagner et al., 2017). However, CYP450 enzymes also produce monohydroxy fatty acids including 19- and 20-HETE.

The soluble epoxide hydrolase (sEH) enzyme hydrolyses epoxides to the corresponding vicinal diols to form the DiHOMEs from LA, DiHETEes from EPA, DiHETrEs from AA and DiHDPAs from DHA. By the use of selective sEH inhibitors, it has in experimental settings been possible to describe potentially important biological functions of these epoxy-fatty acids (Guglielmino et al., 2012). Interestingly, in sputum from asthmatics, decreased formation of pro-resolving LXA₄ has also been associated with increased sEH activity, highlighting sEH inhibitors as a new class of compounds of potential benefit in asthma treatment (Ono et al., 2014). Elimination of epoxide products is generally considered less favorable as those oxylipins have shown anti-inflammatory effects in cardiovascular systems by relaxant effects on vascular tone, but also by cellular proliferation and smooth muscle migration (Thomson et al., 2012).

3.2.8 Oxylipin generation by ROS and RNS

During inflammation, extensive oxidative burst will contribute to the total oxidized lipid production. Due to ROS or RNS free radical induced peroxidation occurring, equal amounts of the *R*- and *S*-enantiomer are produced at any potential chiral centers. To determine the contribution of non-enzymatic *vs.* enzymatically produced oxylipins chiral chromatography must be applied to discriminate each enantiomer and the enantiomeric excess thus calculated. Chiral chromatography can therefore be essential to elucidate the biosynthetic source of production of specific lipid species. For example, 9(*S*)- and 9(*R*)-HETE were shown to be equally produced following lipopolysaccharide and/or zymosan stimulated human whole blood while 12(*S*)- and 15(*S*)-HETE were the main products (Mazaleuskaya et al., 2018).

3.3 OXYLIPIN PROFILING

Quantification of PUFA-derived oxylipins and related compounds requires methods that are selective and sensitive. For histamine metabolite quantification in urine, selectivity is the greatest challenge, while for oxylipins and related eicosanoids sensitivity is more important. To achieve high sensitivity, the solid phase extraction technique enables efficient clean-up of samples from proteins and inorganic salts, and to concentrate the analytes of interest. Following sample clean-up and analyte enrichment, high resolution chromatography coupled to high sensitivity mass spectrometry, *i.e.*, a tandem quadrupole mass spectrometry (LC-MS/MS) has been exclusively used. This makes it possible to determine the concentration of a large number of individual analytes (Yamada et al., 2015; Yang et al., 2009) from biological fluids. By LC-MS/MS, quantification is performed using multiple

reaction monitoring (MRM) transmitting one or more selective fragment ions. In a relatively short time frame, hundreds of fragment ion transitions can be monitored. Electrospray ionization has been most commonly used to ionize oxylipins and related eicosanoids (Murphy et al., 2005). The utility of this analytical technique has been informative in many biological applications where oxylipin profiles have been obtained from urine, blood, cerebrospinal fluid (CSF) and BALF (Balgoma et al., 2016; Rago and Fu, 2013; Strassburg et al., 2012; Wolfer et al., 2015).

3.4 URINE AS A NON-INVASIVE MATRIX

Following kidney filtration of blood, the bladder accumulates filtered blood stream constituents over longer periods and therefore offers a great opportunity to catch locally produced inflammatory mediators (Dahlén and Kumlin, 1998). Local release of PGs, TXs and CysLTs can increase tremendously when cells are activated (up to 100-fold). After being released in the respiratory tract, they are rapidly taken up by the circulation and metabolized systemically, transported by the blood stream and excreted by kidneys. They can therefore be detected at elevated levels in the urine. By sampling urine, one for example avoids the risk of platelet-derived lipid mediator release due to the interplay between platelets and vascular endothelial wall during invasive blood sampling. This has important practical implications in the clinical setting, providing a unique opportunity because urine sampling is non-invasive and contains enriched biological information. Methods for detection of the mast cell mediators histamine, PGD₂ and CysLT in urine have been established by immuno-based assays as well as by mass spectrometry. Following allergen induced bronchoconstriction the subsequent release of PGs and CysLTs is well documented by accurate determination whereas markers from the histamine pathway have been studied only occasionally (O'Sullivan et al., 1998). For this reason, the major histamine metabolite in urine was evaluated in **Paper I** and an extended panel of 13 eicosanoids from six enzymatic pathways, as well as the non-enzymatically produced isoprostanes, were quantified in **Paper IV** (Balgoma et al., 2013).

4 METHODS

4.1 GENERAL

Quantification of small molecular weight compounds in biofluids requires careful selection of sample processing methods and detection techniques. Markers of inflammation are generally found low at baseline, but can increase 100-fold in the local milieu following cellular activation. Furthermore, released mediators also undergo systemic metabolism to different degrees during their transport to the blood stream and following clearance by the kidneys. Therefore, baseline concentrations of analytes in the various biofluids may span a wide dynamic range.

Consequently, low abundant analytes require pre-treatment steps that enrich analyte concentration so that sufficient signal can be detected, *i.e.* above the limit of quantitation (LOQ). Regarding markers of mast cell activation, histamine is reported to be found in the μmol range in urine (Granerus et al., 1999a), while eicosanoids are found at nmol range (Balgoma et al., 2013). The expected range of concentrations of oxylipins in mice BALF is only partly described while their presence in human BALF has been reported (Balgoma et al., 2016; Larsson et al., 2014; Lundström et al., 2012). The concentrations detected following release from isolated lung tissue preparations, when stimulated *in vitro*, remain to be defined.

Given the challenges above, careful consideration has to be taken with regard to sample properties and chemical class of analyte(s), from the start of bioanalytical methods development to the final experimental and biological research application, **Figure 6**.

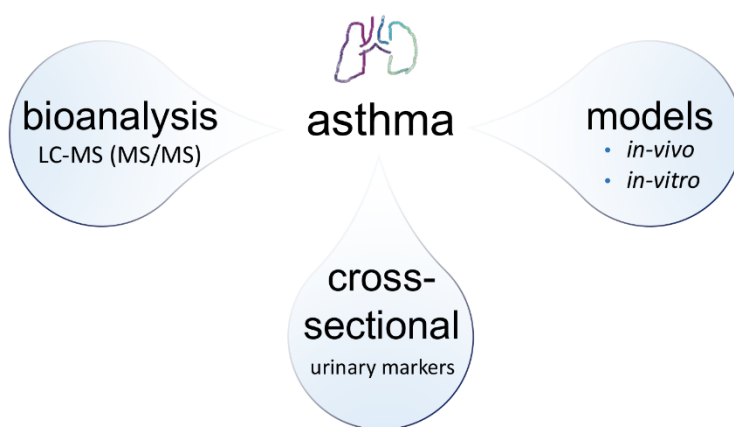


Figure 6. The current thesis work has been centered around developing bioanalytical methods for quantification of inflammatory mediators in a research setting that involves animal and human models as well as clinical studies.

Below follows a description of material and methods used in the different studies included in this thesis. A more detailed description is found in each method section in the published papers and appended manuscript.

4.2 A MODEL OF HDM INDUCED AIRWAY INFLAMMATION IN MICE

In mice models of asthma, ovalbumin (OVA) has commonly been used to establish a type 2 inflammatory response in the BALB/c mice strain. Earlier studies using OVA as allergen have shown changes in a few oxylipins in BALF. For example, LTB_4 and LTC_4 have been associated with eosinophilic infiltration and mucus secretion. After blocking 5-lipoxygenase (5-LOX) activity, or 5-LOX activating protein (FLAP), it was possible to

reduce this LT production (Henderson et al., 1996). Basal lung levels of a limited panel of monohydroxy lipid mediators, such as hydroxyeicosatetraenoic acids (HETEs), have also been obtained and compared between rat and BALB/c mice (Sagliani et al., 2013).

In the human population, HDM is a common aeroallergen and many patients with asthma are allergic to HDM (Gregory and Lloyd, 2011; Tham et al., 2016). In contrast to OVA, HDM triggers inflammation by several mechanisms involving the many complex constituents present in HDM (Asokanathan et al., 2002). In our study, HDM was used as the allergen. We also wanted to use the C57BL/6 strain, as it offers many possibilities to induce genetic modifications. However, in the C57BL/6 mice strain a type 2 inflammatory response is more difficult to achieve than in BALB/c and is often skewed towards type 1. Thus, to induce allergic inflammation by HDM in C57BL/6 we developed a protocol with intense exposure of to HDM during 4 weeks. Of interest, a comprehensive oxylipin profile in BALF in response to HDM exposure in the C57BL/6 mice has not been previously determined.

For **Paper III**, a conditional knock-out mice *Igf1r^{fl/fl}* was bred in a C57BL/6 enriched background for the purpose of studying the conditional deletion of the Insulin Growth Factor 1 (IGF1) receptor (López et al., 2015). However, no induced knock-outs were used in our study and consequently, the phenotype was considered as normal mice. The control mice were challenged with PBS and treated were exposed to 20 µL HDM extract for four weeks, **Figure 7**. Animal handling and experiments were approved by the CIBIR Bioethics Committee (refs. 03/12, Logroño, Spain) and the Regional Committee of Animal Experimentation Ethics (N152/15; Stockholm, Sweden).

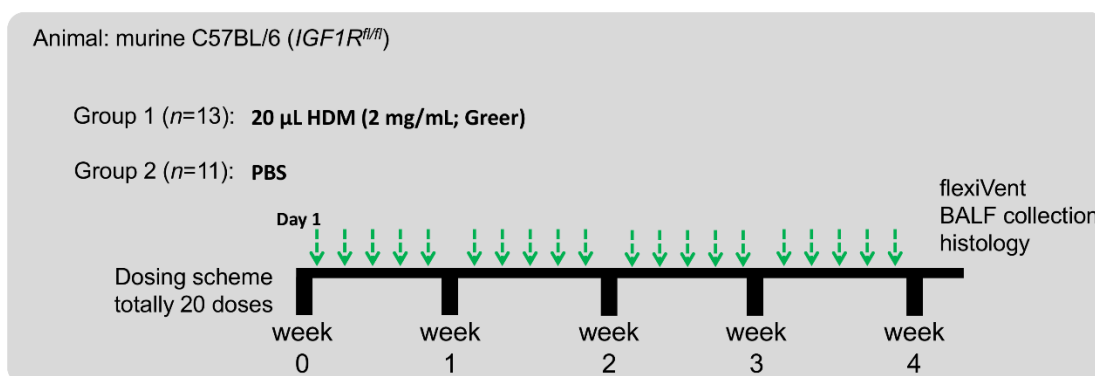


Figure 7: Study design of the murine asthma model in the C57BL/6 mice strain and using house dust mite (HDM) as allergen to induce airway inflammation.

Characterisation of airway hyperresponsiveness was evaluated using a FlexiVent™ system (Scireq, Montreal, Canada) where mice after the 4 weeks of HDM exposure were anesthetized 24-hour after the last HDM challenge. To evaluate the contribution of 3 pro-inflammatory cytokines and 14 enzymes involved in oxylipin production, their corresponding mRNA expression in homogenized whole lung tissue was quantified. Mast cell and eosinophil cell infiltration were calculated using isolated lung lobe tissue.

4.3 CLINICAL STUDIES

Urine samples were collected during a period of 6 and 24 hours in **Paper 1** and spot urine was collected in the U-BIOPRED study in **Paper IV**. To further demonstrate the utility of the method presented in **Paper I**, spot urine samples, or hourly collected urines samples, were collected in the mastocytosis study (Gülen et al., 2017) and the allergen challenge study

(Daham et al., 2014). Ethical permits for studies presented in this thesis were approved by the local Swedish ethics committees as follows; **Paper I** (2009/137, 2009/302-31/3 and 2009/935-32), **Paper IV**, the allergen challenge study and the mastocytosis study (2009/959-31-4 and 2009/1422-32). There was no restriction of diet prior to urine collection. Coffee intake was limited to ≤ 2 cups for subjects enrolled in the mastocytosis and allergen challenge studies.

Urine samples from the U-BIOPRED study were analysed to determine the concentration of urinary eicosanoids in both healthy human subjects and those with mild-to-moderate (MMA) and severe asthma non-smokers (SAn) and severe asthma smokers, or ex-smokers, (SAs/ex). Subjects in the U-BIOPRED study were recruited from 16 European clinical sites. Number of subjects per group is described in **Figure 8** below and inclusion criteria followed the ATS/ERS guidelines (Chung et al., 2014).

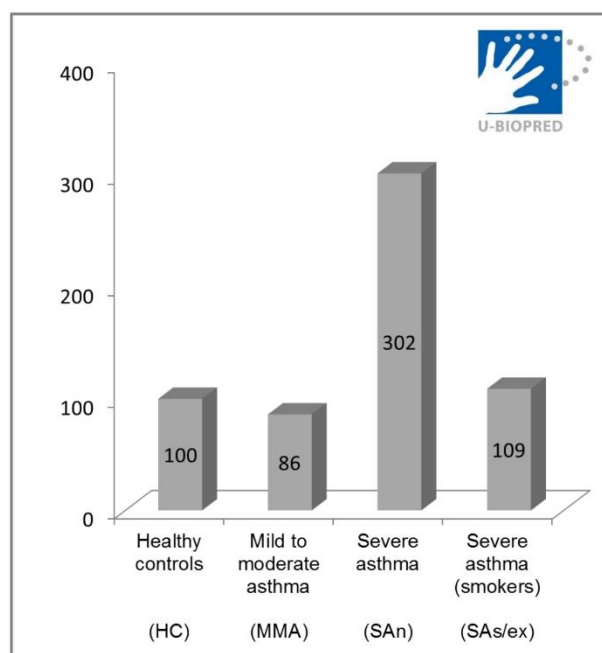


Figure 8. In total, 597 subjects included in U-BIOPRED study were successfully screened to establish urinary eicosanoid profiles, **Paper IV**.

The inclusion criteria for healthy controls was $FEV_1 \geq 80\%$, non-smoking for the past 12 months and no history of asthma or other respiratory diseases. Subjects in the MMA group were non-smokers for the past 12 months and had partial control of their asthma symptoms, as defined by GINA guidelines (Bousquet, 2000), following a dose $< 500 \mu\text{g}$ fluticasone propionate/day. The SAn group subjects were non-smokers for the past 12 months, had uncontrolled symptoms according to GINA, and/or experienced more than two exacerbations per year despite $\geq 1000 \mu\text{g}$ fluticasone propionate/day. All subjects in the HC, MMA and SAn groups reported less than 5 total pack-years. The severe asthma smokers/ex-smokers (SAs/ex) group followed the same criteria as the SAn subjects, but had a history of smoking of > 5 pack-years. Further clinical study details are found in (Shaw et al., 2015).

Extensive clinical evaluation was conducted which included, for example; questionnaires, spirometry, measurement of exhaled NO ($F_{E}NO$), blood, sputum and urine collection. The median (inter quartile range) $FEV_1\%$ in the MMA group was 92 (75-100), 67 (50-85) in SAn and 66 (52-78) in SAs/ex. The overall trend of decreasing lung function ($FEV_1\%$) with asthma severity is shown **Figure 9**.

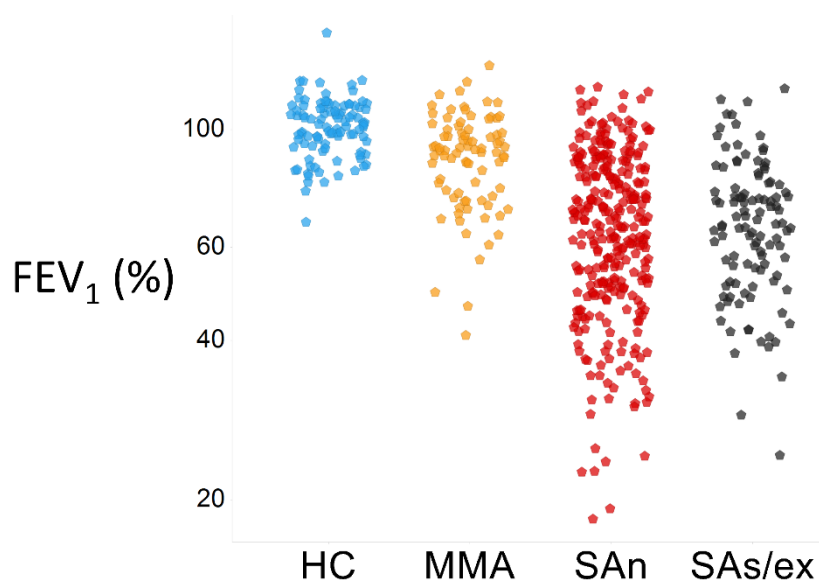


Figure 9. Lung function measured as forced expired volume during 1 second (FEV₁(%)) demonstrated a decline with asthma severity. Healthy control (HC), mild-to-moderate asthma (MMA), severe asthma non-smokers (SAn) and severe asthma smokers/ex-smokers (SAs/ex).

A total of 302 severe asthmatics agreed to perform spirometry, blood and sputum collection and donate urine at a 12-18 month longitudinal visit. A fewer number of subjects underwent bronchoscopy evaluation where bronchial brushings and biopsies were collected. All collected subject data (clinical and omics-platform data) in the U-BIOPRED study was stored and accessible via the TranSMART data base (<http://etriks.org>).

4.4 ORGAN BATH METHODOLOGY

The organ bath methodology is a well-established platform to perform *in vitro* pharmacology using isolated and intact smooth muscle tissues, such as aorta, gastro-intestinal and airway tissue. From these preparations major findings, such as the discoveries of PGs, LTs, NO and endothelin, have been accomplished (von Euler, 1936; Feldberg et al., 1938; Furchgott and Zawadzki, 1980; Palmer et al., 1987). In these preparations, all natural signaling routes are present and the cellular structure and function is similar to the *in vivo* situation. Induction of airway smooth muscle constriction can be performed by the addition of receptor agonists directly, or indirectly, as by allergic stimulation of the mast cell via crosslinking the IgE receptor. Together this enables detailed characterisation of dose-response curves to study basic mechanisms of bronchoconstriction. For example, this experimental system has been able to show that the mast cell stabilizing effect of PGE₂ was mediated via the EP₂ receptor while bronchorelaxant effects were mediated via EP₄ (Säfhholm et al., 2015).

A strength of the organ bath is the possibility to study pharmacological effects in different species, such as human bronchus, guinea pig bronchus and trachea as well as mice trachea. In these preparations, a simultaneous screening of multiple oxylipins has not been reported previously. Developing a workflow to combine data from biochemical pathways with that of functional responses may therefore provide novel insights into signalling pathways involved in mediating airway smooth muscle responses. The surrounding organ bath solvent can be collected and the analytes quantified.

The overall workflow and experiments presented in **Paper II** are illustrated in **Figure 10** below. In brief, healthy human lung tissue was obtained from individuals undergoing

surgical lobectomy at the Karolinska University Hospital. Small airway segments, down to bronchi level 16, were isolated and placed over night at 37 °C in culture plate wells containing Dulbecco's modified Eagle medium (DMEM; Gibco, Auckland, NZ) supplemented with penicillin streptomycin under sterile conditions on the day before organ bath experiments. The use of human tissue was approved by the Stockholm south regional ethical review board (ref. no. 2010/181-31/2).

To address translational aspects of bronchoconstriction, male albino guinea pigs were also tested in the organ bath setting. Animals were OVA sensitized 28 days prior to organ bath experiments by a single intra-peritoneal injection, containing 100 µg. The guinea pigs were sacrificed and trachea was isolated and cut into eight intact rings. The Swedish animal experimentation ethical review board approved the use guinea pig animals (ref. no. N143/14).

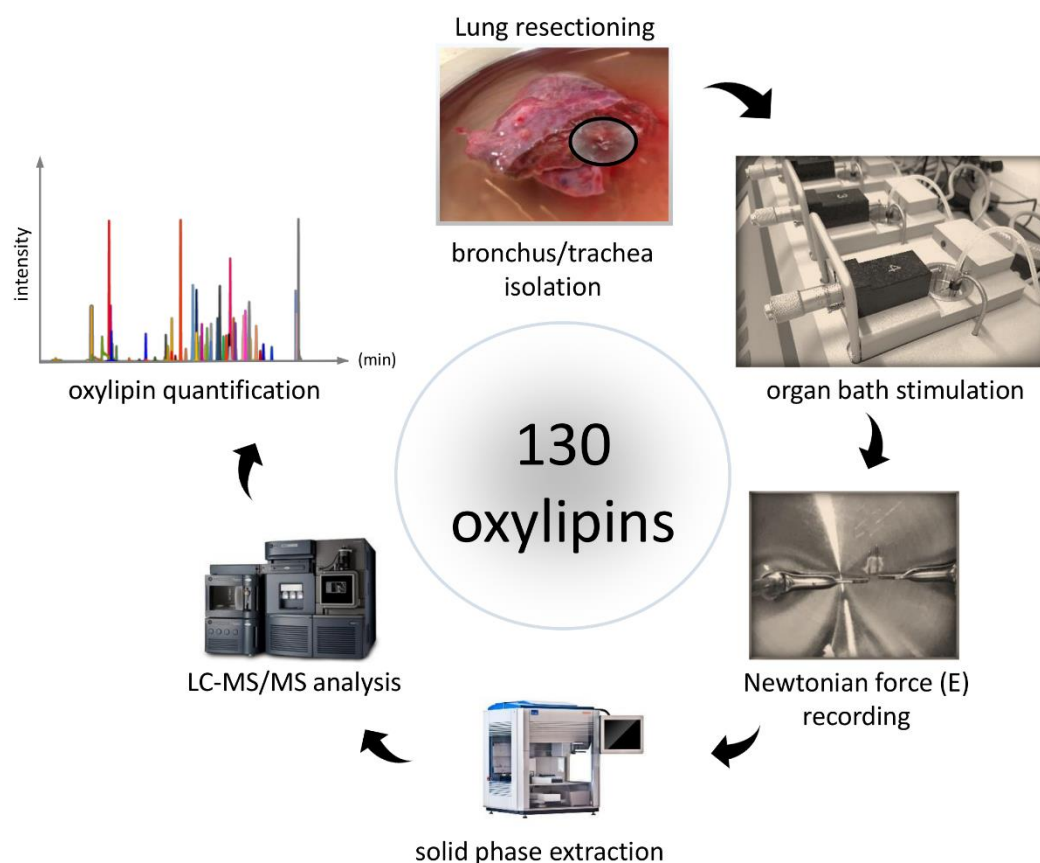


Figure 10. A workflow was developed in **Paper II** to combine physiological and biochemical measurements that mimic bronchoconstriction *in vivo*. Following lobectomy at the Karolinska University Hospital, the human bronchus was isolated and mounted in the organ bath. Mast cell mediated smooth muscle contraction was induced by anti-IgE and the Newtonian force recorded. After 60 min, the surrounding Krebs buffer solvent was withdrawn for SPE processing and subsequent oxylin profiling by LC-MS/MS analysis.

After a period 60 min of tissue stimulation in the organ bath, the surrounding Krebs buffer was removed for oxylin quantification as described in more detail in **Paper II**. In brief, withdrawn Krebs buffer samples were processed by solid phase extraction (SPE) to remove interfering salts, proteins and buffer constituents. Following SPE, each dried extract was reconstituted in 70 µL of 84% methanol and oxylipins quantified by injecting 7.5 µL of each extract into the LC-MS/MS using *Methods A-C*. Further details of *Methods A-C* are presented in **Paper II**.

4.5 OXYLIPIN AND EICOSANOID ANALYSIS

The majority of oxylipins are found present at low concentration in biofluids or in experimental models. Therefore, samples were cleaned up and concentrated using a solid phase extraction (SPE) procedure. This method improved the ability to detect low abundant lipid species, while simultaneously decreasing the number of interfering compounds during the electrospray ionization process. A cleaner extract also provides for increased reproducibility during longer series of sample analysis.

4.5.1 Extraction and analysis of oxylipins

In **Paper II** and **III**, the same automated extraction protocol was employed using the Extrahera robot system (Biotage, Uppsala, Sweden). However, a larger sample volume could be extracted from organ bath samples (up to 3.5 mL), while from the mice 800 μ L could be used. Mice BALF samples were stored five days in -80°C until the day of oxylipin extraction, while for organ bath derived samples they were extracted directly following the end of organ bath experiment.

In **Paper II** and **III**, samples were extracted using 3 mL (3 cc/60mg) Evolute Express ABN cartridges (Biotage, Uppsala, Sweden). To each sample, 10 μ L of internal standard solution was added followed by a 1:1 dilution with an extraction buffer, pH=5.6, 58/42 (v/v) of 0.2 M Na_2HPO_4 /0.1 M citric acid hydrate solution. The Extrahera system use a positive pressure of N_2 gas in the different processing. Evaporation of elution solvent was performed using N_2 gas (TurboVap LV, Biotage, Uppsala, Sweden). Oxylipin extracts were reconstituted in 70 μ L methanol/water (6:1, v/v) and filtered by centrifugation using 0.1 μm polyvinylidenfluorid membrane spin filters. An overview of the three LC-MS/MS methods (A-C) used for **Paper II** and **III** is shown in the **Figure 11** below.

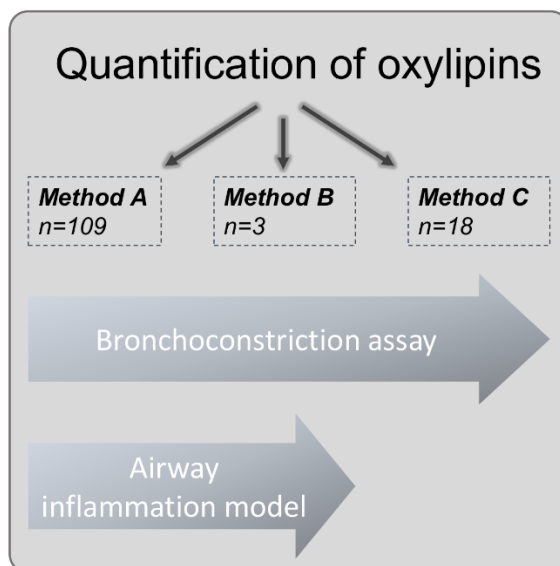


Figure 11. Oxylipins were extracted using SPE and the 70 μ L extract was injected three times using *Methods A-C*. In **Paper II**, all methods were used and for **Paper III** *Method A* and *B*. The number of analytes included are highlighted for each method.

4.5.2 Extraction and analysis of urinary eicosanoids

For quantification of urine eicosanoids in **Paper IV**, both automated extraction using the Extrahera system as well as manual extraction using a vacuum manifold were used. Prior to extracting the urine samples, the UV absorbance at 300 nm was measured and the withdrawn urine volume was adjusted by an established pool of urine reference material

which ensured a more homogenous urine concentration on a sample to sample basis. An overview of the three LC-MS/MS methods used for **Paper IV** is shown in **Figure 12** below.

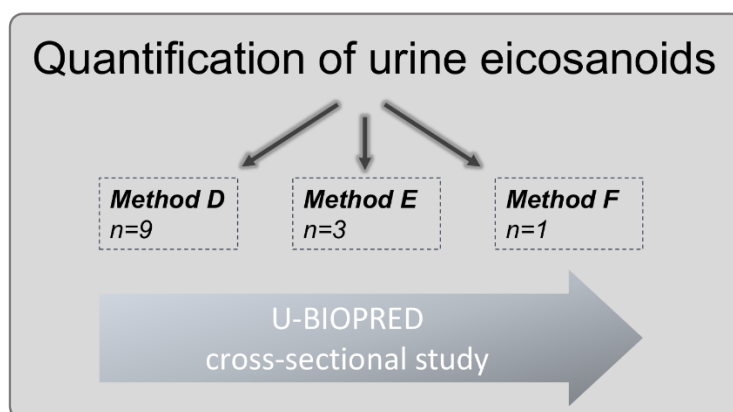


Figure 12. Urine eicosanoids were analysed in **Paper IV** by three different LC-separation methods. Main prostaglandins, 11-dehydro-TXB₂ and isoprostanes were detected by *Method D* and three tautomeric eicosanoids (TXB₂, 2,3-dinor-TXB₂ and 2,3-dinor-6-keto-PGF_{1 α}) by a derivatization step included for *Method E*. LTE₄ was quantified by *Method F* using a separate SPE extraction step.

In principle, the two SPE protocols in **Paper IV** followed the same basic principles as in **Paper II** and **III** with the exception samples for determination of urinary LTE₄ was acidified with 0.1 % aqueous acetic acid prior to SPE loading. In comparison to the positive pressure applied by the Extrahera system, the manual SPE extractions using the glass manifold used reduced pressure, or gravity, throughout the process.

4.6 LC-MS AND LC-MS/MS

4.6.1 Chromatographic separation

For all papers, liquid chromatography was carried out using an ultra-performance liquid chromatography (UPLC) Acquity™ system. In **Paper I**, ion-pairing chromatography was employed using 0.5 mM tridecafluoroheptanoic acid in the aqueous and organic mobile phase and histamine metabolite separation performed on a BEH C₁₈ column, 2.1x 100 mm, 1.7 μ m particle size (Waters, MA). In **Paper II** and **III**, oxylipin separation was carried out using three different methods: general oxygenated lipids (*Method A*), cysteinyl-leukotrienes (*Method B*) and chiral separation of monohydroxy fatty acids (*Method C*). *Method A* and *B* used a BEH C₁₈ 2.1x150 mm, 1.7 μ m column (Waters, MA) and *Method C* a Chiral-Pak AD-RH column, 2.1x150 mm, 5 μ m column (Daicel corporation, France). More details regarding the different methods employed for urine eicosanoids are found in (Balgoma et al., 2013), but included three similar chromatographic methods using the same BEH C₁₈ 2.1x150 mm, 1.7 μ m column.

4.6.2 Mass spectrometric detection

Mass spectrometry data was collected using the two different mass analyzers from the same vendor (Waters, Milford, MA). For **Paper I**, a full scan function was applied using a quadrupole time-of-flight (QTOF Premier™) mass spectrometer and for **Paper II-IV** a triple quadrupole mass analyzer (Xevo TQ and TQ-S™) was operated using multiple reaction monitoring (MRM).

4.6.3 Quantification of analytes.

For **Paper I**, quantification was performed using a calibration curve established by the standard addition method, *i.e.*, 1-methyl-4-imidazoleacetic acid spiked into urine in increasing concentration. In **Paper II-IV**, an external calibration curve was created for each quantified oxylipin in $\geq 84\%$ methanol. To adjust for variability during sample processing and electrospray ionization deuterated standards were added to each sample prior to analysis. For quantification of *tele*-MIAA, either a deuterated structural analog, or amino acid was used. For oxylipins, a mix of 42 deuterated structural standards was used. Urinary mediators normalized to urinary creatinine

To adjust for fluctuations in urine concentration reported, concentrations of *tele*-MIAA and eicosanoids were reported as normalized to mmol/L urine creatinine. In **Paper I**, urine creatinine was determined using a COBAS 6000 C501 clinical chemistry analyser (Roch Diagnostics, Indianapolis, USA) or the compensated Jaffe colorimetric assay. In **Paper IV**, urine creatinine was determined using two LC-MS/MS methods (Balgoma et al., 2013; Fraselle et al., 2015).

4.7 DATA ANALYSIS

4.7.1 Univariate analysis

Univariate statistical methods were applied as appropriate and are described in more detail in each paper (**I-IV**). Urinary *tele*-MIAA and eicosanoids follow a non-normal distribution and were log-transformed prior to analysis of variance (ANOVA). Two-way ANOVA was applied for urinary *tele*-MIAA, controlling for gender and age in **Paper I**, and multiple *t*-testing in **Paper II** and **III**. A Mann-Whitney U-test and Kruskal-Wallis test (one-way ANOVA) were used for urinary eicosanoids. For longitudinal assessment of SAn and SAs/ex participants having urine samples from two time points, a paired *t*-test was applied using the Wilcoxon rank sum test. P-values < 0.05 were considered significant. A false discovery rate correction of 5% was applied after multiple testing of oxylipin differences in **Paper III** to adjust for false positives.

4.7.2 Extreme value analysis

To discover potential associations between urine eicosanoids with clinical or biochemical variables, a selection of objects/subjects having high or low numerical values may highlight an important connection between a molecular mechanism and a clinically relevant symptom. This selection is referred to as an extreme value analysis wherein the selected participants belong to either the high (> 75 percentile) or low (< 25 percentile) concentration of a specific analyte. As exemplified using urinary LTE₄ in **Figure 13**, all participants having urinary LTE₄ concentration above 10.3 ng/mmol and below 3.8 ng/mmol creatinine were selected in the first step. In the second step, the corresponding F_{ENO} values for the selected participants were analysed and statistical significance tested.

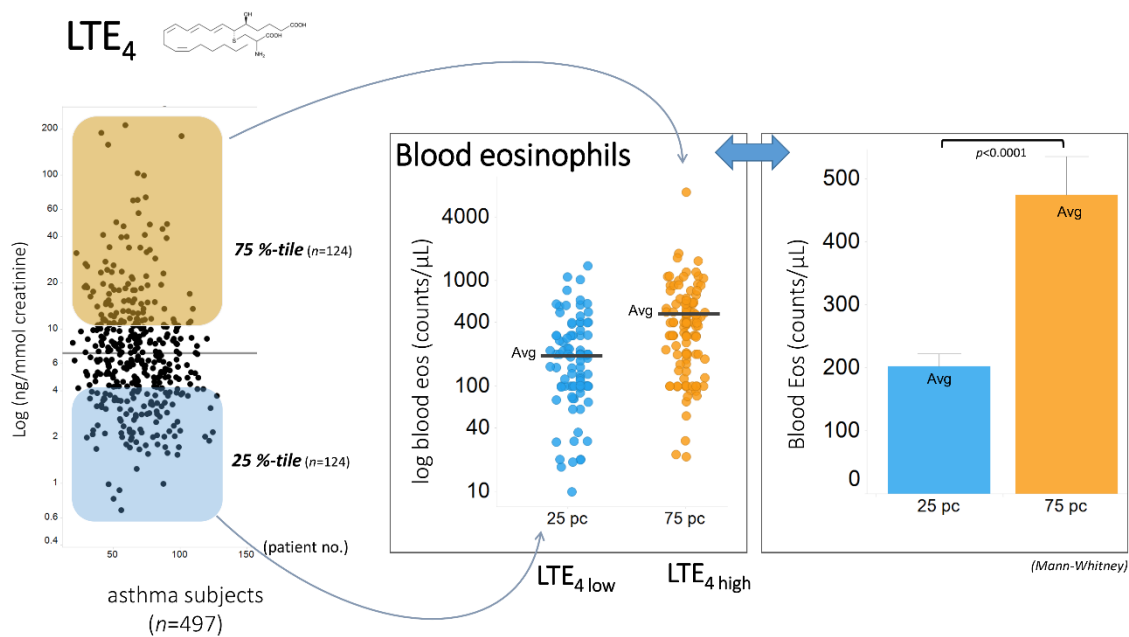


Figure 13. Selection of U-BIOPRED participants for extreme value analysis based on urinary LTE₄ concentration, *i.e.*, by quartiles (25th and 75th). The selected subjects' blood eosinophil count is then plotted as a scatter plot, or as histogram bar plot.

4.7.3 Consensus clustering

Clustering is a class discovery method that attempts to find structure among a group of patients or objects. The input data for any class discovery method is based on a larger number of variables that reflect measured entities. Typically, this could be mRNA, protein or metabolite data. Clustering has been utilized in **Paper IV** to group participants in the U-BIOPRED study who have similar urine eicosanoid profiles. To account for differences in absolute concentration, numerical ranges and variability of each urine eicosanoid data was log₂ transformed and subjected to unit variance scaling (*i.e.*, z-scores) or Pareto scaling. The Euclidean, and the Pearson correlation, distance measure was calculated and used as separate input when searching for similarities between subjects during the clustering computation.

Consensus clustering is a further development of classical clustering by the incorporation of bootstrapping during multiple runs of the clustering (Monti et al., 2003). For each iteration, a certain percent of the total study participants is left out. The clustering is repeated multiple times while testing cluster models of increasing K, *i.e.*, typically K=2 to 10. For each given K, a consensus matrix is generated wherein the frequency of clustered subject pairs is highlighted by a dark blue dot (patient pairs always together), and subject pairs that never cluster together by white dot, **Figure 5A, Paper IV**. A strength of this computational approach is the iterative process in which a given partition of subjects is left out while the clustering is repeated multiple times, enabling the stability of each model (*i.e.*, K₁₋₁₀) to be evaluated. Measures of how often patient pairs are clustered together is referred to as stability and the cumulative distribution function (CDF) plot highlights the stability of each K. A flat line in the CDF function plot infers that patient pairs are always clustered together. When the pairs do not cluster, the CDF function increases.

To aid stability, or robustness, assessment Senbabaoglu et al. (Senbabaoglu et al., 2014) proposed a method in which the percent of ambiguously clustered (PAC) pairs is calculated to localize optimal K. However, the boundaries (at consensus index 0.1 and 0.9) used in the PAC calculation are arbitrary chosen and may negatively compromise the results.

Instead, deviation for ideal stability can be identified by finding the model with minimal area. The area is calculated above and below a pre-defined line in the CDF plot, drawn orthogonal to consensus index 0.5, and can serve as a better estimate of stability (De Meulder et al., 2018).

5 RESULTS DISCUSSION

5.1 QUANTIFICATION OF MAST CELL DERIVED HISTAMINE

The initial focus of **Paper 1** was to develop a simple and fast LC-MS method for determination of the stable end-point metabolite of histamine (*tele*-MIAA) in urine. Once established, this would enable a new sensitive method for detection of increased mast cell activation, *e.g.*, after suspected allergic responses, in the clinical setting.

The baseline concentration of *tele*-MIAA in healthy individuals was determined to be 2.1 and 3.0 $\mu\text{mol}/\text{mmol}$ creatinine, in males and females, respectively. In mastocytosis patients up to 10-fold elevation could be observed. Compared to previous standard method for urinary *tele*-MIAA, the improved precision may account for the possibility to detect minor shifts in baseline excretion and thereby enabling gender differences to be observed. There have been no reports explaining why females and males differ in baseline histamine release and this accordingly remains an open question. Furthermore, geographical differences may reflect different lifestyles where increased intake of fermented food stuff, like fish, cheese, yoghurt and beans, could explain slightly higher excretion of *tele*-MIAA in the Japanese population, **Figure 5, Paper I**.

The ion-pairing chromatography method was superior to the HILIC method when inorganic salt induced matrix effects were prominent. Ion suppression negatively affects the electrospray process and is most apparent in urine analysis and was clearly compromising the initially develop HILIC method, **Figure 4, Paper I**. In most other biological fluids where the aqueous fluid is buffered to maintain constant pH and ionic strength, *i.e.*, plasma and cerebrospinal fluid (CSF) ion suppression effect is more equal comparing sample to sample. Most likely, this is the reason for why Zhang et al. could establish and validate a HILIC method for rapid quantification of acetylcholine, histamine and *tele*-MIAA in rat CSF samples (Zhang et al., 2011).

However, the ion-pairing method demonstrated excellent repeatability (8.4%) across a large number of samples from multiple cohorts, **Table 3, Paper I**. The dilute and inject method for urine samples is an attractive preparation method and offered substantial simplicity while operating in the lab. The relatively high back pressure, with cycle time peak at 14 000 PSI, is demanding for the UPLC and needs to be carefully monitored over time. Furthermore, the cycle time of 30 min, sample to sample, is rather inefficient when only one analyte is reported. On the other hand, the ion-pairing method can be used to separate more than 87 amines present in urine (Waterval et al., 2009).

5.1.1 Kinetic release of histamine following airway challenge test

To study the kinetics of the release of histamine, urine samples from a previously published allergen-bronchoprovocation study (Daham et al., 2014) have been analysed. The data will be compared with the release of eicosanoid metabolites in the same study. To date, there are only some preliminary findings compiled, shown in **Figure 14**. Prior to allergen provocation, indicated by arrows in **Figure 14**, baseline values are in the expected range of normal (1-3 $\mu\text{mol}/\text{mmol}$ creatinine). Post allergen challenge, urinary *tele*-MIAA increased from the expected baseline of 3 $\mu\text{mol}/\text{mmol}$ creatinine up to 11 $\mu\text{mol}/\text{mmol}$ creatinine. The apparent difference in peak urinary excretion of histamine metabolites between the two subjects seemed related to differences in AHR (less release in the most responsive subject), a hypothesis that will be tested in the final analysis of the complete profile of released mast cell mediators.

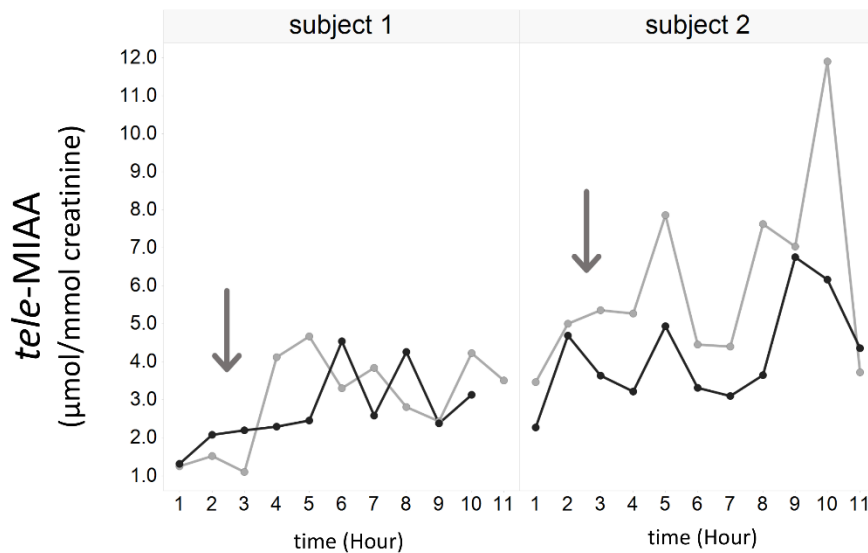


Figure 14. Time-course of urinary *tele*-MIAA excretion following allergen challenge, arrows indicate time of allergen exposure. Urine samples were then collected in hourly intervals. Grey and black lines correspond to placebo or treated (etoricoxib) study arm (blinded groups).

5.1.2 Urinary *tele*-MIAA is elevated among patients diagnosed with system mastocytosis

In a second study, urine *tele*-MIAA was determined from healthy controls, patients with mild-to-moderate asthma and systemic mastocytosis. Spot urine samples were acquired in the morning for assessment of mast cell mediators (Gülen et al., 2017). It could be clarified that patients with systemic mastocytosis not only presented elevated serum tryptase and urinary PGD₂ levels, but also histamine, **Figure 15**.

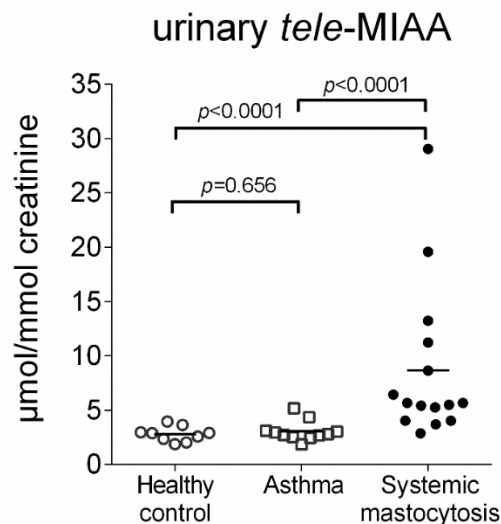


Figure 15: Urinary concentration of the main histamine metabolite 1-methyl-4-imidazoleacetic acid (*tele*-MIAA) in healthy control subjects, asthmatics and subjects with systemic mastocytosis. Data represents average values of two independent time points, with 1-2 hours in between, per subject. Group average concentration is highlighted by black line. Mann-Whitney U-test.

5.1.3 Identification of the “unknown”

Finally, the late eluting isobaric compound, *i.e.*, having the same monoisotopic mass, that was abbreviated “unknown” in **Figure 2a (Paper I)** could subsequent to the publication be successfully identified as 4-amino-propionic acid (or deamino-histidine), **Figure 16**. Instead of having a methyl-group situated on the imidazole ring, as in the case of *pi*- or *tele*-MIAA, de-amino histidine has one additional carbon incorporated on the short carbon chain. The slight increase in carbon chain length probably increased analyte hydrophobicity leading to the observed increase in retention time.

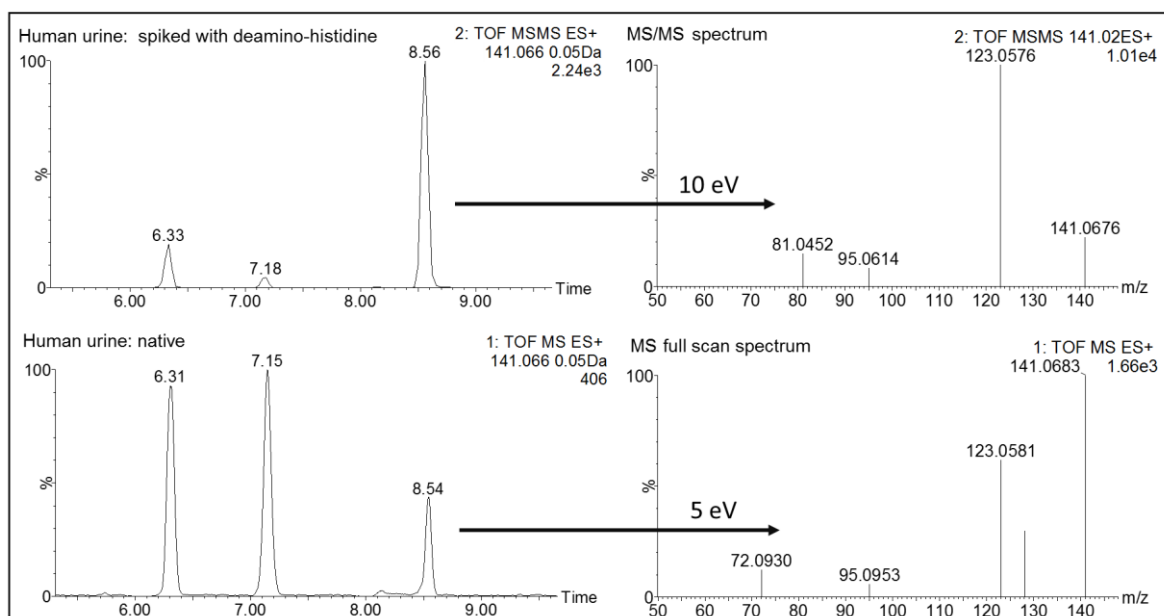


Figure 16. Confirmation of 4-amino-propionic acid (deamino-histidine) retention time ($t_R=8.54$ min) in spiked human urine (left) and verification of its unique product ion $m/z=123.06$ at 10 eV (right).

5.1.4 Limitations of using *tele*-MIAA as surrogate marker of histamine release

There are certain advantages and disadvantages of determining histamine turnover by quantifying *tele*-MIAA in urine. First, urine is a non-invasive matrix and therefore allows for repeated sample collections with time. Sample volume is seldom a limitation and instability at -20 °C, or even at room temperature, of *tele*-MIAA in urine has not been reported. However, interpretation of urinary concentration levels may be influenced by the intake of fermented food stuff, such as cheese, yoghurt and sea food. A high intake of these histamine-rich food stuff may eventually bias the result. The amount of histamine in certain food items has been well described and can therefore be easily avoided. It is notable that the highest concentration of urinary *tele*-MIAA was measured in samples from Japanese subjects, **Figure 5, Paper I**. This could possibly be explained by their different diet, because fermented soy and fish products is more common in Asian countries compared to northern Europe. Despite the influence from histamine rich food stuff the methodology was able to distinguish mastocytosis patients, as those demonstrated up to 10-fold higher concentration of urinary *tele*-MIAA.

5.2 DEVELOPMENT AND APPLICATION OF A COMPREHENSIVE OXYLIPIN PROFILING PLATFORM

A comprehensive platform to quantify 130 oxylipins and related lipid mediators was developed to enable assessment of molecular mechanisms involved in airway inflammation and bronchoconstriction.

5.2.1 Analytical characterisation of oxylipin method

The workflow described in **Paper II** describes three LC-MS/MS methods, which together can screen for multiple oxylipins in extracted samples that were redissolved in only 70 μ L. All three methods (A-C), together enabled the quantification of 112 oxylipins and 18 chiral monohydroxy fatty acids, **Figure 17**.

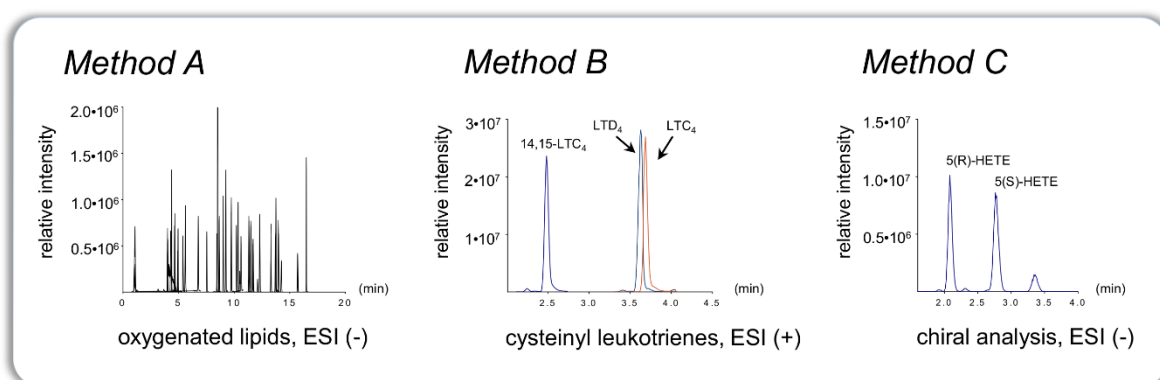


Figure 17. Extracted ion chromatograms of *Method A-C*. In *Method C* chiral separation is illustrated by extracted ion chromatogram of 5(*S*)- and 5(*R*)-HETE.

Measuring multiple analytes in a single analysis is challenging, but we succeeded to maintain separation of all analytes having the same molecular mass and to use multiple reaction monitoring that uniquely could detect each oxygenated lipid species. In *Method A*, the mobile phases used 0.1% acetic acid in H₂O (mobile phase A) and acetonitrile:isopropanol (90:10, mobile phase B), but it was unfortunately suboptimal to achieve elution of TXB₂ as a Gaussian peak. To compensate for its poor peak shape, the peak height was used for quantification. The introduction of 84% organic solvent composition in the final extract to be injected to the LC-MS/MS system showed however a greater improvement in peak shape.

The characterization metrics of the methods demonstrated a lower limit of quantification (LLOQ) of 0.016 to 0.7 ng/mL with a linearity of $R^2 > 0.98$. While the mean SPE recovery of 71% was good, for a few oxylipins the extraction was less efficient, such as for 5(6)-EpETrE (11%) and tetranorPGEM (31%). Low levels of matrix effects (approximately 11%) were observed, and when compared to tissue DMEM media no significant difference was seen.

5.2.2 Organ bath – *in vitro* pharmacology

Mast cell stimulation in human segments was induced by adding anti-IgE, and in guinea pig by ovalbumin (OVA), to the organ bath followed by recording the change in isometric smooth muscle contraction. **Figure 2a (Paper II)** illustrates the rapid increase in contractile force in both human bronchi and guinea pig trachea, reaching E_{max} within 15 min. Before quantifying the parallel release of oxylipins into the surrounding Krebs buffer, a set of experiments was conducted to characterize kinetic release, oxylipin stability and freeze-thaw effects.

5.2.3 Application of oxylipin profiling to study mast cell mediated smooth muscle contraction

The kinetic release experiment demonstrated that the major COX products PGD₂ and PGE₂ were released in increased amounts already after 15 min, but reached the highest concentration after 60 min, **Figure 2c-d (Paper II)**. In order to obtain a strong signal during mass spectrometric detection of oxylipins from the organ bath, the 60 min time point was considered as optimal. The constant supply of O₂/CO₂ into the bath ensures optimal physiological conditions for the lung tissue segments, but was highlighted as a main risk of oxylipin degradation. In the conducted oxylipin stability experiment, the degradation of oxylipins during the time-course of 60 min was individually different with 5(*S,R*)-HETE demonstrating a loss of 92% (**Figure 18**). Interestingly, substantial degradation was also seen for 5 DHA-derived monohydroxy products (HDoHEs) where ≤ 2% of the signal was recovered after 60 min (**Table S-5, Paper II**).

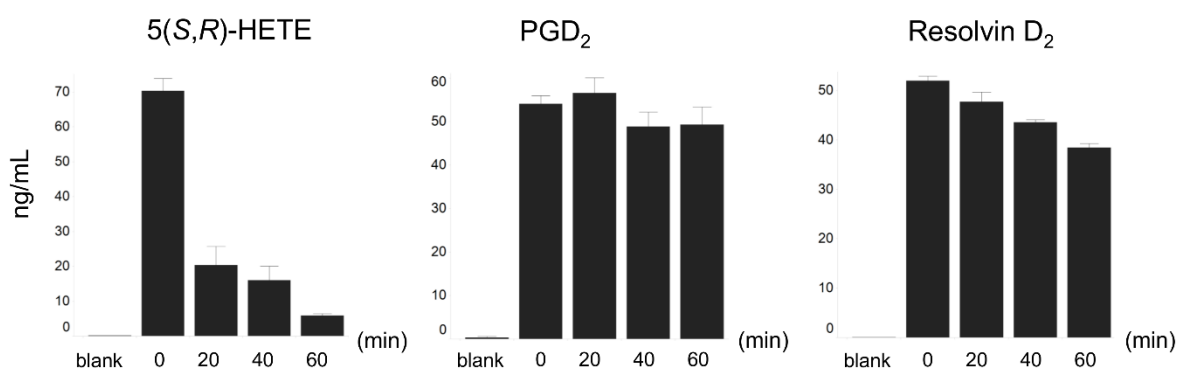


Figure 18. Degradation of 5(*S,R*)-HETE, PGD₂ and Resolvin D₂ following 60 min incubation in the organ bath without tissue present.

When samples were collected after 60 min of anti-IgE or OVA induced smooth muscle contraction, a strong increase in both COX- and FLAP/5-LOX-derived oxylipins was detected (**Figure 3, Paper II**). Simultaneously, the recorded smooth muscle force (E_{\max}) increased from 7 (PBS) to 79% (anti-IgE) in human bronchus preparation. In both species, an increase in E_{\max} was observed following the addition of the COX inhibitor indomethacin. This functional increase in contraction was followed by a reduction in all prostaglandins highlighting the opposing roles PGD₂ (constriction) and PGE₂ (relaxation).

Other COX products, such as 6-keto-PGF_{2 α} , PGF_{2 α} and 12-HHTrE were also significantly decreased. The latter is produced together with TXB₂ by thromboxane A₂ synthase in a 1:1 ratio. TXB₂ itself was not detected, which may be due to its short half-life in biological systems or to the chromatographic method used. There are no reports on the role of 12-HHTrE in mediating smooth muscle contractions. This result was confirmed in guinea pig trachea as well.

The addition of a FLAP/5-LOX inhibitor (MK-886) reduced E_{\max} and levels of all CysLTs in both species. When the dual combination of COX and FLAP/5-LOX inhibition was tested, further reduction in E_{\max} was observed and the corresponding oxylipins decreased. From the third injection using *Method C*, it was possible to define the biosynthetic origin of production of 18 monohydroxy fatty acids from LA, AA, and DHA metabolism. Interestingly, it was shown that DHA derived 14- and 17-HDoHE, together with 12-HETE were solely produced by enzymatic conversion (**Table 1, Paper II**), whereas 5-, 11 and 15-HETE, together with 9- and 13-HODE, under these conditions mainly were produced by autoxidation, *i.e.*, non-enzymatically. Of interest, under these conditions it was not possible

to detect a release of oxylipins with pro-resolving properties, although several were included in *Method A*, such as resolvin D₂.

Following 60 min stimulation of human bronchus tissue, the segments were washed and transferred to DMEM media and incubated over night at 37 °C. When the surrounding tissue media was analysed for oxylipins, a clear signal of elevated PGD₂ and its metabolites was observed indicating a significant activity of mast cells even after 24 hours (**Figure 5, Paper II**).

5.2.4 Characterisation of BALF oxylipins after 4 weeks of HDM exposure in mice

The developed *Methods A* and *B* in **Paper II** were also applied in a mice model of asthma to characterize the oxylipin release profiles in BALF following 4 weeks of HDM exposure, **Figure 7**. 24 hours after the last exposure to HDM, airway hyperresponsiveness was observed by an increase in lung resistance (R_L) and decrease in lung compliance (C_L). Elevation of *Tnf*, *Il10* and *Il13* mRNA transcripts verified that both a type 1 and type 2 inflammatory response was present. In the C57BL/6 strain, a type 2 inflammatory response is usually more difficult to achieve as it is skewed towards type 1. However, the strong infiltration of eosinophils into the lungs and the BALF was convincing (Piñero-Hermida et al., 2017).

In mice BALF, 26 of 59 detectable oxylipins exhibited > 2-fold increase in concentration, **Table 1, Paper III**. In total, 57 oxylipins were detected in control (PBS) mice, which provides the largest descriptive signature of oxylipins in an allergic asthma model, **Figure 4, Paper III**. The majority originated from the LOX pathway, but also COX, CYP450 and sEH products were significantly elevated following HDM exposure.

An attempt was made to relate these elevations to changes at the mRNA level for the responsible enzymes, however the RT-qPCR data did not provide such support, **Figure 3, Paper III**. The findings are consistent with the biochemical dogma that substrate availability is rate limiting for most eicosanoids rather than downstream enzymes. Furthermore, correlating individual oxylipin levels with total number of cells in BALF indicated that the number of infiltrating inflammatory cells substantially contributed to the observed increase of several oxylipins, such as 5,15-DiHETE, **Figure 19**. Similar to 5,15-DiHETE, LTB₄ demonstrated a 20-fold increase in HDM exposed animals, but the correlation to total cells in BALF was negative.

The most significant signature was the strong elevation of multiple LOX (5-LOX, 12/15-LOX) products followed by those generated by COX, CYP450 and sEH. In particular, the 5-LOX products 5-HETE and LTB₄, including its non-enzymatically produced isomers 6-*trans*-LTB₄ and 6-*trans*-12-*epi*-LTB₄. Interestingly, PGD₂ was significantly elevated, while no change in lung mast cell numbers was observed. This is expected, because *de novo* synthesis of PGD₂ occurs when mast cells are activated. The source of PGD₂ production is attributed to mast cells, which possess the necessary PGD-synthase, but other inflammatory cells may contribute as well.

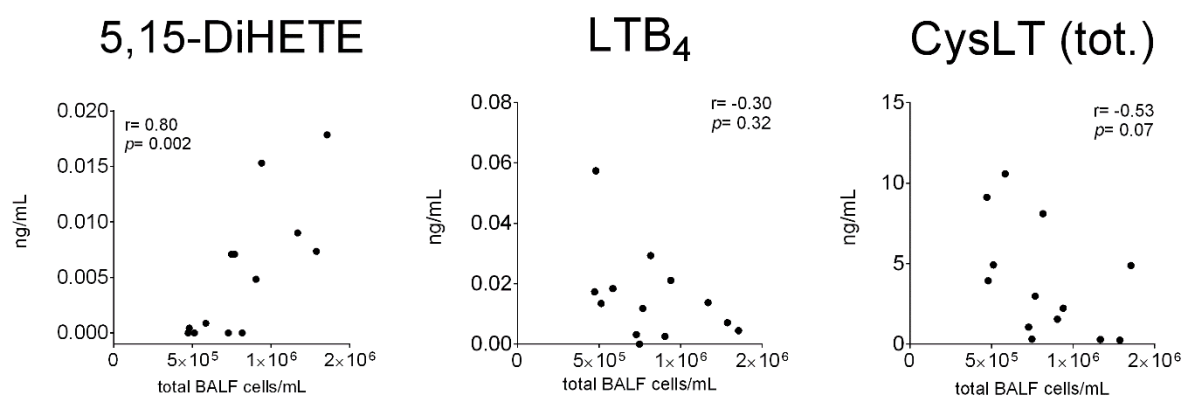


Figure 19. Spearman rank correlation of total inflammatory cells in mice BALF with that of 5,15-DiHETE, LTB₄ and total CysLT(tot.) concentration in HDM exposed animals.

The finding that the highest levels of all detected oxylipins was CysLT(total) at baseline is interesting, and perhaps the unexpected lack of effect following the HDM exposure suggest that a different role may exist in mice compared to humans regarding CysLTs. Furthermore, correlation of CysLTs to total cells in BALF was similarly negative. For example, a LTC₄ synthase knock-out mouse demonstrated a role for CysLTs in cutaneous vascular permeability and mucin release in the nasal mucosa. This effect was suggested to be mediated via LTE₄ binding to the GPR99 receptor (Bankova et al., 2016; Maekawa et al., 2008).

5.3 CHARACTERISATION OF URINARY EICOSANOIDS IN SUBJECTS WITH ASTHMA

The principle focus of the U-BIOPRED study was to integrate molecular biomarker profiles to create a handprint to increase the prediction of treatment efficacy or identifying new drug targets. This unbiased omics-approach acquired targeted and untargeted analysis of mRNA, protein and metabolite data from multiple biological matrices. By combining the collected multivariable data set, it is anticipated to construct a handprint that can sub-group asthmatic subjects by their combined molecular profile. Although the urine eicosanoid metabolite platform is not an unbiased analytical omics-platform, the strength of the selected panel of analytes, **Figure 4**, is that the analytes reflect mediators with established key roles in inflammation, mast cell activation and bronchoconstriction.

5.3.1 Analytical performance

Eicosanoids in the laboratory reference material, included in all batches, demonstrated a coefficient of variation (CV) between 11.7 to 37.9% in the baseline data set. The measurement precision was somewhat better in the longitudinal data set with a CV ranging from 2 to 23.7%. Unfortunately, TXB₂ and 2,3-dinor-6-keto-PGF_{2α} (the main PGI₂ metabolite) demonstrated a baseline CV > 40% and had to be excluded from the data analysis. The eicosanoid data set was collected from multiple batches of sample analysis during a three-year period. Longitudinal urine samples were analysed separately from the baseline, because they were collected 12-18 months after the baseline time point. For improved assessment of intra-individual variability among SA subjects, it may have been more appropriate to have analysed the two samples from each subject in pairs. Despite those shortcomings, the urine eicosanoids data sets (baseline=597 and longitudinal=302) represent the largest comprehensive European data set acquired from subjects with asthma in a cross-sectional study.

5.3.2 Normal baseline excretion of eicosanoid metabolites in HC subjects

The 100 HC subjects together constitute the largest available cohort of healthy individuals defining baseline urinary eicosanoid metabolite concentrations (**Figure 2, Paper IV**). By far the most abundant eicosanoid metabolite in the panel was the major PGE₂ metabolite tetranorPGEM, which was two times higher in males (1510 ng/mmol creatinine) compared to females (701 ng/mmol creatinine), confirming previous observed gender differences (Daham et al., 2014). In the lung, primary PGE₂ is an important smooth muscle tone regulator. In patients with aspirin intolerance, maintaining normal airway tone is critically dependent on PGE₂. It is less clear how useful urinary PGE₂ may be as a marker reflecting total formation in the lung since the kidney and prostate may produce substantial amounts, *e.g.*, radiolabeled infusion of PGE₂ recovers less than 4% in urine (Seyberth et al., 1976). Despite this, we observed 10 ng/mmol creatinine of PGE₂ in HC subjects.

Of the two PGD₂ metabolites, tetranorPGDM was 3 times more abundant than 2,3-dinor-11 β -PGF_{2 α} (188 *vs.* 46 ng/mmol creatinine respectively), which implies that release of PGD₂ can be quantified with greater confidence using tetranorPGDM. It also demonstrated a CV of 15.3% *vs.* 23.3% for 2,3-dinor-11 β -PGF_{2 α} . In the longitudinal samples, 2,3-dinor-11 β -PGF_{2 α} was found 3 times lower than in the corresponding subjects' baseline samples, while tetranorPGDM exhibited the same group median. Prostaglandin F_{2 α} (PGF_{2 α}) binds the TP receptor to induce bronchoconstriction in the lung and there exists limited data regarding downstream metabolites excreted in urine. In its stable form, primary PGF_{2 α} was found at 97 ng/mmol creatinine in urine from HC subjects.

TXA₂ is a potent vasoconstrictor and induces bronchoconstriction also via binding to the TP receptor (Manning et al., 1991; S  fholm et al., 2015). It is highly unstable and therefore rapidly metabolized into inactive TXB₂ (Hamberg et al., 1975). However, the kidney is able to produce TXB₂ and it may therefore be less useful than its two downstream metabolites 11-dehydro-TXB₂ and 2,3-dinor-TXB₂, which exclusively reflect systemic TXA₂ production, primarily via platelet activation (Roberts et al., 1977). In the baseline data set TXB₂ failed to meet QC criteria while its metabolites demonstrated CVs < 38%. Of the two TXs metabolites 2,3-dinor-TXB₂ were most abundant (48-49 ng/mmol creatinine) while 11-dehydro-TXB₂ were found at 6.8 ng/mmol creatinine, consistent with previous reports (Balgoma et al., 2013).

The greatest concentration of the three included isoprostanes was evidenced by 8,12-iso-iPF_{2 α} -VI (392 ng/mmol creatinine) followed by the 2,3-dinor-8-iso-PGF_{2 α} (46 ng/mmol creatinine). Primary 8-iso-PGF_{2 α} is considered to be the gold standard for measuring isoprostanes in urine as a marker of oxidative stress and smoking (Montuschi et al., 2000). It was found at 23 ng/mmol creatinine.

The least abundant eicosanoid was the end-product of the CysLT pathway LTE₄ (3.1 ng/mmol creatinine). LTC₄ and LTD₄ were only occasionally detected reflecting their rapid metabolism in the lung into LTE₄. Apart from CysLTs being known as the most potent bronchoconstrictors and powerful inducers of increased vascular permeability, CysLTs are chemoattractants for eosinophils. In addition, recent data has demonstrated that inhaled LTE₄ or LTD₄ activates the CysLT₁ receptor on mast cells causing release of PGD₂, which could further amplify constriction. This effect was also shown to be completely inhibited by montelukast (Lazarinis et al., 2018).

5.3.3 Metabolite levels relating to per study protocol recruitment

As per study protocol evaluation, the most significant observation was the increasing concentration of LTE₄ with asthma severity ($p < 0.0001$), followed by the two PGD₂ metabolites and 11-dehydro-TXB₂, which were increased in SA, **Figure 20**. Elevated levels of LTs and PGs in asthma have been well documented previously, but for the first time, in a large cohort of subjects, the excreted levels can be directly compared with disease severity. For the remaining eicosanoids, smaller shifts were observed when comparing HC vs. MMA, or MMA vs. SAn or SAs/ex (**Figure 3, Table S2, Paper IV**). Notably, the somewhat large intra-group variability for individual eicosanoids most likely reflects that spot urine samples contain a snap-shot of the total systemic excretion and is most likely influenced by the current degree of inflammatory cell activation, or possibly, how close to an exacerbation the sample was collected.

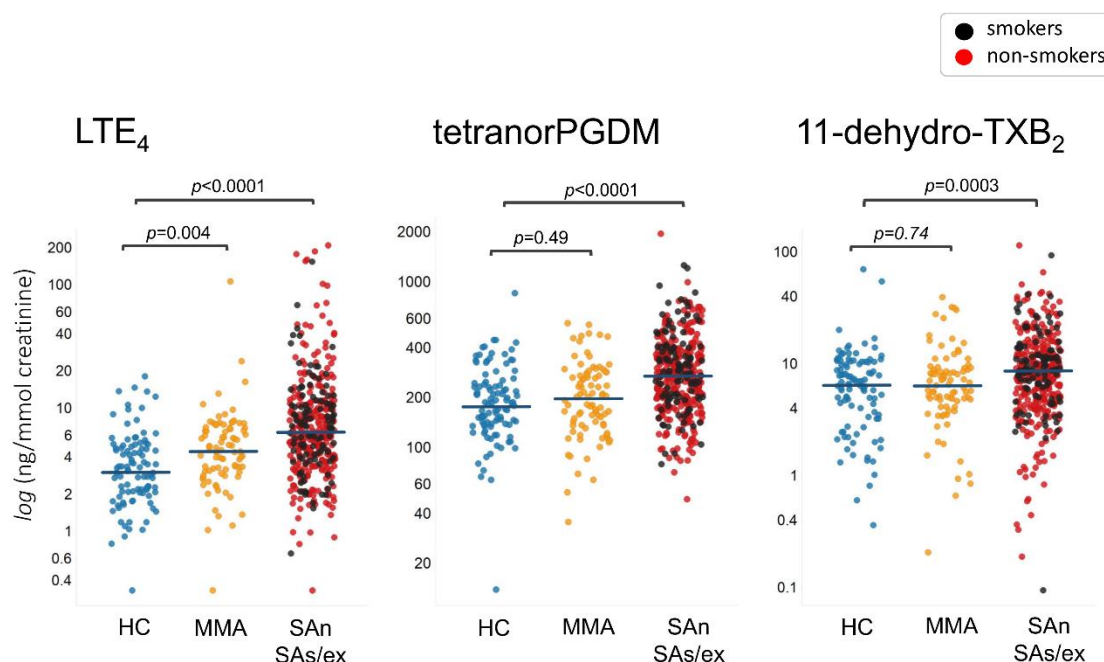


Figure 20. Baseline concentration of urine eicosanoid metabolites from cysteinyl-leukotriene (LTE₄), prostaglandin D₂ (tetranorPGDM) and thromboxane (11-dehydro-TXB₂) pathways demonstrate significantly elevated levels in severe asthma subjects (SAn and SAs/ex). Cohort median indicated by flat lines. Kruskal-Wallis test.

5.3.4 Smoking and oxidative stress

The least abundant isoprostane in urine, 8-iso-PGF_{2 α} , demonstrated increased levels in the SAn group (27 ng/mmol creatinine), with an even stronger increase (30 ng/mmol creatinine) in the smokers group (SAs/ex). The most abundant isoprostane in urine, 8,12-iso-iPF_{2 α} -VI (HC: 392 ng/mmol creatinine) was not different between any groups, despite being reported to be upregulated following allergen provocation (Balgoma et al., 2013), while 2,3-dinor-8-iso-PGF_{2 α} was significantly elevated in the smokers group (**Table S3, Paper IV**).

5.3.5 Effects of oral corticosteroids (OCS) on urine eicosanoid levels

The reported use of oral corticosteroids was about 45% in the two SA groups. We hypothesized that OCS would not affect the detectable concentration of eicosanoids as has previously been published (Gyllfors et al., 2006). Subjects were stratified in the following two ways: 1) medical history of being OCS user (Yes vs. NO), and 2) medical history of being OCS user, plus detection of prednisolone in urine (Yes vs. NO). The presence of

prednisolone was defined by a positive detection of either of prednisone, prednisolone, or related hydroxy metabolites of prednisolone, in urine by a specific LC-MS steroid analysis method, **Table 2, Paper IV**. In any of the two comparisons no significant difference was observed for 9 of 11 eicosanoids. A significant, but small, decrease was observed for 2,3-dinor-TXB₂ and 8,12-iso-iPF_{2α}-VI using either of the two stratification criteria.

5.3.6 Treatment with anti-IgE

Interestingly, fewer subjects with extremely high levels of the two PGD₂ metabolites (2,3-dinor-11β-PGF_{2α} and tetranorPGDM) and LTE₄ were observed in SA subjects reported to take omalizumab (Xolair), **Figure 21** below. This finding strengthens the mechanism that removal of free IgE from the systemic circulation stabilises the mast cells, and perhaps other cell types as well (Hayashi et al., 2016; Holgate et al., 2005), leading to a reduction in amount of allergen stimulation. Verification of this effect is best assessed in follow-up studies with an appropriate study design that includes equally balanced groups. However, despite the rather weak statistical significance in this analysis, it is clear that fewer subjects were found with extremely high urinary levels of prostaglandin D₂ metabolites and LTE₄.

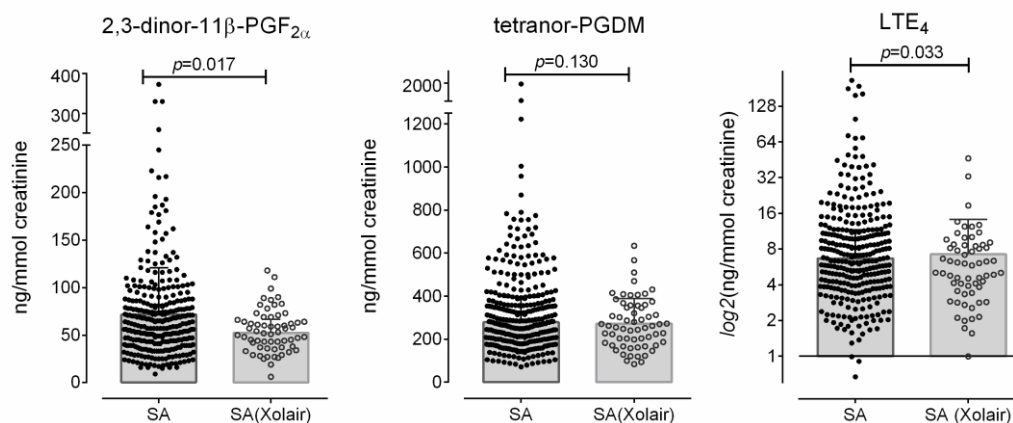


Figure 21. Urinary concentration of prostaglandin D₂ metabolites, 2,3-dinor-11β-PGF_{2α} and tetranorPGDM, and LTE₄ in severe asthma subjects treated with omalizumab (Xolair: $n=62$) vs. no omalizumab ($n=315$) in the U-BIOPRED study indicate a potential mast cell stabilizing effect of omalizumab. Bar graphs are median (+IQR). Mann-Whitney U-test.

5.3.7 Association between eicosanoids and type 2 inflammation

T-cells releasing pro-inflammatory cytokines IL-4, IL-5 and IL-13 have been linked to increased serum IgE, F_ENO and eosinophil counts in blood and sputum. More recently, serum periostin has been evaluated as a maker of type 2 inflammation, but with less success (James et al., 2017; Takayama et al., 2006; Wagener et al., 2015; Woodruff et al., 2007). Those characteristics constitute the type 2 inflammatory pathway and have attained great focus in treating patients with severe asthma. Correlating urinary LTE₄ levels with levels of IL-13, periostin, serum IgE, F_ENO and eosinophil counts for all included study subjects highlight scattered data, but statistically significant, exemplified in **Figure 22**. However, as asthma is a variable disease with episodes of worsening of symptoms, *i.e.*, exacerbations, it can be speculated that subjects with a more pronounced type 2 signature would also present a greater release of eicosanoids.

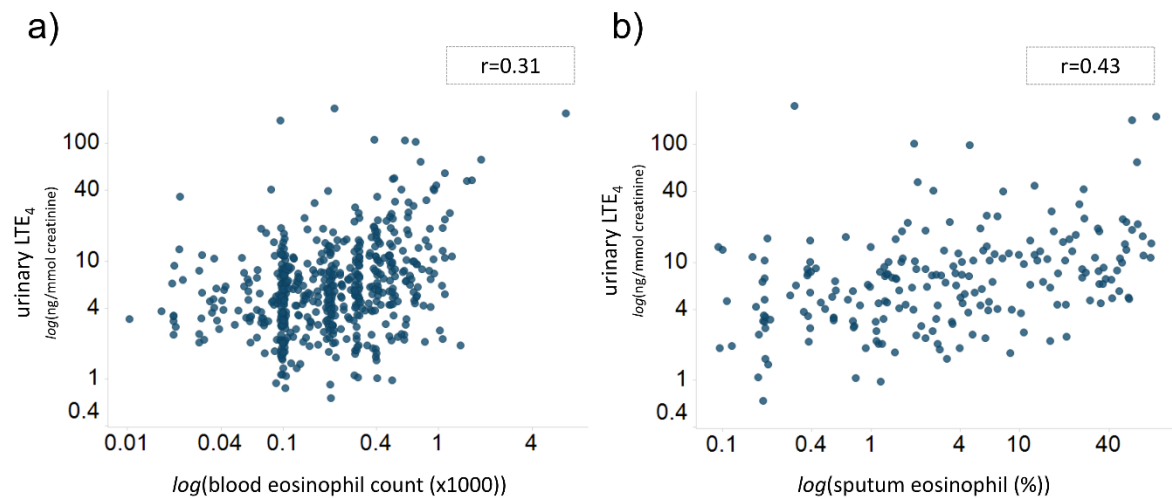


Figure 22. Urinary LTE₄ demonstrates a modest increase with: (a) blood eosinophil count ($n=583$; $p<0.0001$) and (b) sputum eosinophils (%) ($n=262$; $p<0.0001$) among subjects in HC, MMA, SAn and SAs/ex. Spearman rank correlation (non-parametric).

Therefore, subjects were selected according to the described extreme value approach (section 4.8.2), *i.e.*, below the 25th or above the 75th percentile. From the selected panel of type 2 markers, LTE₄ was significantly associated with blood and sputum eosinophils, F_{ENO}, IL-13, IgE and serum periostin. Both PGD₂ metabolites were linked to elevated blood eosinophil counts, sputum eosinophils (2,3-dinor-11 β -PGF_{2 α}) and IL-13, **Figure 4, Paper IV**. This interesting finding suggests that PGs and CysLTs are important mediators in the type 2 inflammatory response and consequently, perturbing those pathways in asthmatics, stratified by elevated PGs and CysLTs, could improve treatment efficacy.

Isoprostanes were combined into a composite variable (using z-scores), but were not found significantly associated with known markers of type 2 inflammation using the extreme value approach. It remains to be evaluated if a significant association exist between isoprostanes and type 1 markers of inflammation (*i.e.*, Th17 or Th6 pathways).

5.3.8 Urinary eicosanoids can distinguish sub-groups of asthma

Referring to the aim of identifying new molecular sub-phenotypes of asthma, we sought to evaluate the strength of clustering the 497 urine eicosanoid profiles from all subjects with asthma to distinguish molecular sub-groups. It was hypothesized that there exist eicosanoid concentration differences among subjects with asthma that go beyond the common cohort classification and which could be used for improved diagnosis.

After evaluation of multiple rounds of unbiased consensus clustering, the final result identified a five-cluster model to be further characterised, **Figure S1, Paper IV**. In this model eicosanoid concentration data was log₂-transformed and z-scored, and by using the Euclidean distance measure as input, the cluster algorithm Partitioning Around the Medoids (PAM) was proven successful. Stability of clustered subject pairs throughout the iterative bootstrapping process allows deviation from ideal stability to be mathematically estimated and visualized, **Figure 23a**. The objective is to reach a stable horizontal line in the CDF plot. Subject pairs, grouped into the five clusters, are visualized in the consensus matrix plot in **Figure 23b**, where dark blue indicates subject pairs who were always clustered together and white, which were never clustered together.

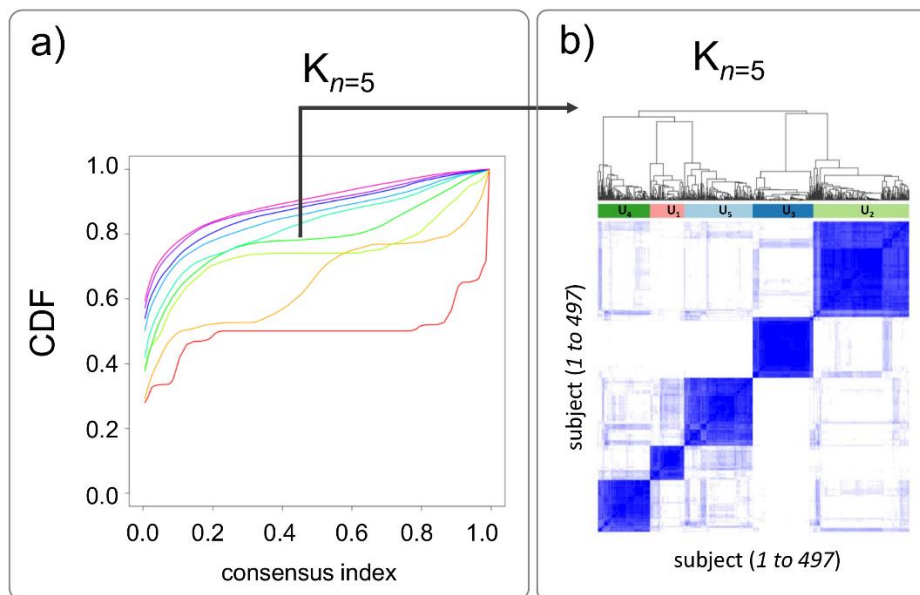


Figure 23. The CDF plot draws a colored line for each tested cluster model ($K_{n=2-10}$) where a moderate flat line is highlighted for $K_{n=5}$ (a). The consensus matrix for the derived five-cluster model (U_1 - U_5) in (b) show patient pairs as dark blue dots if they are always clustered together, while subject pairs never clustered together are white.

For $K_{n=5}$, the last flat line was achieved having index 0.8, although the length was somewhat short. Assessment of clinical and biochemical differences between the obtained sub groups demonstrated several significant and important differences summarized in **Figure 24 (Table S4, Paper IV)**. The mathematically calculated PAC and DIS values provided no conclusive evidence to support a selection of model (K_n) and instead the selection approach had to rely on the statistical differences of the biochemical and clinical variables describing the clusters. Of interest, the median concentration of individual eicosanoids per cluster (U_1 to U_5) markedly differed compared to per protocol cohort medians, **Figure S2, Paper IV**.

In brief, $FEV_1(\%)$ was $< 71\%$ for subjects in cluster U_2 - U_4 and a larger proportion of SAn and SAs/ex subjects were clustered here. Subjects in those clusters reported worse asthma control (ACQ-5) and quality of life (AQLQ) and reported increased use of oral steroids, **Figure 24**, compared to U_1 and U_5 .

Cluster $U_3(n=97)$ was attributed to the largest concentration of PGD_2 metabolites and all isoprostanes, suggesting oxidative stress to be most prominent among this group of subjects. 82% were females, presenting the highest median BMI (30.2) and more frequent exacerbations and emergency visits (73%). Interestingly, their elevated CRP also evidenced systemic inflammation and their lower levels of serum creatinine may reflect a reduced muscle turnover rate, indicative of less physical exercise. This phenotype has been proposed to be driven by molecular mechanisms of non-type 2 origin, reduced FE_{NO} and elevated 8-isoprostanes in exhaled breath (Holguin and Fitzpatrick, 2010). Likewise, in cluster U_3 FE_{NO} was low (23.5 ppb), **Table 3, Paper IV**. Furthermore, PGD_2 were most abundant in cluster U_3 which implies that mast cells play an active role in this phenotype.

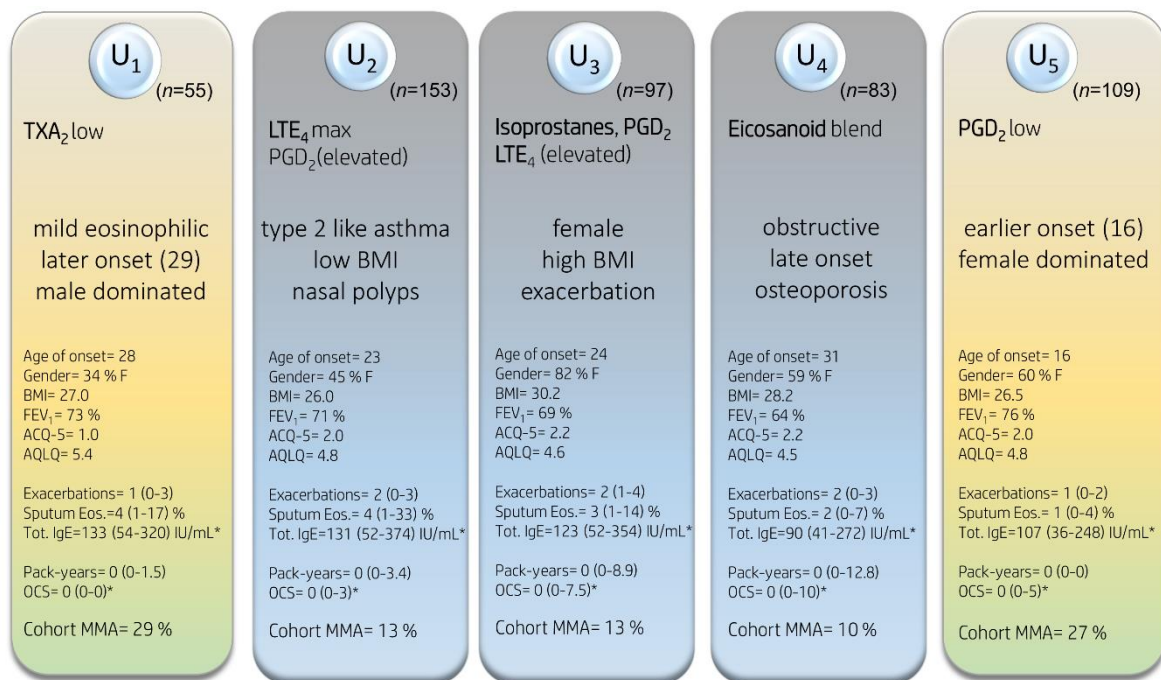


Figure 24. Urinary eicosanoid metabolites and selected clinical and biochemical variables describing the five-cluster model derived from consensus clustering. Values presented as median (IQR). Not statistically different (*).

Cluster U₂ comprised the largest number of subjects ($n=153$) with the largest median concentration of LTE₄ at 8.7 ng/mmol creatinine and with clear type 2 signatures, such as elevated FE_{NO} (28.5 ppb), sputum eosinophils 4% (IQR; 1-33), blood eosinophils (270 cells/ μ L), IL-13 (0.74 pg/mL) and total serum IgE 131 UI/mL (IQR; 52-374).

Cluster U₄ was most obstructive (FEV₁/FVC=0.63) with median FEV₁=63.6%, having later onset of age (31 years) and reported the highest use of OCS (IQR; 0-10). Surprisingly, only a modest increase in isoprostanes was observed while the interquartile range of reported pack-years was the largest (IQR; 0-12.8). As no single eicosanoid metabolite in this cluster presented values markedly different from the other, the eicosanoid pattern for subjects in U₄ was termed “blend”.

The two remaining clusters U₁ and U₅ contained 55 and 109 subjects respectively with 29 and 27% MMA subjects in each, reflecting a less severe disease state. They significantly differed in terms of gender (66% vs. 40% male) and age of onset (29 vs. 16 years). OCS usage was higher in U₅ followed by lower sputum eosinophils 1% (IQR; 0-4). In contrast, subjects in U₁ presented both elevated FE_{NO} (28 vs. 22 ppb) and sputum eosinophils 4% (IQR; 1-17). The eicosanoid profiles showed low levels of TXs (cluster U₁) and PGD₂ metabolites (cluster U₅).

5.3.9 Longitudinal 12 to 18-month follow-up

The variability in clinical symptoms and disease control is a hallmark of asthma. Seasonal changes and comorbidities influence the disease state and adjustment of treatment dose, or frequency, may be required to maintain optimal control. Using a paired *t*-test, a significant reduction in reversibility from 14.6 to 10.2% ($p<0.0001$) was seen at corresponding longitudinal follow-up time point, but not for any of the other clinical variables tested suggesting that subjects remained classified as being severe both at the individual, as well as, the group level.

The eicosanoid metabolite excretion profiles of the 302 asthmatics is shown for tetranorPGDM, LTE₄, 2,3-dinor-TXB₂ and 8,12-iso-iPF_{2α}-IV in **Figure 6A-D (Paper IV)**. For in total 7, out of 11, quantified eicosanoids, including PGE₂ and tetranorPGEM, the median SAn and SAs/ex concentrations remained the same comparing baseline vs. longitudinal time points ($\pm 25\%$ of total SA median). An example of intra-individual variability is shown for the most abundant isoprostane in urine, 8,12-iso-iPF_{2α}-VI, in **Figure 25** below. A relative comparison of median cluster concentration values clarified that the contribution of pathways to the observed cluster levels was preserved, **Figure 6E-F, Paper IV**. Potential reasons for the four eicosanoids exhibiting differences remains to be evaluated.

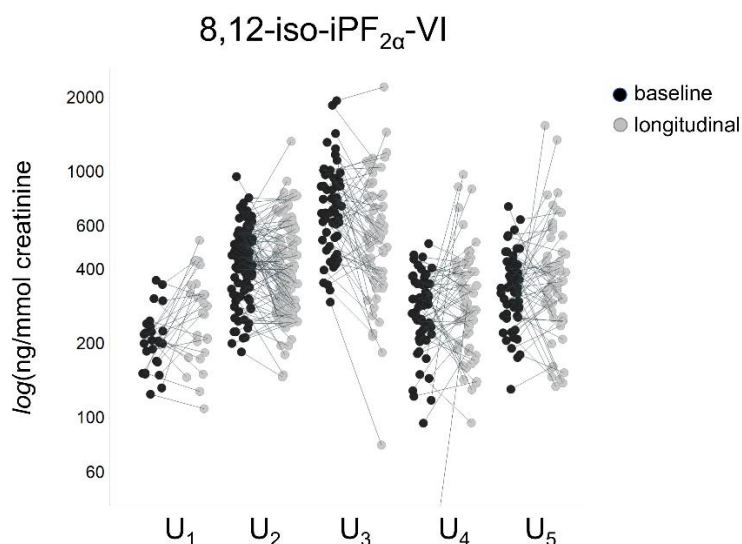


Figure 25. Concentration of 8,12-iso-iPF_{2α}-VI in 302 severe asthma subjects at the baseline and longitudinal time point highlight a reproducible cluster pattern. Longitudinal data is plotted according to the baseline derived five-cluster model. One imputed value in U₄ is located outside y-axis range.

5.4 METHODOLOGICAL CONSIDERATIONS

In this thesis, quantification of inflammatory mediators in clinical material and experimental models has exclusively relied on the use of LC-MS and LC-MS/MS technology. It offers some advantages compared to immunological assays, where cross-linking between molecules and antibodies are used for detection. Immunoassays requires development of antibodies with high specificity to avoid unwanted cross-reactivity against similar chemical structures or epitopes. While mass spectrometry analyzers offer simplicity by keeping sample preparation to a minimum, ionization of intact molecules by the soft electrospray ionization (ESI) technique has generally a poor efficiency. Consequently, assessing the limit of detection is important for each LC-MS and LC-MS/MS method. Despite this limitation, it was possible to report the largest number of oxylipins to be detected in mice BALF, and from isolated lung tissue segments, to date.

Although urine is a non-invasive matrix available in larger volumes, the biochemical composition, with large amounts of inorganic salt components, is an analytical challenge. There are few strategies to handle the large amounts of salt and the great variability that can exist between urine samples from patients. In this thesis, in principle three approaches were tested for quantification of analytes in urine, of which HILIC was the least suitable. In **Paper I**, we could show that 1:9 diluted urine generated irreproducible standard addition calibration curves for *tele*-MIAA due to severe matrix effects using the developed HILIC method. One alternative option to circumvent this could have been to first use SPE to clean up the samples, and to remove interfering salt components. On the other hand, that would also extend sample

processing time, consumables and laboratory costs. Instead, the final ion-pairing separation method utilized a 50-time dilution of urine and provided increased retention and separation of structural isomers. While the retention factor increased from $k'=3$ (HILIC) to $k'=24$ using ion-pairing separation it also minimized the risk co-elution of inorganic salt, as those are not retained during reverse phase separation. For the relatively low abundant eicosanoid metabolites quantified in **Paper IV** SPE was necessary to enrich individual eicosanoid concentration. Also, the included SPE wash step most likely aided the removal of interfering salt components while enabling repeated collection of a purer extract subjected to LC-MS/MS analysis.

Simplifying sample handling and reducing analysis time can be a bottleneck in bioanalysis of large number of samples. The long analysis time in the final ion-pairing method decreased throughput, but kept sample handling at a minimum by the simple dilute and shoot approach. For future improvements, mobile phase consumption could be reduced by switching analytical column size to 1 mm instead of 2.1 mm allowing a reduced flow rate (below < 0.2 mL/min) to be used and the total consumed mobile phase volume can be estimated to be 60% lower, which would also cause less negative environmental impact.

In **Paper II**, chromatographic aspects that could improve the operation and speed, while reducing solvent use, in *Method A-B* would be to decrease column length from 150 to 100 mm. The estimated theoretical decrease in chromatographic resolution would be only 18%, but may negatively affect separation between stereoisomers, such as 15-*epi*-LXA₄ and LXA₄, who were not completely baseline separate in *Method A*. Furthermore, enhanced chromatographic resolution in *Method C* (chiral monohydroxy fatty acids) could be obtained by using 3 μ m instead of 5 μ m particle size columns, and may consequently result in less interference between co-eluting 12(*S*)-HETE ($m/z=319.05$) and 11(12)-EpETrE ($m/z=319.2$).

The majority of oxylipin and eicosanoid species are found present in the nanomolar range in biological fluids, and consequently following SPE, the reconstituted sample volume was kept below 70 μ L for both urine, BALF and organ bath samples. In such small volumes, not all sample constituents are soluble, leading to fewer number of ions detected. Using a higher reconstitution volume could therefore be more efficient and possibly lead to improved limit of detection and reproducibility. The electrospray response may also increase if less competition exist between analytes during the ionization process (King et al., 2000). A larger reconstitution volume would also allow for other methods to be applied on the same extract, such as untargeted lipidomics or metabolomics methods (Fauland et al., 2011; Reinke et al., 2017), or improved quantification of tautomeric structures by derivatization, *i.e.*, TXB₂, 2,3-dinor-TXB₂ and 2,3-dinor-6-keto-PGF_{2 α} .

The extreme-value analysis was performed using a selection of subjects based on concentration quartiles of $<25\%$ and $>75\%$. It is possible that using a different cut-off range could provide further significance in the proceeding statistical analysis. For example, different cut-off values could be tested using an iterative approach and the response variable evaluated for improved statistical significance.

Urinary LTE₄ was shown to be associated with type 2 inflammatory markers. To further increase confidence in these results markers of eosinophil activation, such eosinophil cation protein (ECP), eosinophil peroxidase (EPO) or urinary 3-bromotyrosine, could confirm if the activated eosinophils were the primary cause of largely elevated LTE₄ (Kita, 2011). 3-bromotyrosine has been suggested as a marker of eosinophil induced peroxidation and elevated levels of this halogenated metabolite have shown promising results in at least two other studies (Cowan et al., 2015; Wedes et al., 2009). Furthermore, a recent study by

Mani (Mani et al., 2016) suggest that 3Br-HPA, may be an even more promising non-invasive urinary marker of eosinophil activation.

The relatively few number of sputum samples (222), of the 497 included asthmatics in the U-BIOPRED study, is a weakness that negatively influences the power of interpretation regarding the five-cluster model, and consequently the low number of sputum samples may account for the lack of statistical significance in the corresponding group comparison. However, data from other type 2 markers, *i.e.*, IL-13, IgE, F_ENO and blood eosinophils supported the conclusions made.

Class discovery methods have a risk of overfitting, *i.e.*, when larger proportions of random noise are introduced in a model. Therefore, splitting up the 497 subjects into a training and a validation set is one strategy to increase confidence in cluster analysis. However, clustering of training ($n=333$) and validation ($n=164$) sets separately reduced the number of subjects in the validation set to an extent that no flat line in the consensus CDF plot could be obtained. Although the U-BIOPRED study contains the largest cohort of severe asthmatics with available urine samples, an increased number of subjects may have provided further power in the consensus clustering and improved quality of the cluster interpretations. A larger data set also offers other data analysis methods based on machine learning to be applied (De Meulder et al., 2018). In this context, it is worth mentioning that unsupervised data analysis methods were initially evaluated, such as principle component analysis (PCA) (Jackson, 1991) or topological data analysis (TDA) (Lum et al., 2013). From these results it was observed that reduction of variance by PCA did not provide any subgroupings apart from a minor trend from HC to MMA and SA with great overlap. TDA is an alternative reduction method and was able to generate a complex network of subject nodes located in multiple sub-clusters. When contrasting the network by eicosanoid concentrations, or clinical variables, only modest interpretations could be made. No further refinement of the TDA settings was evaluated, but is of continued interest to explore, as the method as such, could suggest additional separation or subgroupings.

Aspects defining an individual's lifestyle, such as diet and exercise, will be reflected in the metabolic profile of each subject, but controlling for lifestyle factors in clinical studies is difficult. However, increasing the number of included subjects may at least increase statistical power. Interestingly, in the U-BIOPRED data storage platform TransSMART, additional data related to comorbidities, or possible lifestyle factors, is available and should be further used to compare specific subgroups.

Finally, urine samples are easy accessible and constitute an attractive non-invasive matrix for markers of disease to be detected. However, diurnal variability and comorbidities affects the observed concentration of analytes in urine to a various degree and clarifying the role of specific dietary components will eventually add value for the future utility of mediators quantified in this thesis. Perhaps food stuffs rich in histamine, such as fermented food like, cheese, yoghurt and fish serve as a practical example of how lifestyle can influence systemic markers. Thus, it can be speculated that reasons for elevated *tele*-MIAA in the Japanese population, compared to US and Sweden, originates from this cause.

Finally, all reported concentrations of mediators in urine were normalised according to standard clinical praxis, *i.e.*, to urinary creatinine. However, it is well established in athletes that muscle turnover elevates serum creatinine, and consequently, urinary creatinine may therefore bias, or skew, the final result. Alternative normalisation methods, such as optical density, *i.e.*, specific gravity measurement, may eventually be a more attractive alternative.

6 CONCLUSIONS

In the current thesis it was successfully demonstrated that release of inflammatory mediators can be quantified using liquid chromatography coupled to mass spectrometry analysis of human urine samples, mice BALF and in samples from *in vitro* organ bath experiments. Specifically, it can be concluded that:

- Reduced risk of ion suppression during electrospray ionization is achieved for urinary *tele*-MIAA by ion-pairing chromatography compared to HILIC. (**Paper I**)
- Females excrete more histamine and less tetranorPGEM than men. (**Paper I and IV**)
- Inhibition of COX and FLAP/5-LOX in human and guinea pig lung tissue preparations results in similar physiological and biochemical responses supporting guinea pig as a relevant translational model for further interventional studies. (**Paper II**)
- Significant elevation of PGD₂ and its metabolites 24-hour after allergic stimulation of human bronchus indicates that mast cells are active longer than expected and that it may be related to late phase reactions in the clinical setting. (**Paper II**)
- Both human and guinea pig isolated airways evidence 15-LOX activity, as demonstrated by the production of solely the 14(*S*)- and 17(*S*)-HDoHE isomers (100% enantiomeric excess). (**Paper II**)
- HDM induced airway inflammation in C57BL/6 mice is associated with a distinct elevation of multiple LOX-derived oxylipins in BALF. (**Paper III**)
- CysLTs may have a different role and regulation in mice compared to humans. (**Paper III**)
- OCS treatment does not decrease release of eicosanoids in severe asthma, which highlights the need for alternative targets for treatment. (**Paper IV**)
- Female-high BMI phenotype has high levels of isoprostanes and PGD₂. (**Paper IV**)
- Clustering of urine eicosanoid profiles contains sufficient resolution to distinguish clinically relevant sub-phenotypes of asthma. (**Paper IV**)

7 GENERAL DISCUSSION

Airway inflammation is a central pathobiological phenomenon in severe asthma. It causes severe impairment of lung function and results in hyperreactivity and variable airway obstruction. It is well known that multiple lipid mediators contribute to these events and therefore constitute targets for drug treatment. However, since not all asthma patients respond to current treatments, increased knowledge about oxylipin profiles among uncontrolled patients can allow better diagnosis to be established and perhaps in the future lead to better choice of treatment.

As to the presented aims of this thesis, the principle results can be divided into two parts, methodological and biological. The methodological achievements were significant in **Paper I** and **II**, where the greatest effort was centered around the development of LC-MS and LC-MS/MS methods, but also to develop an interdisciplinary workflow that enabled biochemical and physiological measurements from lung tissue to be combined. The second achievement focused on the utility of bioanalytical methods to characterize and describe changes in endogenous inflammatory mediators in urine, BALF and organ baths following inflammatory cell activation, airway inflammation and bronchoconstriction. The developed methods have been utilized in a broad research environment that comprise *in vitro* and *in vivo* models of human, guinea pig and mice lung tissue. In addition, the utility of non-invasive urine samples, from clinical phase I and II studies and a cross-sectional research study, has been examined.

Using urinary *tele*-MIAA as a safety biomarker was successfully demonstrated in a clinical trial program monitoring the global systemic histamine turnover. Compared to an earlier study where no gender differences were seen, our data support that females excrete more histamine than men from three independent clinical studies, but it is unclear what causes this difference and if it has clinical relevance (Granerus et al., 1999a). With large increases in urinary *tele*-MIAA following mast cell activation, this gender difference probably has a negligible effect. However, limiting a high intake of histamine rich food stuffs is suggested to be important during repeated sampling schemes where excreted concentrations approach normal baseline levels.

The methods characterised in **Paper II** were able to quantify 130 different oxylipins and included a chiral separation method for monohydroxy fatty acids, **Figure 1, Paper II**. From one single extracted sample, three injections could be made from only 70 μ L of reconstituted sample extracts. The reversed phase separation methods in *Method A* had a 17.5 min cycle time, which is slower than a previously published method adopted in 6 min (Song et al., 2013). However, shortening the cycle time generates more co-eluting peaks that can lead to inaccurate results. A technical improvement was the use of an automated SPE system where positive pressure was used, rather than negative pressure or gravity. Although experiments were conducted to demonstrate improved reproducibility by this automation, no difference in CV's was observed. Instead, the total processing time was decreased to 105 min, as compared to manual extractions taking 130 min.

Previous functional experiments in the organ bath setting have verified the release of a few oxylipins, such as TXs, CysLTs and PGs (Dahlén et al., 1983). With our developed *Method A-C*, a large increase in total number of oxylipins (130) was possible to detect. This expands the utility. Furthermore, a release of 57 oxylipins from human tissue and 42 from guinea pig was observed after mast cell stimulation. In human lung tissue, inhibition of the primary COX and FLAP/5-LOX resulted in altered smooth muscle tone while significant biochemical PG and LT pathways changed accordingly. The same basic functional and biochemical changes were confirmed in the guinea pig trachea. Of interest, the FLAP/5-LOX

induced changes of the CYP products EpETrEs and EpOMEs deserve further investigation because these epoxides may have an active role in several inflammatory diseases (Wagner et al., 2017). Another interesting finding from human bronchi was the elevated PGD₂ and its metabolites following incubation of bronchi in tissue media for 24 hours, suggesting that mast cells have a prolonged activation state.

During inflammation, infiltrating leukocytes exert their actions in part by the release of oxidative species. Although isoprostanes are considered markers of free radical induced lipid peroxidation/oxidative reactions several of the PUFA-derived oxylipins can be formed by autoxidation, wherein equal amounts of the *R* and *S*-enantiomer is produced (Mazaleuskaya et al., 2018). However, both 5-LOX and 15-LOX enzymes stereo-selectively produce hydroxylated lipids with the *S*-configuration. For the first time, the chirality of organ bath released monohydroxy fatty acids from DHA and AA was shown by using *Method C* (**Figure 4, Table 1, Paper II**). In the same organ bath experiments, 9- and 13-HODE together with 5-, 11- and 15-HETE were shown to be products of autoxidation. It is concluded that calculated enantiomeric excess increase our understanding of biosynthetic production of specific oxylipins and therefore remain to be a fundamental aspect to consider in order to define biochemical mechanisms.

In asthma, eosinophilic inflammation is a hallmark of the type 2 inflammatory response. The murine BALB/c strain is commonly used to obtain a type 2 inflammatory response, while the C57BL/6 strain is more skewed towards type 1. However, our repeated intranasal HDM exposure in the C57BL/6 successfully demonstrated infiltration of eosinophils, together with elevated *Tnf*, *Il10* and *Il13* mRNA transcripts showing that HDM triggers both type 1 and a type 2 response. By using this HDM model and applying *Method A* and *B*, a comprehensive pattern of altered oxylipins described the total presence of 57 oxylipins in BALF, of which 26 were strongly elevated with HDM exposure. Although a release pattern is informative, it is still purely descriptive, because no intervention was added. However, to further elucidate the reason for the many elevated oxylipins in BALF, whole lung tissue mRNA was investigated to answer whether an elevation of the responsible oxylipin producing enzymes could be the cause. We found no such evidence and concluded that increased levels of oxylipins must be attributed to either more infiltrating cells, or an enhanced level of cellular activation. One weakness in this conclusion is that the corresponding proteins in the lung tissue were not quantified.

Furthermore, the observed excessive production of LTB₄ is a strong pro-inflammatory signal because it promotes neutrophil aggregation, chemotaxis, and superanion oxide production. This finding would suggest a prompt oxidative environment and induction of isoprostanes. However, of the 5 isoprostanes that could be detected, only 5-iPF_{2α}-VI was present. A more detailed assessment of free-radical induced peroxidation could preferably have been done by applying chiral separation of monohydroxy fatty acids using *Method C*, but at the time of analysis *Method C* was not established.

Of interest, further investigative studies in the murine allergic asthma model would preferably be to evaluate the effect of specific interventions, such as by inhibiting oxylipin producing enzymes or their receptors, or to add oxylipins with anti-inflammatory, or pro-resolving, properties to study resolution of inflammation. Several oxylipins that are included in *Method A* have pro-resolving properties and may therefore be more likely to be detected during a recovery phase where animals are not exposed to HDM. By including such a group in parallel, wherein animals are sacrificed at a later time point of for example 3-7 days, an altered release profile in BALF may reveal a switch from oxylipins with pro-inflammatory properties into those which counteract inflammation, or even promote resolution. Specialized

pro-resolving lipid mediators (SPM) are a newly described group of oxylipins capable of reducing infiltrating leukocytes, increasing macrophage efferocytosis and thereby actively promote a return to tissue homeostasis (Levy and Serhan, 2014). Recent data also suggest that this group of oxylipins may in part be involved in failure to resolve airway inflammation in asthmatics (Barnig et al., 2018). Although the established platform in **Paper II** is capable of quantifying 6 different SPMs, further development of specific SPM extraction methods, together with mass spectrometry detection, is encouraged. Collectively, this would enable the later phase of the inflammatory process to be studied in greater detail.

It is increasingly recognized that patients with severe asthma respond insufficiently to treatment and have inadequate control of their symptoms. There exists an unmet need to identify and develop new clinical and molecular markers of the disease. The use of type 2 markers has substantially contributed to treat one subgroup of asthmatics, but still, a substantial number of subjects present a different endotype, or fall in between type 2 and non-type 2. While the call for improved clinical evaluation and molecular characterization of subjects with asthma is most apparent, the eicosanoid panel explored in **Paper IV** constitute a significant contribution to molecular sub-phenotyping that may help reshape current clinical views and improve diagnosis.

The U-BIOPRED study enabled subjects with mild-to-moderate and severe asthma to be extensively evaluated and the release profiles of 11 urine eicosanoid metabolites from five enzymatic and one non-enzymatic pathway were defined. Until now, baseline values most often originated from smaller studies with fewer subjects included and with often only one representative eicosanoid per pathway. In fact, HC subjects in U-BIOPRED provided the first large reference cohort of expected normal excretion levels in spot urine and evidenced release of LTs to be very low during non-inflamed conditions. With increasing asthma severity, the end-product of CysLTs, LTE₄, demonstrated the most significant increase with asthma severity among the 11 included metabolites, and in some subjects reached urinary levels 50 times higher than the median baseline value in healthy controls.

The associated link between subjects with high LTE₄ (and tetranorPGDM) and high levels of type 2 markers further evidence the involvement of mast cells in asthma. LTE₄ not only promotes eosinophil recruitment, but was recently attributed extended properties as it was shown to stimulate mast cells during allergen challenge (Lazarinis et al., 2018).

Corticosteroids down regulate important immune functions and are generally effective in patients with eosinophilic asthma. Also, inhaled steroids have been shown to reduce mast cell numbers in the airway smooth muscle and epithelium of asthmatics, but not in the submucosa (James et al., 2012). An objective measure of adherence to steroid treatment has not been previously reported, but was addressed in the U-BIOPRED study. However, only a minor difference was observed in two urinary eicosanoid metabolite levels when SA subjects were stratified according to reported OCS use, or when a positive detection of urinary prednisolone was added as a combined criterion. This extends and confirms previous data supporting that most eicosanoid pathways per se are relatively steroid insensitive (Gyllfors et al., 2006; O'Shaughnessy et al., 1993). However, reduction in cell numbers with long term treatment may indirectly affect eicosanoid metabolite levels.

With respect to the aims of the U-BIOPRED study, unbiased consensus clustering demonstrated that urinary eicosanoid metabolites contain sufficient resolution to subdivide asthmatics. Furthermore, the clinical and biochemical properties of cluster U₂ to U₄ partly overlapped with previously reported clinical clustering (Haldar et al., 2008; Lefaudeux et al.,

2016; Wu et al., 2014). Perhaps most clear was the contribution of LTE₄, PGD₂ metabolites and isoprostanes to these clusters.

It has been reported that elevated BMI is associated with reduced levels of F_ENO (Holguin et al., 2013; Komakula et al., 2007; van Veen et al., 2008). Cluster analysis in the studies by Haldar et al., SARP and U-BIOPRED have also reported a subgroup consisting of females with high BMI, late onset of disease, being associated with reduced F_ENO and poor asthma control, despite reporting use of high doses of oral steroids (Haldar et al., 2008; Lefaudeux et al., 2016; Moore et al., 2010). Interestingly, our cluster U₃ attained a similar description, with the addition of markedly elevated PGD₂ metabolites, isoprostanes and LTE₄ together with the highest CRP values. It has also been suggested that this phenotype may have a shift in ω 6/ ω 3 diet (Holguin and Fitzpatrick, 2010; Wendell et al., 2014). Even though 82% of the subjects in our U₃ cluster were females, tetranorPGEM was not the eicosanoid causing the separation of this cluster. While subjects in cluster U₂ presented clear type 2 cellular and molecular signatures, subjects in cluster U₄ had more characteristics of chronic obstructive pulmonary disease (COPD) by the low FEV₁, FEV₁/FVC ratio, latest age of onset, reporting the highest dose of OCS and more frequent osteoporosis.

Interestingly, the two clusters having 27 and 29% MMA subjects presented some small differences in terms of gender, type 2 signature, age of onset, ACQ-5 and OCS usage. Their levels of urinary eicosanoid metabolites were closer to the HC group, but differed by lower TXs in U₁ and lower PGs in U₅. A similar clinical description was presented for two of the clusters published by Haldar et al.

In follow-up studies the predictive power of urine eicosanoid metabolites, together with cluster significant clinical variables, should be tested to further validate those findings, preferably using different class discovery algorithms. The longitudinal follow-up of clinical status supported that the recruited severe asthma group were stable. Similarly, the reproduced eicosanoid pathway profiles, contributing to the clusters, were in principle maintained in the urine, **Figure 6E-F, Paper IV**. However, median SA concentration of 4 out of 11 eicosanoid metabolites was different, of which 3 belonged to the structural family of PGF_{2 α} -related metabolites. This could best be re-evaluated by a paired analysis of both samples (baseline and longitudinal). In addition, it is suggested to further evaluate the observed intra-individual variability, which varied depending on eicosanoid metabolite and specific cluster, by taking clinical and other biomarkers into consideration.

8 FUTURE PERSPECTIVES

After many years of describing and treating asthma as a single disease, it is now well appreciated that patients with asthma present different clinical and molecular signatures (Wenzel, 2016). However, it has also been demonstrated that a significant overlap between those signatures is prominent. This has led to an emerging need to find better markers of disease and treatment response.

In this thesis, in addition to the basic research studies aimed to define oxylipin release in tissue preparations and in the chronic allergic asthma model in mice, a substantial effort has been made to demonstrate the importance and utility of eicosanoids in asthma. Clearly, our data support that eicosanoid production increases with asthma severity and is capable of distinguishing clinically relevant subgroups. However, the eicosanoid metabolite signals in the clinical setting also demonstrate significant overlap. To address this problem, a next step is to characterize the diagnostic accuracy of these metabolite markers in validation experiments including repeated measurements over time. Such an experiment would further describe the consistency in the excreted eicosanoid patterns and enable the assessment of individual variability.

In addition, repeated collection of urine samples would allow improved understanding of daily to monthly fluctuations, while variability in clinical symptoms is recorded. Furthermore, to address the need for better markers of treatment response, our urinary metabolite panel could be further explored when evaluating drug targets during drug development. Also, it may be that improved diagnostic accuracy is achieved by the inclusion of additional markers, thereby recreating a more complex panel of measured variables of different molecular origin, not limited to eicosanoid metabolism.

It was apparent that when comparing the urinary clusters, the concentration distribution of eicosanoids metabolites is largely overlapping. If this is due to spot urine sampling, or simply to a diverse range of inflammatory cell activation, is unclear. In future studies, it would be helpful to include eosinophil activation markers, such as EPX and ECP. It is unlikely that a single eicosanoid variable will reach sufficient predictive power to distinguish individual patients. Instead, additional molecular and clinical markers will be necessary in order to increase selectivity and statistical power. The reality of molecular sub-phenotyping is that a diverse set of markers will be required in order to deconvolute complex heterogeneous diseases and accurately segregate different cellular and molecular processes. Indeed, a personalized approach to diagnose and treat asthma is the way towards achieving improved asthma control and reduced inflammation in the airways. Large-scale integration of clinical and molecular variables (*i.e.*, system medicine) is therefore one way to identify an optimal set of predictive variables.

While the utility of the bioanalytical method employed in **Paper IV** was demonstrated in the context of population-based molecular phenotyping, our published workflow (**Paper II**) allows a comprehensive number of inflammatory mediators to be screened in controlled experimental models. We applied them in guinea pig and mice models, but the bioanalytical methods may of course be used in many other models. Varying sensitisation/exposure protocols and sampling schemes will enable anti-inflammatory and pro-resolving hypothesis testing before proceeding to clinical evaluation. One such experiment would be to use a LOX knock-out in the C57BL/6 mice to characterise the associated cellular and molecular changes. Of further interest, is how changes in inflammatory events track with time in HDM induced eosinophilic inflammation, such as the progression from an acute to a chronic inflammatory state.

There are lifestyle factors contributing to clinical asthma that have not been addressed in the presented studies. Two such important factors are dietary intake of fatty acids (*e.g.*, $\omega 3$ and $\omega 6$) and dietary glycemic load. The dietary ratio of $\omega 6/\omega 3$ has been linked to multiple diseases with ongoing chronic inflammation (Calder, 2011; Simopoulos, 2008). With the ability to quantify 130 oxylipins, new opportunities exist to investigate dietary interventions with greater resolution, thereby enabling new molecular pathways to be associated with dietary effects.

One may think that inflammation happens only occasionally, but in fact chronic inflammation is increasingly recognised as an underlying, or main, cause of several diseases in which PUFAs are suggested to play a role (Minihane et al., 2015). In this thesis, inflammation and bronchoconstriction in the airways served as the primary biological events under investigation. The properties of the samples selected for the current studies guided the development of the bioanalytical methods described herein. However, the utility of the methods extends beyond that of urine, BALF or Krebs buffer and may be used for the analysis of the same compounds in plasma, sputum, lung tissue biopsies, cell cultures or exhaled breath condensates. The latter being another non-invasive sampling technique able to directly reflect inflammatory processes in the lung (Sanak et al., 2011). The choice of matrix will most likely differ, depending on research question and analyte of interest.

Finally, it is difficult to overstate the importance of research into the fundamental role of PUFA-associated biology in the inflammatory process. For decades, research involving the study of PUFAs and their many metabolites has provided important knowledge, that for example has led to effective anti-inflammatory treatments (*e.g.*, NSAIDs, low dose aspirin, anti-leukotrienes and prostacyclin). As chronic inflammation is a growing phenomenon in the human population, it has been recognised as one important factor in multiple diseases, and as such, the bioanalytical methods developed in this thesis, primarily for inflammation in the airways, could have a more widespread area of usefulness in other inflammatory diseases. The different innate immune functions we have are the result of an evolutionary preserved host defense mechanism that protects us. They are fundamental, but complex, biological processes under strict regulation. In view of this, histamine, oxylipins and related eicosanoids are control systems of considerable importance. Low molecular analytes are exciting entities, but are technically advanced to capture. However, their role and function in immunology cannot be emphasized enough and will keep us motivated for continued future work. To quote Roy J Sobermans statement published in Journal of Clinical Investigation 2003:

“...the combination of 20 carbons and four unsaturated double bonds has proved to be one of the most flexible molecular combinations yet described and has provided the stimulus for over 50 years of intense investigation. As more roles and interactions for arachidonic acid products are identified, it is clear that this molecule will remain an integral component of biomedical research for at least another 50 years.”

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Astma är en lungsjukdom som förekommer bland ca 10% av befolkningen. Astma har en komplex sjukdomsbild där både frekvensen och graden av symptom varierar över tid, vilket gör det svårt att ställa diagnos. Eftersom astma kännetecknas av inflammation i luftvägarna, och i vissa fall överkänslighet, är en av behandlingsstrategierna vid astma att dämpa inflammationen med hjälp av inhalerade, och/eller orala kortikosteroider. Vid en akut astmaattack försvåras andningen påtagligt då glattmuskulaturen drar ihop luftvägarna så att mindre luft kommer ned i lungorna, vilket försämrar gasutbytet och minskar syrenivån. Detta fenomen utgör den andra viktiga behandlingsformen vid akut luftrörsobstruktion, varvid luftrörsvidgande medicin ges, av typen β 2-stimulerare, som relaxerar glattmuskeln.

En subgrupp av astmatiker har en mer svårbehandlad astma och kräver högre doser eller ytterligare tilläggsbehandling. Trots ibland höga doser av kortikosteroider, tillsammans med annan tilläggsbehandling, saknar dessa patienter en fungerande kontroll på sina symptom och upplever en försämrad livskvalitet. Dessa patienter uppsöker oftare akut sjukhusvård. På grund av de många olika symptom som astmatiker uppvisar är det idag ännu oklart vilka molekylära mekanismer som kan tänkas ligga bakom varje enskilt fall. Det finns också en stor okunskap kring vad som molekylärt skiljer en patient med svårbehandlad astma från en annan. Förutom att lungfunktionen är påtagligt sämre vet man att komplexa inflammatoriska processer i luftvägarna är viktiga bakomliggande faktorer. Följaktligen finns ett stort behov av att bättre kunna diversifiera och diagnosticera astmatiker utifrån nya molekylära mätvariabler och med hjälp av nya metoder.

En del inflammatoriska processer kan idag bara delvis förklaras av att ett förhöjt antal immunförsvarsceller, så som mastceller, eosinofiler och neutrofiler, som infiltrerar luftvägarna, eller att de cellerna utsätts för en ökad stimulering och därmed aktivering. Vid dessa olika aktiveringar frisätts potenta inflammatoriska signaler (mediatorer) så som histamin eller fettsyramolekylerna prostaglandiner och cysteinyl-leukotriener (CysLT) vilka orsakar både inflammation i, och kontraktion av, luftvägarna. En central fråga i dessa processer är; vilka celler och signaler är det som initierar och reglerar inflammation, samt vilka har en direkt, eller indirekt, påverkan på luftrörskontraktion?

I denna avhandling har arbetet fokuserat på att utveckla känsliga metoder för att mäta ett stort antal inflammatoriska signaler som frisätts från immunologiska och strukturella celler med hjälp av vätskekromatografi kopplat till mass-spektrometri (LC-MS). I det första (I) delarbetet utvecklades en metod för att mäta halten av den totala histaminfrisättningen indirekt, via den metabolite som histamine bryts ned till. Metoden kunde användas för att påvisa frånvaro av anafylaktiska reaktioner i kliniska studier på människa och samtidigt påvisa en högre histaminfrisättning vid systemisk mastcellsaktivering.

I det andra (II) delarbetet utvecklades en metod för att kunna mäta 130 fettsyramolekyler. Analysmetoden kunde identifiera en frisättning av 57, respektive 42, olika ω 3- och ω 6-fettsyrametaboliter från isolerad human (samt marsvin) lungvävnad efter allergisk stimulering. Anti-inflammatorisk behandling med COX och FLAP/5-LOX hämmare reducerade nivån av fettsyror producerade från dessa två enzymer och påverkade glattmuskeltonus. Kombinerad blockering av de två enzymerna reducerade nivåerna av utsöndrade pro-inflammatoriska fettsyror och reducerade glattmuskeltonus med ca 40 %.

I det tredje (III) delarbetet etablerades en astmamodell i mus, där humant kvalster initierade eosinofil inflammation och hyper-reaktivitet i luftvägarna. En förhöjd koncentration av flertalet lipoxygenasfettsyror med både pro- och anti-inflammatoriska

egenskaper påvisades, dock utan förändring av de basalt höga nivåerna av CysLTs, vilket indikerar en annan fysiologisk roll för CysLTs i mus än i människa.

I delarbete **IV** analyserades urinprover från svårbehandlade astmatiker i en stor Europeisk studie (U-BIOPRED) som syftade till att hitta subgrupper av astmatiker som kunde grupperas utifrån ett molekylärt perspektiv. Patientprofiler baserat på 11 pro-inflammatoriska eicosanoider utsöndrade i urinen användes för klusteranalys där 5 kliniskt relevanta subgrupper kunde identifieras med olika lungfunktion, könsfördelning, eosinofil infiltration, CRP koncentration samt självuppskattad symtomkontroll och livskvalitet.

Sammanfattningsvis redovisar avhandlingens resultat en metod för att mäta histaminfrisättningen i samband med mastcellsaktivering i kliniska studier med hög tillförlitlighet. Resultaten visar också hur ett stort antal ω 3- och ω 6-fettsyrametaboliter medierar kontraktion av glattmuskelvävnad samt inflammation i luftvägarna. Därutöver kunde en pro-inflammatorisk fettsyrametabolit-profil i urinen identifiera olika molekylära subgrupper bland astmatiker som också påvisade ett unikt kliniskt uttryck.

Slutligen, bioanalytiska mätmetoder för bestämning av inflammatoriska mediatorers förekomst i biologiska system möjliggör identifiering av biologiska mekanismer i astmamodeller samt identifiering av nya molekylära subgrupper av astmatiker som har ett unikt kliniskt relevant uttryck.

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11 REFERENCES

- Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *The Lancet* 2008;372:1107–19. doi:10.1016/S0140-6736(08)61452-X.
- Asokanathan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, et al. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol Baltim Md 1950* 2002;169:4572–8.
- Balgoma D, Larsson J, Rokach J, Lawson JA, Daham K, Dahlén B, et al. Quantification of lipid mediator metabolites in human urine from asthma patients by electrospray ionization mass spectrometry: controlling matrix effects. *Anal Chem* 2013;85:7866–74. doi:10.1021/ac401461b.
- Balgoma D, Yang M, Sjödin M, Snowden S, Karimi R, Levänen B, et al. Linoleic acid-derived lipid mediators increase in a female-dominated subphenotype of COPD. *Eur Respir J* 2016;47:1645–56. doi:10.1183/13993003.01080-2015.
- Bankova LG, Lai J, Yoshimoto E, Boyce JA, Austen KF, Kanaoka Y, et al. Leukotriene E4 elicits respiratory epithelial cell mucin release through the G-protein-coupled receptor, GPR99. *Proc Natl Acad Sci U S A* 2016;113:6242–7. doi:10.1073/pnas.1605957113.
- Barnig C, Frossard N, Levy BD. Towards targeting resolution pathways of airway inflammation in asthma. *Pharmacol Ther* 2018;186:98–113. doi:10.1016/j.pharmthera.2018.01.004.
- Bell MC, Busse WW. Severe Asthma: An Expanding and Mounting Clinical Challenge. *J Allergy Clin Immunol Pract* 2013;1:110–21. doi:10.1016/j.jaip.2013.01.005.
- Bigler J, Boedigheimer M, Schofield JPR, Skipp PJ, Corfield J, Rowe A, et al. A Severe Asthma Disease Signature from Gene Expression Profiling of Peripheral Blood from U-BIOPRED Cohorts. *Am J Respir Crit Care Med* 2017;195:1311–20. doi:10.1164/rccm.201604-0866OC.
- Black JL, Johnson PRA. Factors controlling smooth muscle proliferation and airway remodelling. *Curr Opin Allergy Clin Immunol* 2002;2:47–51.
- Bousquet J. Global initiative for asthma (GINA) and its objectives. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* 2000;30 Suppl 1:2–5.
- Bradding P, Arthur G. Mast cells in asthma - state of the art. *Clin Exp Allergy* 2016;46:194–263. doi:10.1111/cea.12675.
- Brash AR, Jackson EK, Saggese CA, Lawson JA, Oates JA, FitzGerald GA. Metabolic disposition of prostacyclin in humans. *J Pharmacol Exp Ther* 1983;226:78–87.
- Buczynski MW, Dumlao DS, Dennis EA. Thematic Review Series: Proteomics. An integrated omics analysis of eicosanoid biology. *J Lipid Res* 2009;50:1015–38. doi:10.1194/jlr.R900004-JLR200.
- Busse WW, Lemanske RF, Gern JE. Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet Lond Engl* 2010;376:826–34. doi:10.1016/S0140-6736(10)61380-3.
- Calder PC. Fatty acids and inflammation: The cutting edge between food and pharma. *Eur J Pharmacol* 2011;668:S50–8. doi:10.1016/j.ejphar.2011.05.085.

- Canning BJ, Chou Y. Using guinea pigs in studies relevant to asthma and COPD. *Pulm Pharmacol Ther* 2008;21:702–20. doi:10.1016/j.pupt.2008.01.004.
- Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014;43:343–73. doi:10.1183/09031936.00202013.
- Cowan DC, Taylor DR, Peterson LE, Cowan JO, Palmay R, Williamson A, et al. Biomarker-based asthma phenotypes of corticosteroid response. *J Allergy Clin Immunol* 2015;135:877–883.e1. doi:10.1016/j.jaci.2014.10.026.
- Daham K, James A, Balgoma D, Kupczyk M, Billing B, Lindeberg A, et al. Effects of selective COX-2 inhibition on allergen-induced bronchoconstriction and airway inflammation in asthma. *J Allergy Clin Immunol* 2014;134:306–13. doi:10.1016/j.jaci.2013.12.002.
- Dahlén SE, Hansson G, Hedqvist P, Björck T, Granström E, Dahlén B. Allergen challenge of lung tissue from asthmatics elicits bronchial contraction that correlates with the release of leukotrienes C4, D4, and E4. *Proc Natl Acad Sci U S A* 1983;80:1712–6.
- Dahlén SE, Hedqvist P, Hammarström S, Samuelsson B. Leukotrienes are potent constrictors of human bronchi. *Nature* 1980;288:484–6.
- Dahlén SE, Kumlin M. Can asthma be studied in the urine? *Clin Exp Allergy J Br Soc Allergy Clin Immunol* 1998;28:129–33.
- Dahlén SE, Kumlin M, Granström E, Hedqvist P. Leukotrienes and other eicosanoids as mediators of airway obstruction. *Respir Int Rev Thorac Dis* 1986;50 Suppl 2:22–9.
- De Meulder B, Lefaudeux D, Bansal AT, Mazein A, Chaiboonchoe A, Ahmed H, et al. A computational framework for complex disease stratification from multiple large-scale datasets. *BMC Syst Biol* 2018;12:60. doi:10.1186/s12918-018-0556-z.
- Dennis EA, Norris PC. Eicosanoid storm in infection and inflammation. *Nat Rev Immunol* 2015;15:511–23. doi:10.1038/nri3859.
- Diczfalusy U, Alexson SE. Identification of metabolites from peroxisomal beta-oxidation of prostaglandins. *J Lipid Res* 1990;31:307–14.
- Dumlao DS, Buczynski MW, Norris PC, Harkewicz R, Dennis EA. High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylethanolamines. *Biochim Biophys Acta* 2011;1811:724–36. doi:10.1016/j.bbalip.2011.06.005.
- Duvall MG, Barnig C, Cernadas M, Ricklefs I, Krishnamoorthy N, Grossman NL, et al. Natural killer cell-mediated inflammation resolution is disabled in severe asthma. *Sci Immunol* 2017;2. doi:10.1126/sciimmunol.aam5446.
- von Euler US. On the specific vaso-dilating and plain muscle stimulating substances from accessory genital glands in man and certain animals (prostaglandin and vesiglandin). *J Physiol* 1936;88:213–34.
- Fadeel B, Ahlin A, Henter JI, Orrenius S, Hampton MB. Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. *Blood* 1998;92:4808–18.
- Fauland A, Köfeler H, Trötz Müller M, Knopf A, Hartler J, Eberl A, et al. A comprehensive method for lipid profiling by liquid chromatography-ion cyclotron resonance mass spectrometry. *J Lipid Res* 2011;52:2314–22. doi:10.1194/jlr.D016550.
- Feldberg W, Holden HF, Kellaway CH. The formation of lysocithin and of a muscle-stimulating substance by snake venoms. *J Physiol* 1938;94:232–48.

- Fireman P. Understanding asthma pathophysiology. *Allergy Asthma Proc* 2003;24:79–83.
- Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith MJ. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 1980;286:264–5.
- Fraselle S, De Cremer K, Coucke W, Glorieux G, Vanmassenhove J, Schepers E, et al. Development and validation of an ultra-high performance liquid chromatography-tandem mass spectrometry method to measure creatinine in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 2015;988:88–97. doi:10.1016/j.jchromb.2015.02.026.
- Frölich JC, Wilson TW, Sweetman BJ, Smigel M, Nies AS, Carr K, et al. Urinary prostaglandins. Identification and origin. *J Clin Invest* 1975;55:763–70. doi:10.1172/JCI107987.
- Fuchs B, Braun A. Improved mouse models of allergy and allergic asthma - Chances beyond ovalbumin. *Curr Drug Targets* 2008;9:495–502. doi:10.2174/138945008784533589.
- Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980;288:373–6.
- Granerus G, Lönnqvist B, Stenström M. No sex difference in the urinary excretion of the histamine metabolite methylimidazoleacetic acid (MeImAA) when corrected for creatinine excretion. *Inflamm Res Off J Eur Histamine Res Soc Al* 1999a;48 Suppl 1:S92-93.
- Granerus G, Lönnqvist B, Wass U. Determination of the histamine metabolite tele-methylimidazoleacetic acid and of creatinine in urine by the same HPLC system. *Inflamm Res Off J Eur Histamine Res Soc Al* 1999b;48:75–80.
- Granström E, Kindahl H, Swahn ML. Profiles of prostaglandin metabolites in the human circulation. Identification of late-appearing, long-lived products. *Biochim Biophys Acta* 1982;713:46–60.
- Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol* 2011;32:402–11. doi:10.1016/j.it.2011.06.006.
- Guglielmino K, Jackson K, Harris TR, Vu V, Dong H, Dutrow G, et al. Pharmacological inhibition of soluble epoxide hydrolase provides cardioprotection in hyperglycemic rats. *Am J Physiol-Heart Circ Physiol* 2012;303:H853–62. doi:10.1152/ajpheart.00154.2012.
- Gülen T, Möller Westerberg C, Lyberg K, Ekoff M, Kolmert J, Bood J, et al. Assessment of in vivo mast cell reactivity in patients with systemic mastocytosis. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* 2017;47:909–17. doi:10.1111/cea.12914.
- Gyllfors P, Dahlén S-E, Kumlin M, Larsson K, Dahlén B. Bronchial responsiveness to leukotriene D4 is resistant to inhaled fluticasone propionate. *J Allergy Clin Immunol* 2006;118:78–83. doi:10.1016/j.jaci.2006.03.040.
- Haeggström JZ, Funk CD. Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. *Chem Rev* 2011;111:5866–98. doi:10.1021/cr200246d.
- Haeggström JZ, Tholander F, Wetterholm A. Structure and catalytic mechanisms of leukotriene A4 hydrolase. *Prostaglandins Other Lipid Mediat* 2007;83:198–202. doi:10.1016/j.prostaglandins.2007.01.006.
- Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, et al. Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* 2008;178:218–24. doi:10.1164/rccm.200711-1754OC.

- Hamberg M, Hedqvist P, Rådegran K. Identification of 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) as a major metabolite of arachidonic acid in human lung. *Acta Physiol Scand* 1980;110:219–21. doi:10.1111/j.1748-1716.1980.tb06656.x.
- Hamberg M, Samuelsson B. On the metabolism of prostaglandins E₁ and E₂ in man. *J Biol Chem* 1971;246:6713–21.
- Hamberg M, Svensson J, Samuelsson B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci U S A* 1975;72:2994–8.
- Haworth O, Levy BD. Lipoxins, resolvins and protectins: new leads for the treatment of asthma. *Expert Opin Drug Discov* 2008;3:1209–22. doi:10.1517/17460441.3.10.1209.
- Hayashi H, Mitsui C, Nakatani E, Fukutomi Y, Kajiwara K, Watai K, et al. Omalizumab reduces cysteinyl leukotriene and 9 alpha,11 beta-prostaglandin F-2 overproduction in aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol* 2016;137:1585–7. doi:10.1016/j.jaci.2015.09.034.
- Heederik D, von Mutius E. Does diversity of environmental microbial exposure matter for the occurrence of allergy and asthma? *J Allergy Clin Immunol* 2012;130:44–50. doi:10.1016/j.jaci.2012.01.067.
- Henderson WR, Lewis DB, Albert RK, Zhang Y, Lamm WJ, Chiang GK, et al. The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J Exp Med* 1996;184:1483–94.
- Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev* 2011;242:205–19. doi:10.1111/j.1600-065X.2011.01030.x.
- Holgate ST, Djukanović R, Casale T, Bousquet J. Anti-immunoglobulin E treatment with omalizumab in allergic diseases: an update on anti-inflammatory activity and clinical efficacy. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* 2005;35:408–16. doi:10.1111/j.1365-2222.2005.02191.x.
- Holguin F, Comhair SAA, Hazen SL, Powers RW, Khatri SS, Bleecker ER, et al. An association between L-arginine/asymmetric dimethyl arginine balance, obesity, and the age of asthma onset phenotype. *Am J Respir Crit Care Med* 2013;187:153–9. doi:10.1164/rccm.201207-1270OC.
- Holguin F, Fitzpatrick A. Obesity, asthma, and oxidative stress. *J Appl Physiol Bethesda Md* 1985 2010;108:754–9. doi:10.1152/jappphysiol.00702.2009.
- Jackson J. *A User's Guide to Principal Components*. vol. 1991. Wiley; 1991.
- James A, Gyllfors P, Henriksson E, Dahlén SE, Adner M, Nilsson G, et al. Corticosteroid treatment selectively decreases mast cells in the smooth muscle and epithelium of asthmatic bronchi. *Allergy* 2012;67:958–61. doi:10.1111/j.1398-9995.2012.02836.x.
- James A, Janson C, Malinovschi A, Holweg C, Alving K, Ono J, et al. Serum periostin relates to type-2 inflammation and lung function in asthma; data from the large population-based cohort Swedish GA(2)LEN. *Allergy* 2017. doi:10.1111/all.13181.
- Janssen LJ. Isoprostanes: an overview and putative roles in pulmonary pathophysiology. *Am J Physiol-Lung Cell Mol Physiol* 2001;280:L1067–82. doi:10.1152/ajplung.2001.280.6.L1067.
- Jevnikar Z, Östling J, Ax E, Calvén J, Thörn K, Israelsson E, et al. Epithelial IL-6 trans-signaling defines a new asthma phenotype with increased airway inflammation. *J Allergy Clin Immunol* 2018. doi:10.1016/j.jaci.2018.05.026.

- King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T. Mechanistic investigation of ionization suppression in electrospray ionization. *J Am Soc Mass Spectrom* 2000;11:942–50. doi:10.1016/S1044-0305(00)00163-X.
- Kita H. Eosinophils: multifaceted biological properties and roles in health and disease. *Immunol Rev* 2011;242:161–77. doi:10.1111/j.1600-065X.2011.01026.x.
- Knox AJ, Pang L, Johnson S, Hamad A. Airway smooth muscle function in asthma. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* 2000;30:606–14.
- Komakula S, Khatri S, Mermis J, Savill S, Haque S, Rojas M, et al. Body mass index is associated with reduced exhaled nitric oxide and higher exhaled 8-isoprostanes in asthmatics. *Respir Res* 2007;8:32. doi:10.1186/1465-9921-8-32.
- Kuhn H, Banthiya S, van Leyen K. Mammalian lipoxygenases and their biological relevance. *Biochim Biophys Acta* 2015;1851:308–30. doi:10.1016/j.bbali.2014.10.002.
- Kumlin M, Dahlén B, Björck T, Zetterström O, Granström E, Dahlén SE. Urinary excretion of leukotriene E4 and 11-dehydro-thromboxane B2 in response to bronchial provocations with allergen, aspirin, leukotriene D4, and histamine in asthmatics. *Am Rev Respir Dis* 1992;146:96–103. doi:10.1164/ajrccm/146.1.96.
- Kumlin M, Hamberg M, Granström E, Björck T, Dahlén B, Matsuda H, et al. 15(S)-hydroxyeicosatetraenoic acid is the major arachidonic acid metabolite in human bronchi: association with airway epithelium. *Arch Biochem Biophys* 1990;282:254–62.
- Kuo C-HS, Pavlidis S, Loza M, Baribaud F, Rowe A, Pandis I, et al. T-helper cell type 2 (Th2) and non-Th2 molecular phenotypes of asthma using sputum transcriptomics in U-BIOPRED. *Eur Respir J* 2017;49. doi:10.1183/13993003.02135-2016.
- Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol* 2015;16:45–56. doi:10.1038/ni.3049.
- Larsson N, Lundström SL, Pinto R, Rankin G, Karimpour M, Blomberg A, et al. Lipid mediator profiles differ between lung compartments in asthmatic and healthy humans. *Eur Respir J* 2014;43:453–63. doi:10.1183/09031936.00209412.
- Lazarinis N, Bood J, Gomez C, Kolmert J, Lantz A-S, Gyllfors P, et al. Leukotriene E4 induces airflow obstruction and mast cell activation through the cysteinyl leukotriene type 1 receptor. *J Allergy Clin Immunol* 2018. doi:10.1016/j.jaci.2018.02.024.
- Lefaudeux D, De Meulder B, Loza MJ, Peffer N, Rowe A, Baribaud F, et al. U-BIOPRED clinical adult asthma clusters linked to a subset of sputum -omics. *J Allergy Clin Immunol* 2016. doi:10.1016/j.jaci.2016.08.048.
- Lemanske RF, Busse WW. Asthma: clinical expression and molecular mechanisms. *J Allergy Clin Immunol* 2010;125:S95-102. doi:10.1016/j.jaci.2009.10.047.
- Leslie CC. Regulation of the specific release of arachidonic acid by cytosolic phospholipase A2. *Prostaglandins Leukot Essent Fatty Acids* 2004;70:373–6. doi:10.1016/j.plefa.2003.12.012.
- Levy BD, Serhan CN. Resolution of acute inflammation in the lung. *Annu Rev Physiol* 2014;76:467–92. doi:10.1146/annurev-physiol-021113-170408.
- Lewis RA, Soter NA, Diamond PT, Austen KF, Oates JA, Roberts LJ. Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE. *J Immunol Baltim Md* 1950 1982;129:1627–31.

- López IP, Rodríguez-de la Rosa L, Pais RS, Piñeiro-Hermida S, Torrens R, Contreras J, et al. Differential organ phenotypes after postnatal Igf1r gene conditional deletion induced by tamoxifen in UBC-CreERT2; Igf1r fl/fl double transgenic mice. *Transgenic Res* 2015;24:279–94. doi:10.1007/s11248-014-9837-5.
- Lum PY, Singh G, Lehman A, Ishkanov T, Vejdemo-Johansson M, Alagappan M, et al. Extracting insights from the shape of complex data using topology. *Sci Rep* 2013;3:1236. doi:10.1038/srep01236.
- Lundström SL, Yang J, Källberg HJ, Thunberg S, Gafvelin G, Haeggström JZ, et al. Allergic asthmatics show divergent lipid mediator profiles from healthy controls both at baseline and following birch pollen provocation. *PloS One* 2012;7:e33780. doi:10.1371/journal.pone.0033780.
- Macklem PT. The physiology of small airways. *Am J Respir Crit Care Med* 1998;157:S181-3. doi:10.1164/ajrccm.157.5.rsaa-2.
- Maekawa A, Kanaoka Y, Xing W, Austen KF. Functional recognition of a distinct receptor preferential for leukotriene E4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors. *Proc Natl Acad Sci U S A* 2008;105:16695–700. doi:10.1073/pnas.0808993105.
- Maltby NH, Taylor GW, Ritter JM, Moore K, Fuller RW, Dollery CT. Leukotriene C4 elimination and metabolism in man. *J Allergy Clin Immunol* 1990;85:3–9.
- Mancini JA, Abramovitz M, Cox ME, Wong E, Charleson S, Perrier H, et al. 5-lipoxygenase-activating protein is an arachidonate binding protein. *FEBS Lett* 1993;318:277–81.
- Mani AR, Moreno JC, Visser TJ, Moore KP. The metabolism and de-bromination of bromotyrosine in vivo. *Free Radic Biol Med* 2016;90:243–51. doi:10.1016/j.freeradbiomed.2015.11.030.
- Manning PJ, Stevens WH, Cockcroft DW, O’Byrne PM. The role of thromboxane in allergen-induced asthmatic responses. *Eur Respir J* 1991;4:667–72.
- Maric J, Ravindran A, Mazzurana L, Björklund ÅK, Van Acker A, Rao A, et al. Prostaglandin E2 suppresses human group 2 innate lymphoid cell function. *J Allergy Clin Immunol* 2018;141:1761-1773.e6. doi:10.1016/j.jaci.2017.09.050.
- Mazaleuskaya LL, Salamatipour A, Sarantopoulou D, Weng L, FitzGerald GA, Blair IA, et al. Analysis of HETEs in human whole blood by chiral UHPLC-ECAPCI/HRMS. *J Lipid Res* 2018;59:564–75. doi:10.1194/jlr.D081414.
- Metcalf DD, Pawankar R, Ackerman SJ, Akin C, Clayton F, Falcone FH, et al. Biomarkers of the involvement of mast cells, basophils and eosinophils in asthma and allergic diseases. *World Allergy Organ J* 2016;9. doi:10.1186/s40413-016-0094-3.
- Milne GL, Yin H, Hardy KD, Davies SS, Roberts LJ. Isoprostane generation and function. *Chem Rev* 2011;111:5973–96. doi:10.1021/cr200160h.
- Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, et al. Low-grade inflammation, diet composition and health: current research evidence and its translation. *Br J Nutr* 2015;114:999–1012. doi:10.1017/S0007114515002093.
- Miranda C, Busacker A, Balzar S, Trudeau J, Wenzel SE. Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. *J Allergy Clin Immunol* 2004;113:101–8. doi:10.1016/j.jaci.2003.10.041.

- Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A Large-Scale, Consortium-Based Genomewide Association Study of Asthma. *N Engl J Med* 2010;363:1211. doi:10.1056/NEJMoa0906312.
- Moncada S, Higgs EA, Vane JR. Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation. *Lancet Lond Engl* 1977;1:18–20.
- Monti S, Tamayo P, Mesirov J, Golub T. Consensus clustering: A resampling-based method for class discovery and visualization of gene expression microarray data. *Mach Learn* 2003;52:91–118. doi:10.1023/A:1023949509487.
- Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, et al. Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am J Respir Crit Care Med* 2000;162:1175–7. doi:10.1164/ajrccm.162.3.2001063.
- Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 2010;181:315–23. doi:10.1164/rccm.200906-0896OC.
- Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. *Jama-J Am Med Assoc* 2001;286:954–9. doi:10.1001/jama.286.8.954.
- Murphy RC, Barkley RM, Zemski Berry K, Hankin J, Harrison K, Johnson C, et al. Electrospray ionization and tandem mass spectrometry of eicosanoids. *Anal Biochem* 2005;346:1–42. doi:10.1016/j.ab.2005.04.042.
- Noonan MJ, Chervinsky P, Brandon M, Zhang J, Kundu S, McBurney J, et al. Montelukast, a potent leukotriene receptor antagonist, causes dose-related improvements in chronic asthma. Montelukast Asthma Study Group. *Eur Respir J* 1998;11:1232–9.
- Ono E, Dutilleul S, Kazani S, Wechsler ME, Yang J, Hammock BD, et al. Lipoxin generation is related to soluble epoxide hydrolase activity in severe asthma. *Am J Respir Crit Care Med* 2014;190:886–97. doi:10.1164/rccm.201403-0544OC.
- O'Shaughnessy KM, Wellings R, Gillies B, Fuller RW. Differential effects of fluticasone propionate on allergen-evoked bronchoconstriction and increased urinary leukotriene E4 excretion. *Am Rev Respir Dis* 1993;147:1472–6. doi:10.1164/ajrccm/147.6_Pt_1.1472.
- O'Sullivan S, Roquet A, Dahlén B, Dahlén S, Kumlin M. Urinary excretion of inflammatory mediators during allergen-induced early and late phase asthmatic reactions. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* 1998;28:1332–9.
- Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;327:524–6. doi:10.1038/327524a0.
- Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *Lancet Lond Engl* 2017. doi:10.1016/S0140-6736(17)33311-1.
- Patrono C, Ciabattoni G, Pugliese F, Pierucci A, Blair IA, FitzGerald GA. Estimated rate of thromboxane secretion into the circulation of normal humans. *J Clin Invest* 1986;77:590–4. doi:10.1172/JCI112341.
- Pavord ID, Beasley R, Agusti A, Anderson GP, Bel E, Brusselle G, et al. After asthma: redefining airways diseases. *Lancet Lond Engl* 2018;391:350–400. doi:10.1016/S0140-6736(17)30879-6.

- Piñeiro-Hermida S, Gregory JA, López IP, Torrens R, Ruíz-Martínez C, Adner M, et al. Attenuated airway hyperresponsiveness and mucus secretion in HDM-exposed Igf1r-deficient mice. *Allergy* 2017. doi:10.1111/all.13142.
- Rådmark O, Werz O, Steinhilber D, Samuelsson B. 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends Biochem Sci* 2007;32:332–41. doi:10.1016/j.tibs.2007.06.002.
- Rago B, Fu C. Development of a high-throughput ultra performance liquid chromatography-mass spectrometry assay to profile 18 eicosanoids as exploratory biomarkers for atherosclerotic diseases. *J Chromatogr B Analyt Technol Biomed Life Sci* 2013;936:25–32. doi:10.1016/j.jchromb.2013.08.001.
- Reinke SN, Gallart-Ayala H, Gómez C, Checa A, Fauland A, Naz S, et al. Metabolomics analysis identifies different metabolotypes of asthma severity. *Eur Respir J* 2017;49. doi:10.1183/13993003.01740-2016.
- Roberts LJ, Sweetman BJ. Metabolic fate of endogenously synthesized prostaglandin D2 in a human female with mastocytosis. *Prostaglandins* 1985;30:383–400.
- Roberts LJ, Sweetman BJ, Payne NA, Oates JA. Metabolism of thromboxane B2 in man. Identification of the major urinary metabolite. *J Biol Chem* 1977;252:7415–7.
- Rosenthal LA, Avila PC, Heymann PW, Martin RJ, Miller EK, Papadopoulos NG, et al. Viral Respiratory Infections and Asthma: the Course Ahead. *J Allergy Clin Immunol* 2010;125:1212. doi:10.1016/j.jaci.2010.04.002.
- Säfholm J, Manson ML, Bood J, Delin I, Orre A-C, Bergman P, et al. Prostaglandin E2 inhibits mast cell-dependent bronchoconstriction in human small airways through the E prostanoid subtype 2 receptor. *J Allergy Clin Immunol* 2015;136:1232-1239.e1. doi:10.1016/j.jaci.2015.04.002.
- Sagliani KD, Dolnikowski GG, Hill NS, Fanburg BL, Levy BD, Preston IR. Differences between basal lung levels of select eicosanoids in rat and mouse. *Pulm Circ* 2013;3:82–8. doi:10.4103/2045-8932.109918.
- Samuelsson B. ISOLATION AND IDENTIFICATION OF PROSTAGLANDINS FROM HUMAN SEMINAL PLASMA. 18. PROSTAGLANDINS AND RELATED FACTORS. *J Biol Chem* 1963;238:3229–34.
- Samuelsson B, Morgenstern R, Jakobsson P-J. Membrane prostaglandin E synthase-1: a novel therapeutic target. *Pharmacol Rev* 2007;59:207–24. doi:10.1124/pr.59.3.1.
- Sanak M, Gielicz A, Bochenek G, Kaszuba M, Nizankowska-Mogilnicka E, Szczeklik A. Targeted eicosanoid lipidomics of exhaled breath condensate provide a distinct pattern in the aspirin-intolerant asthma phenotype. *J Allergy Clin Immunol* 2011;127:1141-U400. doi:10.1016/j.jaci.2010.12.1108.
- Senbabaoglu Y, Michailidis G, Li JZ. Critical limitations of consensus clustering in class discovery. *Sci Rep* 2014;4:6207. doi:10.1038/srep06207.
- Serhan CN. Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. *FASEB J Off Publ Fed Am Soc Exp Biol* 2017;31:1273–88. doi:10.1096/fj.201601222R.
- Seyberth HW, Sweetman BJ, Frolich JC, Oates JA. Quantifications of the major urinary metabolite of the E prostaglandins by mass spectrometry: evaluation of the method's application to clinical studies. *Prostaglandins* 1976;11:381–97.

- Shaw DE, Sousa AR, Fowler SJ, Fleming LJ, Roberts G, Corfield J, et al. Clinical and inflammatory characteristics of the European U-BIOPRED adult severe asthma cohort. *Eur Respir J* 2015. doi:10.1183/13993003.00779-2015.
- Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med* 2008;233:674–88. doi:10.3181/0711-MR-311.
- Smith WL. The eicosanoids and their biochemical mechanisms of action. *Biochem J* 1989;259:315–24.
- Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* 2000;69:145–82. doi:10.1146/annurev.biochem.69.1.145.
- Song J, Liu X, Wu J, Meehan MJ, Blevitt JM, Dorrestein PC, et al. A highly efficient, high-throughput lipidomics platform for the quantitative detection of eicosanoids in human whole blood. *Anal Biochem* 2013;433:181–8. doi:10.1016/j.ab.2012.10.022.
- Song W-L, Wang M, Ricciotti E, Fries S, Yu Y, Grosser T, et al. Tetranor PGDM, an abundant urinary metabolite reflects biosynthesis of prostaglandin D2 in mice and humans. *J Biol Chem* 2008;283:1179–88. doi:10.1074/jbc.M706839200.
- Spector A, Yorek M. Membrane Lipid-Composition and Cellular Function. *J Lipid Res* 1985;26:1015–35.
- Strassburg K, Huijbrechts AML, Kortekaas KA, Lindeman JH, Pedersen TL, Dane A, et al. Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery. *Anal Bioanal Chem* 2012;404:1413–26. doi:10.1007/s00216-012-6226-x.
- Takahashi K, Pavlidis S, Ng Kee Kwong F, Hoda U, Rossios C, Sun K, et al. Sputum proteomics and airway cell transcripts of current and ex-smokers with severe asthma in U-BIOPRED: an exploratory analysis. *Eur Respir J* 2018;51. doi:10.1183/13993003.02173-2017.
- Takayama G, Arima K, Kanaji T, Toda S, Tanaka H, Shoji S, et al. Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *J Allergy Clin Immunol* 2006;118:98–104. doi:10.1016/j.jaci.2006.02.046.
- Tham EH, Lee AJ, Van Bever H. Aeroallergen sensitization and allergic disease phenotypes in Asia. *Asian Pac J Allergy Immunol* 2016;34:181–9. doi:10.12932/AP0770.
- Thomson SJ, Askari A, Bishop-Bailey D. Anti-inflammatory effects of epoxyeicosatrienoic acids. *Int J Vasc Med* 2012;2012:605101. doi:10.1155/2012/605101.
- Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;39:44–84. doi:10.1016/j.biocel.2006.07.001.
- van Veen IH, Ten Brinke A, Sterk PJ, Rabe KF, Bel EH. Airway inflammation in obese and nonobese patients with difficult-to-treat asthma. *Allergy* 2008;63:570–4. doi:10.1111/j.1398-9995.2007.01597.x.
- Wagener AH, de Nijs SB, Lutter R, Sousa AR, Weersink EJM, Bel EH, et al. External validation of blood eosinophils, FE(NO) and serum periostin as surrogates for sputum eosinophils in asthma. *Thorax* 2015;70:115–20. doi:10.1136/thoraxjnl-2014-205634.
- Wagner KM, McReynolds CB, Schmidt WK, Hammock BD. Soluble epoxide hydrolase as a therapeutic target for pain, inflammatory and neurodegenerative diseases. *Pharmacol Ther* 2017;180:62–76. doi:10.1016/j.pharmthera.2017.06.006.

Waternival WAH, Scheijen JLJM, Ortmans-Ploemen MMJC, Habets-van der Poel CD, Bierau J. Quantitative UPLC-MS/MS analysis of underivatized amino acids in body fluids is a reliable tool for the diagnosis and follow-up of patients with inborn errors of metabolism. *Clin Chim Acta Int J Clin Chem* 2009;407:36–42. doi:10.1016/j.cca.2009.06.023.

Wedes SH, Khatri SB, Zhang R, Wu W, Comhair SAA, Wenzel S, et al. Noninvasive markers of airway inflammation in asthma. *Clin Transl Sci* 2009;2:112–7. doi:10.1111/j.1752-8062.2009.00095.x.

Welsch DJ, Creely DP, Hauser SD, Mathis KJ, Krivi GG, Isakson PC. Molecular cloning and expression of human leukotriene-C4 synthase. *Proc Natl Acad Sci U S A* 1994;91:9745–9.

Wendell SG, Baffi C, Holguin F. Fatty acids, inflammation, and asthma. *J Allergy Clin Immunol* 2014;133:1255–64. doi:10.1016/j.jaci.2013.12.1087.

Wenzel SE. Emergence of Biomolecular Pathways to Define Novel Asthma Phenotypes. Type-2 Immunity and Beyond. *Am J Respir Cell Mol Biol* 2016;55:1–4. doi:10.1165/rcmb.2016-0141PS.

Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;18:716–25. doi:10.1038/nm.2678.

Wolfer AM, Gaudin M, Taylor-Robinson SD, Holmes E, Nicholson JK. Development and Validation of a High-Throughput Ultrahigh-Performance Liquid Chromatography-Mass Spectrometry Approach for Screening of Oxylipins and Their Precursors. *Anal Chem* 2015;87:11721–31. doi:10.1021/acs.analchem.5b02794.

Wood LG, Gibson PG, Garg ML. Biomarkers of lipid peroxidation, airway inflammation and asthma. *Eur Respir J* 2003;21:177–86.

Woodruff PG, Boushey HA, Dolganov GM, Barker CS, Yang YH, Donnelly S, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc Natl Acad Sci U S A* 2007;104:15858–63. doi:10.1073/pnas.0707413104.

Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* 2009;180:388–95. doi:10.1164/rccm.200903-0392OC.

Wu W, Bleecker E, Moore W, Busse WW, Castro M, Chung KF, et al. Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. *J Allergy Clin Immunol* 2014;133:1280–8. doi:10.1016/j.jaci.2013.11.042.

Yamada M, Kita Y, Kohira T, Yoshida K, Hamano F, Tokuoka SM, et al. A comprehensive quantification method for eicosanoids and related compounds by using liquid chromatography/mass spectrometry with high speed continuous ionization polarity switching. *J Chromatogr B Analyt Technol Biomed Life Sci* 2015;995–996:74–84. doi:10.1016/j.jchromb.2015.05.015.

Yang J, Schmelzer K, Georgi K, Hammock BD. Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem* 2009;81:8085–93. doi:10.1021/ac901282n.

Yu L, Liu Q, Canning BJ. Evidence for autocrine and paracrine regulation of allergen-induced mast cell mediator release in the guinea pig airways. *Eur J Pharmacol* 2018;822:108–18. doi:10.1016/j.ejphar.2017.11.017.

Zhang Y, Tingley FD 3rd, Tseng E, Tella M, Yang X, Groeber E, et al. Development and validation of a sample stabilization strategy and a UPLC-MS/MS method for the simultaneous quantitation of acetylcholine (ACh), histamine (HA), and its metabolites in rat cerebrospinal fluid (CSF). *J Chromatogr B Analyt Technol Biomed Life Sci* 2011;879:2023–33. doi:10.1016/j.jchromb.2011.05.030.