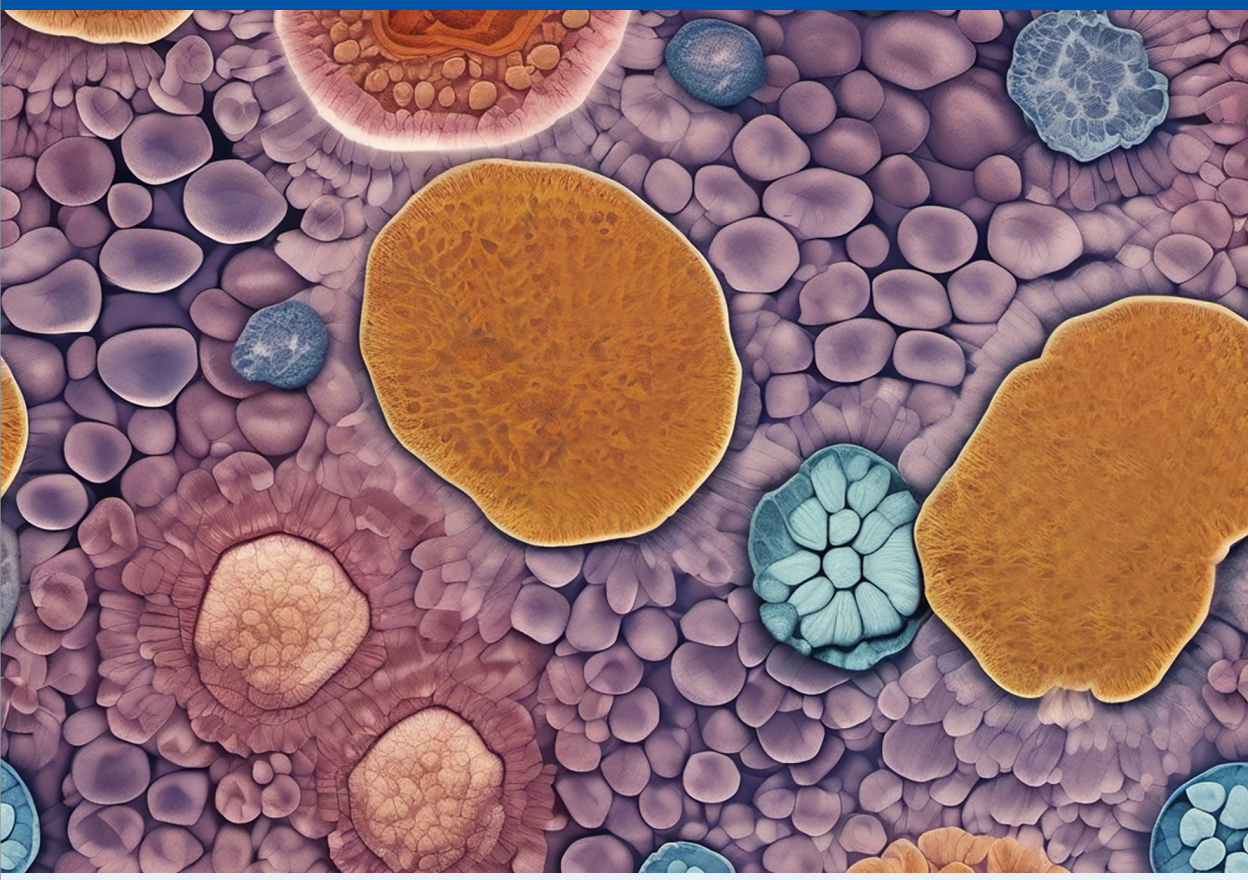
Thesis for doctoral degree (Ph. D.)
2024

Exploring eicosanoids as biomarkers in severe chronic rhinosinusitis with nasal polyps

AXEL NORDSTRÖM



SOPHIA HEMMET
UNIVERSITY

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AXEL NORDSTRÖM

Academic dissertation for the Degree of Doctor of Philosophy in Health Care Sciences at Sophiahemmet University will be defended in public

Wednesday, May 15 2024, at 9.30

in Erforssalen, House R, Sophiahemmet University, Valhallavägen 91, Stockholm

SOPHIA HEMMET
UNIVERSITY

Principal Supervisor:
Maria Kumlin, Professor
Department of Health Promoting Science,
Sophiahemmet University

Co-supervisor(s):
Marie Svedberg, Associate Professor
Department of Health Promoting Science,
Sophiahemmet University

Mattias Jangard, PhD
Department of Otorhinolaryngology,
Sophiahemmet Hospital

Michael Ryott, PhD
Department of Otorhinolaryngology,
Sophiahemmet Hospital

Opponent:
Gunnar Nilsson, Professor
Division of Immunology and Allergy,
Karolinska Institutet

Examination Board:
Per-Johan Jakobsson, Professor
Division of Rheumatology,
Karolinska Institutet

Mats Holmström, Professor
Department of CLINTEC, Division of ENT diseases, Karolinska Institutet

Ann-Charlotte Falk, Associate Professor
Department of Health Promoting Science,
Sophiahemmet University

EXPLORING EICOSANOIDS AS BIOMARKERS IN SEVERE CHRONIC RHINOSINUSITIS WITH NASAL POLYPS

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Sophiahemmet University Dissertations
No 5

Sophiahemmet University

ISBN 978-91-988733-8-2 (print)
ISBN 978-91-988733-9-9 (pdf)
ISSN 2004-7479 (print), 2004-7460 (online)

Published by Sophiahemmet
Printed in Sweden by Universitetsservice US-AB, Stockholm 2024

Till mommo och tammo

Populärvetenskaplig sammanfattning

Kronisk bihåleinflammation (eng: chronic rhinosinusitis; CRS) är ett av de mest vanligast förekommande långvariga inflammatoriska tillstånden. Att leva med CRS präglas av uttalade bihålebesvär med konstant nästäppa, rinnsnuva och luktbortfall. Historiskt har en uppdelning skett i CRS, med (eng: CRSwNP) eller utan näspolyper (CRSsNP). Näspolyper är sfäriska utväxter av nässlemhinnan som ytterligare försämrar livskvalitén, framförallt då en total avsaknad av lukt ofta ses. Kirurgisk borttagning av näspolyperna utförs, i många fall, upprepade gånger hos de med svårbehandlad CRSwNP på grund av att näspolyperna återutvecklas efter operation. Inflammationen i nässlemhinnan är oftast av typ 2. Nya så kallade biologiska läkemedel har utvecklats som riktar sig mot ämnen och vita blodkroppar som orsakar typ 2 inflammation. Olyckligtvis gagnas inte alla patienter av denna riktade behandling, och det har visat sig svårt att förutspå vilka som får lindring av dessa nya mediciner.

Eikosanoider bildas från fleromättade fettsyror och är biologiskt aktiva ämnen som fungerar som inflammatoriska budbärare. Flera studier visar att eikosanoider är involverade i utveckling och sjukdomsförlopp vid CRSwNP. Dessutom finns ett tydligt samband mellan en obalans i bildningen av vissa eikosanoider och typ 2 inflammation. Tidigare forskning har dock sällan betraktat eikosanoider som möjliga biomarkörer för CRSwNP. En biomarkör är en mätbar indikator på ett biologiskt skeende och används ofta för att diagnostisera eller följa sjukdomsförlopp.

Syftet med denna avhandling var att undersöka eikosanoider som potentiella biomarkörer och karaktärisera förändringar i hälsorelaterad livskvalitet samt graden av luktförlust hos patienter med svårbehandlad CRSwNP. Projektet innefattade immunologiska analyser av ett flertal inflammatoriska budbärare, inklusive ett urval av eikosanoider, i prover från nässlemhinna, i nässekret och i urin. Genom mätning i prov från nässlemhinna, gällande aktivitet av gener för enzymer och receptorer för eikosanoider, kunde ytterligare information fås om eikosanoidernas betydelse vid CRS. Hälsorelaterad livskvalitet bedömdes genom patienters svar på frågeformulären SNOT-22 och RAND-36. Dessutom utfördes patientnära analyser som mätning av vita blodkroppar, utandad kväveoxid och lukttest.

I det första delarbetet (**I**) visades att nivåer av eikosanoider i nässekret var associerade med en ökad polyptillväxt hos patienter med CRSwNP. Nasala

nivåer av en av eikosanoiderna, leukotrien E_4 (LTE_4), korrelerade dessutom med försämrad förmåga att känna lukt hos dessa patienter.

I det andra delarbetet (**II**) observerades en ökning i nivåer av nasalt LTE_4, från sex till 12 månader efter kirurgi, hos patienter med återkommande polyper (dvs, återväxt av polyper). Dessutom identifierades olika subgrupper av patienter och dessa karaktäriserades av distinkta förändringar i nivåer av eikosanoider. Patienter med återkommande polyper var klart vanligare i en av dessa subgrupper.

Även i delarbete **III** identifierades subgrupper av patienter. Skillnaden var att dessa subgrupper baserades på mätningar i slemhinneprover i stället för nässekret, men liknande förändringar i nivåer av eikosanoider kunde förknippas med återkommande polyper även här. Nivåer av eikosanoider i slemhinna och nässekret jämfördes sedan för varje patient och visade en relativt bra överenstämmelse mellan dessa prover. Detta resultat indikerar att undersökning av inflammation kan utföras i nässekret i stället för vävnadsprover, vilket är fördelaktigt då samling av nässekret inte kräver ett invasivt ingrepp.

I delarbete **IV** fann vi att patienter med återkommande näspolyper hade förhöjda nivåer av en viss typ av vit blodkropp (eosinofiler) i blod, och att deras luktsinne var betydligt försämrat både före och efter utförd bihåleoperation. Denna upptäckt antyder att luktförlust kan vara det första symtomet vid återfall av sjukdom i CRSwNP. Även om hälsorelaterad livskvalitet inte var påtagligt försämrad hos patienter med återkommande polyper jämfört med patienter utan återkommande polyper, så verkade graden av luktbortfall stämma väl överens med upplevd livskvalitet i patientgruppen. Detta bekräftar att förlust av luktsinne har en betydande inverkan på patientens hälsorelaterade livskvalitet vid CRSwNP.

Sammanfattningsvis bidrar resultaten från denna avhandling till en ökad förståelse för de karaktäristiska drag som är relevanta för att identifiera svårbehandlad CRSwNP. Kliniska faktorer som kan vara av största vikt att följa i sjukdomsförloppet inkluderar distinkta förändringar i nivåer av särskilda eikosanoider i bihålan, ett totalt luktbortfall och en ökad infiltration av eosinofiler. En undersökning av patients luktsinne och analys av sådana biomarkörer kan således vara användbara för att förutsäga snabb tillväxt av polyper och därmed identifiera fall av svårbehandlad CRSwNP. I förlängningen kan sådana förutsägelser vägleda i val av behandling för dessa patienter - kirurgi eller medicinsk behandling med de nya biologiska läkemedlen.

Abstract

Chronic rhinosinusitis (CRS) is one of the most common inflammatory chronic conditions, leading to a persistent nasal congestion, nasal discharge, and a loss of smell. Despite sinus surgery and frequent use of oral corticosteroids, a large proportion of individuals with CRS are difficult to treat and have recurrent inflammation. They are usually referred to as individuals with recalcitrant disease and having recurrent nasal polyps (NPs; CRSwNP). The disease poses a significant impact on the patients´ health-related quality of life (HrQoL), mainly because of a complete loss of smell. Pharmacological treatment with biological therapies has recently been developed, targeting mediators of the type 2 inflammatory response. However, not everyone benefits from the biological therapy, and it has proven difficult to identify and characterise patients that are responsive to these new medications. Eicosanoids, being arachidonic acid derived bioactive lipid mediators, has been shown to be implicated in CRSwNP. Although there is a clear link between an imbalanced biosynthesis of pro- and anti-inflammatory eicosanoids and type 2 inflammation, to date research has not focused on them as biomarkers in CRSwNP.

The overall aim of this thesis was to explore the potential role of eicosanoids as biomarkers and characterise changes over time in HrQoL as well as the degree of smell loss in patients with severe recalcitrant CRSwNP. The project involved immunoassay analysis of levels of various inflammatory mediators, including a selection of eicosanoids, in nasal tissue, nasal secretions and urine from patients with CRSwNP as well as gene expression analyses regarding biosynthetic enzymes and receptors for eicosanoids in nasal tissues. HrQoL was assessed with SNOT-22 and RAND-36, along with point-of-care tests as eosinophil blood count, fractional exhaled nitric oxide (FeNO) and smell tests with Burghart Sniffin' Sticks.

Levels of eicosanoids in nasal secretions were found to associate with the disease severity, defined as the extent of NP growth (**paper I**). One of the eicosanoids, leukotriene E_4 (LTE_4), were correlated to the degree of smell loss (**paper I**). An increase in LTE_4 between six and 12 months after surgery was demonstrated in patients with recurrent NPs (**paper II**). Recurrent NPs were identified endoscopically 12 months after surgery and a distinct eicosanoid profile involving LTE_4, prostaglandin D_2 and 15(S) hydroxyeicosatetraenoic acid was found to be more common in those with recurrence (**paper II**). A similar eicosanoid profile, based on measurements from nasal tissue samples instead, was also associated with NP recurrence (**paper III**).

Levels of eicosanoids in nasal tissue and nasal secretions were correlated suggesting that analysis of biomarkers in nasal secretions reflects release from the nasal tissue (**paper III**). Patients with recurrent NPs had elevated blood eosinophil counts before their surgery, and their sense of smell was significantly impaired both before and after (**paper IV**). This finding suggests that loss of smell may be the first symptom during recurrence. Although measures of HrQoL could not distinguish patients with recurrent NPs, there was a strong correlation to the degree of smell loss suggesting that loss of smell has a significant impact on the HrQoL (**paper IV**).

In summary, the results from this thesis contribute to an extended knowledge regarding characteristics relevant for identifying severe recalcitrant CRSwNP. Characteristics of interest included a distinct eicosanoid profile, severe loss of smell and eosinophil involvement, all of which may be possible prognostic markers for severe recalcitrant CRSwNP with rapid NP growth. It may be concluded that such biomarkers can guide the choice of treatment for these severely ill patients – repeated surgery or pharmacological treatment with the newly developed biological therapies.

List of scientific papers

The following papers are included in this thesis and will be referred to by their roman numeral.

I. Nordström A, Jangard M, Svedberg M, Ryott M, Kumlin M. **Levels of eicosanoids in nasal secretions associated with nasal polyp severity in chronic rhinosinusitis**. Prostaglandins, leukotrienes, and essential fatty acids. 2022;184:102474.

II. Nordström A, Jangard M, Svedberg M, Ryott M, Kumlin M. **Distinct eicosanoid patterns in severe recalcitrant nasal polyposis**. International forum of allergy & rhinology. 2023;13(11):2043-54.

III. Nordström A, Jangard M, Ryott M, Tang X, Svedberg M, Kumlin M. **Mucosal LTE_4, PGD_2 and 15(S)-HETE as potential prognostic markers for polyp recurrence in chronic rhinosinusitis**. Submitted

IV. Nordström A, Jangard M, Ryott M, Svedberg M, Kumlin M. **Assessment of health-related quality of life and olfactory loss in relation to nasal polyp recurrence after endoscopic sinus surgery in patients with chronic rhinosinusitis- a real life-study**. Manuscript

Scientific paper not included

Nordström A, Jangard M, Svedberg M, Kullenberg H, Ryott M, Kumlin M. **Hot saline irrigation in comparison to nasal packing after sinus surgery**. Laryngoscope investigative otolaryngology. 2021;6(6):1267-74.

Contents

List of abbreviations

AA	Arachidonic acid
COX	Cyclooxygenase
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
CRTH2	Chemoattractant receptor homologue expressed on Th2 cells
CysLT	Cysteinyl-leukotriene
EDN	Eosinophil derived neurotoxin
ENT clinic	Ear, nose, and throat clinic
EPOS	European Position Paper on Rhinosinusitis and Nasal Polyps
EP receptor	E-prostanoid receptor
EUFOREA	European Forum for Research and Education in Allergy and Airways diseases
Feno	Fractional exhaled nitric oxide
FESS	Functional endoscopic sinus surgery
HETE	Hydroxyeicosatetraenoic acid
HPGDS	Hematopoietic Prostaglandin D Synthase
HrQoL	Health related quality of life
IL	Interleukin
LM score	Lund-Mackay score
LTC4S	Leukotriene C4 synthase
LOX	Lipoxygenase
MGST2	microsomal glutathione S-transferase 2
OCS	Oral corticosteroid
PG	Prostaglandin
RAND-36	RAND corporation 36-item health survey
SNOT-22	Sinonasal outcome test-22

Introduction

My background in biomedicine and toxicology has led to a fascination for inflammatory reactions. Inflammation is an essential part of our immune system consisting of an intricate network of cellular and molecular interactions. Its main purpose is to eliminate invading treats, repair and maintain tissue homeostasis, but in certain cases the immune system gets chronically active. This leads to a sustained activation of immune cells and often an excessive release of various inflammatory signalling molecules that can be harmful to us.

This thesis is a patient-oriented research project focusing on the care and treatment of individuals with severe chronic rhinosinusitis (CRS). The main objective was to identify and characterise the disease in individuals with difficult-to-treat CRS in terms of health-related quality of life, loss of smell and by analysing a range of inflammatory biomarkers.

CRS has historically been divided into patients with (CRSwNP) and without nasal polyps (CRSwNP). Antibiotics were commonly administered to those with CRSsNP, while oral corticosteroids (OCS) were given to those with CRSwNP. Looking back, it is now clear that this was a rather simplistic view of CRS as it was based on assumptions that CRSsNP was the cause of an unresolved bacterial infection and that CRSwNP was some form of systemic allergy. Later, the view of CRS evolved into a chronic condition with a multifactorial ethology driven by various environmental stressors and pathogens. At that time, research focused on causal factors to find new treatment targets. Given the diversity of environmental factors and the late disease-onset of CRS, this was proven to be a difficult task. Instead of focusing on causation, current research has shifted towards understanding the resulting inflammation, identifying specific molecular pathways activated in the sinuses. This has led to the development of biological therapies targeting specific inflammatory processes, offering potential for personalized medicine based on molecular biomarkers found in an individual patient.

In efforts to improve the quality of life in patients with CRS, academic research and in particular the pharmaceutical industry play an important role in finding new ways to control the disease. A major step towards improving the patient's well-being involves reducing the need for surgery, which induces stress and anxiety, as well as minimising treatment with corticosteroids.

1 Background

1.1 CHRONIC RHINOSINUSITIS

Chronic rhinosinusitis (CRS) is a heterogenous inflammatory condition of the sinonasal cavities that causes excessive swelling and mucus secretion of the sinuses. This can lead to a variety of persistent sinonasal symptoms, with nasal congestion/obstruction, facial pain or pressure, nasal discharge, and impaired sense of smell and taste being some of the most common ones. CRS is believed to be one of the most common chronic inflammatory diseases and affects around 10% of adults in the general population worldwide (1). However, prevalence of CRS may vary substantially between countries. In a study provided by the Global Asthma and Allergy European Network a wide range of estimated prevalences in Europe was reported, from 6.9% to 27.1% (2).

Sinonasal symptoms experienced by individuals with CRS commonly interfere with their day-to-day life (3) and has been shown to significantly reduce the health-related quality of life (HrQoL) (4). Although there is considerable individual variation, previous studies have shown that sleep quality, facial pain, cognitive function, productivity, and loss of smell have the greatest impact on the quality of life in CRS (5-7). Management and controlling of the disease progression is not achieved in a large proportion of patients with CRS (around 40%) despite the use of recommended treatment strategies (8). One of the current forms of treatment is surgical intervention, which is commonly perceived as a stressful and uncomfortable experience (9). Generally, these patients require frequent contact with the health care system, partly at primary health centres but mainly at specialist clinics *i.e.*, ear, nose, and throat (ENT) clinics. Therefore, CRS causes a substantial economic burden to the healthcare system. When including both direct (*e.g.* outpatients visits and surgeries) and indirect costs (*e.g.* sick days and loss of productivity), a total of $30 billion dollars is spent annually on CRS in the US (10). The socio-economic burden of CRS is less studied in Europe but has been estimated at £16.8 billion per year in the UK where each individual with CRS costs on average £3872 more than those of the general population (11).

1.1.1 Diagnosis and current treatment

According to the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS), CRS is defined as an inflammation of the nose and the paranasal sinuses followed by sinonasal symptoms that persist for at least

12 consecutive weeks. At least two of the major sinonasal symptoms are required for diagnosis of CRS, one of which needs to be nasal discharge or nasal congestion/obstruction, whereas other symptoms include facial pain/pressure and loss of smell. Nasal endoscopy is also performed to find signs of ongoing inflammation in the mucosa such as oedema/obstructions, mucopurulent discharge, and nasal polyps (NPs). NPs are noncancerous round/teardrop-shaped out-growths of the sinonasal mucosa (Figure 1). The expansion of these growths can contribute significantly to the nasal blockage experienced in patients.

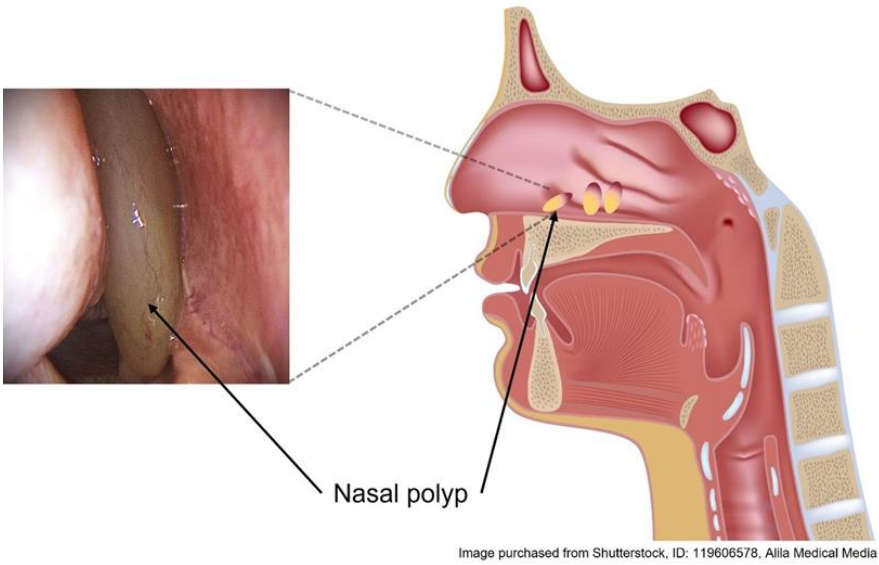


Figure 1. Lefthand side: A photograph depicting a nasal polyp growing in the inferior meatus of the sinus cavity. Righthand side: An illustrative image of the sinonasal cavities.

The identification of growing NPs in the sinuses are classically used to divide patients into those with (CRSwNP) and without NPs (CRSsNP). During this procedure, the physicians also establish whether the disease is unilateral or bilateral, which is important since CRSwNP affects both right and left sinuses, *i.e.,* always bilateral. Of note, the onset of CRSwNP is usually in the middle-age, around 40-50 years of age (12), and has been shown to be more common among males (13).

The first line of treatment consists of saline irrigation and intranasal corticosteroid (INCS), and if needed an adjuvant therapy of short courses of per oral corticosteroids (OCS) or antibiotics. As a last resort in case of inadequate control of the disease, functional endoscopic sinus surgery (FESS) is performed for the removal of inflamed tissue to restore the ventilation and mucus drainage of the sinuses. Surgery can also help INCS sprays to reach deeper into the sinus cavity, which is impossible in cases of total nasal congestion.

Patients that still have chronic inflammation of the mucosa and ongoing symptoms despite appropriate medical and surgical treatment are referred as difficult-to-treat or recalcitrant CRS. These individuals have uncontrolled disease defined as having at least three of the following criteria after surgery: mucopurulent nasal discharge, congestion, or facial pain present on most days of the week, sleep disturbances/fatigue, impaired sense of smell, an inflamed mucosa and failure to alleviate previously mentioned symptoms with additional short courses of OCS (14). Recurrent disease after surgery is far more common in those with CRSwNP relative to CRSsNP (15). Recurrent NPs has been estimated to occur in 30% of patients with CRSwNP already at six months post-surgery (16) or in 40 % 18 months post-surgery (15, 16). The current doctoral thesis focuses primarily on patients with difficult-to-treat CRSwNP and will be referred to as recalcitrant CRSwNP from now on.

1.1.2 Associated comorbidities

Lower airway inflammation is frequently occurring in CRS with up to 30-40% of patients with CRS having comorbid asthma (17, 18). Previous reports suggest that there is a significant impact on lung function in patients with CRS, even in those without bronchial symptoms and no asthma diagnosis (19). The prevalence of co-existing asthma is around 35% in CRSsNP and increases further in CRSwNP to 55-65% (18). This is usually a late-onset and non-atopic asthma that develops around 40-50 years of age and is especially observed among patients with CRSwNP. Comorbid asthma may further worsen the progression of CRS, especially in those with CRSwNP (20). More importantly, these individuals are in need of OCS treatment more frequently and have an increased risk of recalcitrant disease (20, 21).

The progression of CRS may also lead to increased burden of asthma since poor asthma control including increased frequency of asthma exacerbations is often reported (22, 23). While adequate asthma control may reduce the need for CRS intended medication (21), sinus surgery has been shown to attenuate the

asthma burden (24). Thus, an adequate control of both asthma and CRS is to prefer as inflammation in the upper and lower airways appears to be interlinked. This naso-bronchial interaction has been extensively studied for many years, commonly called "the global airways disease concept" (25). The mechanism behind this interaction is not fully understood. This may be due to mucosal barrier disruptions, present in both the upper and lower airways, ultimately leading to passage of exogenous particles causing a systemic inflammation that affects distant regions (25).

Some patients with comorbid asthma also develop an intolerance to cyclooxygenase-1 inhibitors, *i.e.*, aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs), leading to the diagnosis aspirin/NSAID exacerbated respiratory disease (AERD/NERD). AERD is characterised by the triad of CRSwNP, moderate-to-severe asthma, and acute hypersensitive reactions in the upper and lower respiratory tract following ingestion of NSAIDs. For a reliable diagnosis, an aspirin challenge is required. However, in some cases diagnosis can be based on prior clinical history of NSAID-induced respiratory reactions, if deemed reliable, in combination with frequent NP recurrence and asthma (26). Approximately 16% of patients with CRSwNP has AERD, and these individuals usually feature the most severe manifestations of nasal polyposis, recalcitrant disease, and asthmatic burden (27, 28).

A co-existence of allergy may also be of concern in CRS with a prevalence ranging from 25% to 65% (4, 17, 18, 29). Due to this variation, the prevalence of allergy in CRS is currently uncertain. As pointed out in previous reports (17), one probable cause for this inconsistency is that selection bias is present in some studies by physicians with a special interest in allergy leading to an overestimated reporting of allergy. Another issue is that the term atopy and allergic rhinitis is used interchangeably, as stated by others (30). Since the allergic inflammation induces swelling and impaired mucociliary clearance of the mucosa, it is logical to assume that allergic rhinitis would contribute to the development of CRS. However, the role of allergy in CRS is currently unclear and under debate as a large proportion of available data in the literature is contradictive and unconclusive (29, 30).

1.1.3 Health-related quality of life and disease severity

Severe CRS is based on the extent of depriving symptoms or HrQoL impairment. HRQoL may be defined as the perceived state of physical, mental, and social well-being, along with its influence on functional capacity. It is a multidimensional concept of health widely employed to evaluate how diseases

or treatments affect patients' perceptions of their symptoms, functioning, and psychological health (31).

Sinonasal outcome test-22 (SNOT-22) is a validated questionnaire and the most utilised tool to assess HrQoL in patients with CRS (14). It consists of 22 questions on the severity of rhinological symptoms, ear/facial symptoms, sleep, and psychological dysfunction (e.g., ability to focus, productivity, sadness, and embarrassment due to CRS). The total SNOT-22 score is from 0 to 110 where higher scores indicate worse psycho-social functioning and/or symptom severity (32). According to the European Forum for Research and Education in Allergy and Airways diseases (EUFOREA) and EPOS 2020, an individual with a SNOT-22 score >35 (33) or >40 (14) should be regarded as someone with severe CRS.

The 36-item short form survey (SF-36) and RAND 36-item health survey (RAND-36) are other available questionnaires for the assessment of QoL, though less commonly used in CRS. Identical questions are addressed in SF-36 and RAND-36, but minimal differences exist in how certain questions are scored. Otherwise, they are equivalent. These surveys covers eight different aspects of HrQoL formulated as general health: limitations on physical activity, issues with work or daily activities due to detrimental physical health, bodily pain or limitations due to pain, perception on current and future health, fatigue, social functioning (*e.g.*, ability to interact with others, engagement in activities and social roles), interference with daily activities due to emotional distress, and mental health (*e.g.*, anxiety and sadness).

The assessment of severity also extents to objective markers that evaluates the paranasal inflammation and obstructions with sinonasal endoscopy and computer tomography (CT) scans. Nasal polyp (NP) score is a physician-reported tool to grade the extent and severity of NP growth in the paranasal sinuses with endoscopy. The NP score ranges from 0 to 8 and higher scores indicate higher severity. The Lund-Mackay (LM) score is a tool to assess the severity of opacification in each sinus cavity using CT scan. For an individual with CRS, an opacified sinus would reflect mucosal swelling caused by the inflammation. Various sinonasal regions are graded yielding a score from 0 to 24. From the EUFOREA expert panel, a NP score of ≥ 4 was suggested as a definition of severe CRSwNP combined with higher symptom or HrQoL scores, *e.g.*, SNOT-22 score >35 (33).

A loss of smell is an important factor that signifies uncontrolled and severe CRSwNP (34). It is also one of the most detrimental factors impairing the

HrQoL among CRSwNP patients (34). In European clinics, the Burghart Sniffin' Sticks smell test is currently the most utilised tool to assess the degree of smell loss (35). The test considers three different aspects of olfactory function: threshold (ability to detect faint smells), discrimination (ability to distinguish between odours) and identification (ability to identify an odour) (36).

Comorbid asthma and AERD is not a direct measure of disease severity but is commonly used in clinics as a strong indicator for the more severe forms of CRSwNP (34). For those with asthma, fractional exhaled nitric oxide (FeNO) is a readily available tool and an adjunctive point-of-care test to detect a certain type of lower airway inflammation, namely a type 2 inflammation. The involvement of eosinophilic granulocytes (both in tissue and blood) may be regarded as an indirect, but not direct measure of disease severity. Associations between eosinophil count and other markers of disease severity (*e.g.*, scores of LM and smell tests) have been reported (37, 38). However, the correlation to HrQoL is quite low in some reports (39), and completely lacking in others (40). Eosinophil granular proteins may feature stronger correlations with HrQoL (41, 42), but standardised analytic methods as well as established cut-off values for these biomarkers is currently lacking.

1.2 THE SINONASAL IMMUNE RESPONSE

The nasal mucosa consists of an epithelial lining of structural cells tightly bounded in cell-cell connections forming the first line of defence as a physical barrier against pathogens and pollutant compounds (43). These are columnar epithelial cells (ciliated and non-ciliated cells), goblet cells (mucus secreting cells), and basal cells (multipotent stem cells) growing as a sheath. Mucus is secreted on top of the epithelial lining designed to trap inhaled materials for subsequent removal by an action called mucociliary clearance. If pathogens do circumvent the epithelial barrier, immune cells in the underlying tissue gets activated to combat the threat. Some of these cells include granulocytes (eosinophils, neutrophils, basophils and mast cells), macrophages, dendritic cells, and innate lymphoid cells (ILCs).

The immune system can be divided into two parts, the innate and the adaptive immune system. The innate system involves the mucosal epithelial barrier as well as the immune cells mentioned above. It is the initial and rapid response against an invading pathogen. The other part, the adaptive immune system, involves the activation of lymphocytes called T- and B-cells. Lymphocytes are

slower but mediates a more specific, effective, and long-lasting response against the pathogen.

For the acquirement of adaptive immunity (immunological memory), antigen-presenting cells (APCs) of the innate immune system (*e.g.*, dendritic cells) are needed to fully activate the lymphocytes (44). In short, APCs internalize pathogens and display short peptides (antigens) of the pathogen to lymphocytes. This interaction causes the lymphocyte to differentiate into an activate immune cell that can recognise a specific part of the pathogen with high affinity. During an infection, the epithelial cells and immune cells release a variety of signalling molecules (cytokines and arachidonic acid metabolites) that in turn regulate the differentiation of lymphocytes and determine the type of immune response needed (45).

The main purpose of the host defence is to eliminate foreign material entering our bodies. However, dysregulations in our immune system may result in an excessive and aberrant inflammatory response resulting in tissue damage and more inflammation in a vicious cycle. The aetiology of CRS is not fully understood but is thought to be multifactorial, resulting from a dysfunctional interaction between environmental factors and the mucosal immune system. The key component in CRS is a dysfunctional mucosal barrier (43, 46). Numerous antigens may therefore continuously expose the underlying parts of the mucosa leading to repeated activation of the immune system. Other factors worth mentioning are anatomical abnormalities, microbiome dysbiosis, and certain infections which commonly precedes the NP formation (47). The inflammation that follows involves an influx of multiple immune cells. In severe cases of CRS, there is a pronounced increase in eosinophils, ILC2s, mast cells, and a distinct set of lymphocytes known as T-helper type 2 cells (48).

1.3 CYTOKINES, CHEMOKINES AND GROWTH FACTORS

Cytokines and chemokines are small extracellular proteins that modulate inflammation and act as regulators or mediators of the intricate signalling networks between the cells in our human body. Their function is to ensure homeostasis in physiological processes as well as to amplify and orchestrate a directed inflammatory response during acute and chronic conditions.

There is a growing body of evidence to suggest that the inflammation in CRS is heterogenous and does not involve one single pathophysiological mechanism but several (49-52). Endotypes are defined as different subtypes of a disease, each with a unique inflammatory pattern that represents different underlying

inflammatory mechanisms, clinical outcomes, and treatment responses. The current endotypes described within CRS is partly and, in some cases, fully based on cytokines and chemokines that reflect different kinds of adaptive immune responses (48). Primarily, these are adaptive immune responses represented by the activation of T-helper type 1 (Th1), type 2 (Th2) or type 17 (Th17) cells, denoted as type 1, type 2, and type 3 inflammatory endotypes, respectively.

1.3.1 Th1

The cytokine IL-12 promotes the differentiation of Th1 cells leading to production of the major effector cytokine, Interferon- γ (IFN-γ), as well as tumour necrosis factor-α (TNF-a) and IL-2 (53). However, IL-2 may not be a Th1-specific cytokine, as it is a regulator of growth and differentiation for lymphocytes in general (54). Other sources of IFN-γ includes ILC1s during the Th1 response in CRS (55). ILCs are similar to Th cells since they mirror the canonical cytokine production of Th cells but lack antigen receptors and are innate immune cells.

Classically, the differentiation of Th1 cells is driven by the presence of intracellular viruses and bacteria. Th1-related cytokines induce a cell-mediated response by activating cells such as macrophages and neutrophils to increase their phagocytotic ability and production of reactive oxygen species (56). Although not specific to the Th1-response, neutrophils and macrophages may in turn release several pro-inflammatory cytokines including IL-1β and IL-6 (57). CRSsNP has been viewed as a Th1-dominant disease with less pronounced clinical symptoms. However, recent evidence suggests that CRSsNP is as heterogenous as CRSwNP (52) In addition, a pure Th1 endotype might be rare and instead mixed with the other Th responses (49). Hence, the clinical outcome of Th1 is not completely clear but may feature the mildest endpoint in the spectrum of CRS diseases (58).

1.3.2 Th2

The Th2 immune response is mainly driven by IL-4, IL-5, and IL-13, produced, and released by Th2, ILC2s and mast cells (55). The differentiation of Th2 cells is a dominant feature in allergic reactions. IL-5 is the major factor that stimulates eosinophil survival and proliferation, whereas IL-4 and IL-13 stimulate isotype switching towards IgE, mucus secretion, and remodelling of the submucosa (59). IgE binds to its high-affinity receptor (FcεRI), expressed on mast cells, evoking degranulation and release of potent mediators: histamine, proteases, and cytokines. In addition, this also triggers biosynthesis of arachidonic acid metabolites with bronchoconstrictive and mucus stimulatory

actions (48). Epithelial basal cells from NPs are prone to express IL-4-driven transcription programs which include eosinophilic chemoattractants, and these cells are unable to fully differentiate into functional epithelia (46). This finding suggests a pronounced epithelial dysfunction during a Th2 inflammation. In addition, epithelial-derived alarmin cytokines (IL-25, IL-33, and thymic stromal lymphopoietin) may further increase the type 2 inflammatory response (59). These cytokines cause mast cell activation and hyperplasia, as well as stimulates ILC2s and Th2 cells to produce type 2 cytokines. Several factors during the Th2 inflammation can damage the epithelial mucosal lining. The most prominent cause being the release of prestored cytotoxic granule proteins from eosinophils (*e.g.*, eosinophil derived neurotoxin and eosinophil cationic protein).

The eosinophilic Th2 inflammation is generally more common in CRSwNP, associated with severe smell loss, comorbid asthma and/or AERD as well as disease recurrence (49, 50, 60).

1.3.3 Th17

Cytokines IL-23, IL-6 and transforming growth factor-β (TGF- β) drives the differentiation of Th17 which in turn starts to produce IL-17 and IL-22. Classically, these cells are activated by the presence of extracellular bacteria and fungi. IL-17 and IL-22 induce strong neutrophil migration towards the airway epithelium. By the release of IL-8, the recruitment and activation of neutrophils is further enhanced (61). Like the other Th responses, IL-17 is also released by ILCs, namely ILC3s. Other relevant factors involved in the neutrophilic growth are GM-CSF and IL-6 secreted by epithelial and surrounding immune cells (62). The combination of having both neutrophilic and eosinophilic tissue inflammation was suggested to cause the highest burden of sinonasal symptoms (63). A Th17-dominant inflammation has been reported to be associated with signs of purulent nasal discharge which are common symptoms of bacterial infection in CRS (49).

1.4 EICOSANOIDS

Eicosanoids are potent bioactive lipid mediators and regulators of inflammation that operate in concordance with the cytokines. They are produced by multiple cells and are essential for basal physiological functions. Eicosanoids are oxidised derivates of 20-carbon polyunsaturated fatty acids (PUFAs). Arachidonic acid (AA, 20;4ω6; 4 double bonds with the last one situated at the 6th carbon from the ω end) is the main precursor for the synthesis of these bioactive oxylipids. Other substrates include dihomo-y-linoleic acid (DGLA, 20:3ω6) and eicosapentaenoic acid (EPA, 20:5ω3) which is much less utilized

for the biosynthesis of eicosanoids due to lower availability in the cell membrane (64). AA is obtained through dietary intake or through the intake of the cis-linoleic acid (18:2ω6)(6ω-series essential fatty acid) which can be converted to AA (65). In parts of the world where fish makes up a large part of the diet the presence of EPA is much greater in the cell membrane and hence eicosanoids with 5 double bonds are more common (66).

The biosynthesis of eicosanoids starts with the release of AA which resides within the phospholipid bilayer of the cell membrane (Figure 2). The ester bond between the glycerol skeleton of the phospholipid and arachidonic acid is hydrolysed, mainly by phospholipase A2, releasing AA for further conversion to eicosanoids.

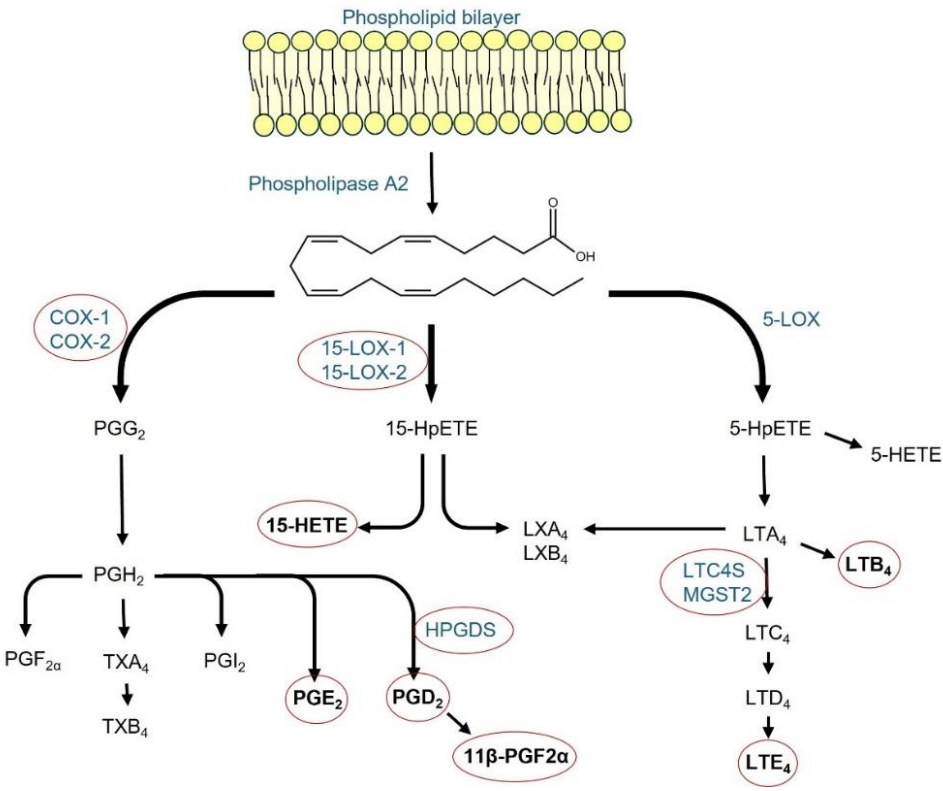


Figure 2. Schematic overview of the arachidonic acid metabolism involving the cyclooxygenase-1 and -2 (COX-1 and -2), 15-lipoxygenase-1 (15-LOX-1 and -2) and 5-LOX enzymatic pathways. This thesis mainly focused on the metabolites and enzymes marked with a red circle.

The AA may then undergo metabolism via a variety of enzymatic pathways which includes cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome p450 (Cyp450) enzymes to form various bioactive eicosanoids. The current thesis focused on a specific set of metabolites from the LOX and COX enzymatic pathways. These were leukotriene (LT) E_4, LTB_4, prostaglandin (PG) D_2, PGE_2, and 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE). The selection of the eicosanoids was based on their potential interactions with the Th2 immune response; the dominant inflammatory feature in CRSwNP, but also due to their involvement in neutrophilic inflammation as well as AERD pathogenesis, a subtype of CRS associated with disease recurrence (67).

1.4.1 Leukotrienes

Leukotrienes (LTs) were originally found in leukocytes, and were shown to have three conjugated double bonds, *i.e.*, a triene structure (68). LTs are biosynthesised de novo in response to cellular activation. It is the 5-LOX enzyme that initially oxygenate AA into 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which is enzymatically or non-enzymatically reduced to 5-HETE or further dehydrated into the unstable epoxide intermediate, LTA_4 (69).

Depending on the cell type, this intermediate may be conjugated with glutathione (a tripeptide chain of cysteine, glutamate, and glycine), a reaction catalysed by LTC_4 synthase (LTC4S), yielding LTC_4. Another enzymatic pathway towards the LTC_4 generation may occur, enabled by microsomal glutathione s-transferase 2 (MGST2) (70). LTC_4 is transported out of the cell and undergoes further metabolism by γ-glutamyl transpeptidase (peptide cleavage freeing the glutamate) and dipeptidase (further peptide cleavage freeing the glycine) yielding LTD_4 and LTE_4, respectively. Collectively, LTC_4, LTD_4 and LTE_4 are called cysteinyl-LTs (CysLTs) as they all have the cysteine amino acid. Previous reports have shown that LTE_4, the predominant CysLT excreted into urine, is highly stable for measurements representing the whole body production of CysLTs, which also reflecting airway inflammation (71).

Multiple cells with hematopoietic origin can produce CysLTs. However, in CRSwNP, mast cells and eosinophils are believed to be the main contributors of these lipid mediators (67). They have a diverse range of effects in the airways; increased vascular permeability, mucus secretion, bronchoconstriction as well as eosinophil chemoattraction. These effects are primarily induced through binding to their receptors, CysLT1 and CysLT2. An overproduction of CysLTs is a key feature in AERD and gets further intensified during NSAID/aspirin induced hypersensitive reactions (72).

Alternatively, the 5-LOX-generated LTA_4 is hydrolysed by LTA_4 hydrolase yielding LTB_4 (73), as seen in figure 2. The actions of LTB_4 include recruitment and activation of a number of different leukocytes. In addition, LTB_4 is especially chemotactic for neutrophils which is also the main cellular source of LTB_4 (56).

1.4.2 Prostaglandins

Prostaglandins (PGs) were first found in seminal vesicles and thought to originate from prostate glands (74). The biosynthesis of PGs involves a two-step enzymatic reaction catalysed by PG endoperoxide H synthase, commonly called the COX enzyme (64). The AA undergoes a cyclooxygenase reaction to form PGG_2 and then a peroxidase reaction to form PGH_2. There are two isoforms of the COX enzyme, namely COX-1 and COX-2. The main functional difference between these two are that COX-1 is constitutively expressed to maintain basal physiological functions, whereas COX-2 is inducible by *e.g.*, bacterial antigens or IL-1β (75).

The intermediate PGH_2, is then further converted by any of the available prostanoid synthases, producing either PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 or thromboxane A_2 (64). The prostanoid synthases are expressed differently depending on cell type and tissue location. For instance, there is evidence to suggest that mast cells are the main source of PGD_2 in the airways (76) which is also likely the case in CRSwNP since the enzyme hematopoietic prostaglandin D synthase (HPGDS) was primarily expressed by nasal mast cells (46, 77). PGD_2 can elicit its effects through binding to the receptors TP, DP1 and DP2 (also called chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)). Although, primarily considered a thromboxane receptor, PGD_2 may stimulate the bronchial smooth muscle to contract via the TP receptor, causing bronchoconstriction. On the contrary, stimulatory actions on DP1 may cause broncho- and vasodilation (76). CRTH2 enhances the production of type 2 cytokines and promotes the recruitment of type 2 inflammatory cells including Th2 cells, ILC2s, and eosinophils (78). CRTH2 was also found to be essential for the activation of ILC2s (79). Further metabolism of PGD_2 by 11-ketoreductase yields 11β-PGF2α, a biologically active lipid mediator that can be readily measured in urine as the main C20 PGD_2 metabolite (80). An overproduction of PGD_2 is apparent in AERD, as previously shown by an increased urinary 11β-PGF2α (81).

PGE_2 can be produced by a number of cells expressing various PGE_2 synthases (cytosolic PGES, membrane-bound PGES-1 and -2), although epithelial cells, fibroblasts and macrophages produce prominent quantities (64). PGE_2 evokes a diverse range of effects through binding to either of the E-prostanoid (EP) receptors, EP1, EP2, EP3 and EP4. Generally, PGE_2 is considered to be a pro-inflammatory mediator inducing fever, pain, and vascular permeability improving the recruitment of leukocytes (82). However, the PGE_2 is rather ubiquitous with both pro- and anti-inflammatory effects which depends on the type of cell, the type of tissue, and the type of EP receptors present (83). PGE_2 has been suggested to enhance the production of pro-inflammatory cytokines from macrophages and stimulate Th17 development through EP2/EP4 signalling in rheumatoid arthritis (84). On the other hand, PGE_2 is anti-inflammatory and bronchoprotective in the airways for patients with allergic asthma as well as AERD after allergen exposure or aspirin provocation (85, 86). The binding of PGE_2 to EP2 on mast cells suppresses 5-LOX, lowering the levels of bronchoconstrictive and pro-inflammatory CysLTs. AERD features a diminished PGE_2 production and impaired EP2 signalling (72). Hence, a complete depletion of PGE_2 through COX-1 inhibition (by *e.g.*, aspirin), combined with an already dysfunctional biosynthesis and signalling of PGE_2 could account for the hypersensitive airway reactions and the increased CysLT production observed in AERD (83).

1.4.3 HETEs

There are several isoforms of the enzyme LOX (*e.g.*, 5-, 12- and 15-LOX) which oxygenate AA forming HETEs with a specific configuration (S isomers). However, they are also produced by Cyp450 enzymes which may lead to the formation of R isomers of the HETE metabolites. In addition, 15(R)-HETE is primarily a byproduct of a COX reaction (87).

The oxygenation of AA by 15-LOX generates the unstable and reactive 15(S)-HpETEs which is reduced to 15(S)-HETE; the primary metabolite of 15-LOX. The level of 15(S)-HETE generally reflects the enzymatic activity of the 15-LOX (88). In a proposed transcellular metabolism between epithelial cells and mast cells, 15-HETE was converted to 15-oxo-ETE by 15-hydroxy PG dehydrogenase in large amounts in AERD patients (77).

15(S)-HETE is one the precursors to lipoxins (LXs), a conversion catalysed by 5-LOX, to LXA_4 and/or LXB_4. Other pathways involve the conversion of the AA by 5-LOX from leukocytes into LTA_4, and subsequently by 12-LOX in platelets into LXA_4 (89). Lipoxins are vasodilatory, regulate leukocyte

trafficking, and induces resolution of the inflammation (90). Of note, other PUFA substrates, namely EPA, is also important for the generations of specialized pro-resolving mediators (SPMs), including LXs.

In recent years, 15-LOX and its products have come into focus due to their involvement in the pathogenesis of severe type 2 asthma and CRSwNP (91, 92). Increased levels of 15-HETE were reported to correlate with the severity of asthma and tissue eosinophilia (88). A gene variant with a loss of function for 15-LOX was protective against growth of NPs (93). Moreover, 15-LOX expression is driven by the induction of IL-4/IL-13 signalling in the airways (94), and there is high expression in epithelial cells from NPs (46, 77). Esterified AA (*i.e.,* still bound to the cell membrane) may undergo oxygenation reactions by 15-LOX forming 15-HETE-phosphatidyl ethanolamine (15-HETE-PE) in the airways (92). In a study on epithelial cells from NPs, both 15-LOX and 15-HETE-PE induced the expression of eotaxin-3, an eosinophilic chemoattractant (95).

1.5 LITERATURE REVIEW OF CURRENT BIOLOGICAL THERAPY

The first biological therapy for severe allergic asthma entered the market almost 15 years ago as an anti-IgE treatment. (96). Specifically, biologics are humanised recombinant monoclonal antibodies (mAbs) designed to target specific cytokines, receptors, or other macromolecules associated with the type 2 inflammation. They are currently intended as add-on treatment for those with severe recalcitrant CRSwNP when conventional therapy have failed to control the disease (97). Indications for biological treatments are recurrent NP after FESS in combination with at least three of the following conditions: need for systemic OCS (≥2 courses/year), a type 2 inflammation as evidenced by the number of eosinophils in tissue (≥10 cells/high-power field), blood (≥150 cells/μl) or serum IgE levels (≥100 total IgE IU/ml), worsened HrQoL (≥40 in SNOT-22), anosmic (as determined by the clinic's smell test) and comorbid asthma (98). The main efficacy results from the currently available biologics are summarised below. Other biologics that are under investigation but not currently approved for the treatment of CRSwNP are also briefly described below.

Omalizumab (Xolair®, anti-IgE mAb): In 2020, the European medicines agency (EPA) approved omalizumab to be used as an add-on treatment for adults with severe CRSwNP (99). This is an anti-IgE mAb that selectively binds to free IgE, blocking their ability to bind to the high-affinity receptor FcεRI (100). The inhibition decreases the levels of free IgE and lowers the expression

of FcεRI on basophils and mast cells (101). The dose and dosing frequency of omalizumab is based on pretreatment serum total levels of IgE and bodyweight. A subcutaneous injection every two weeks (2QW) or every fourth week (4QW) for 16 weeks showed a significant reduction in NP score (mean change -2.67) in a study of 23 participants (100). Moreover, a reduction in LM score (mean change -4) and symptom scores (ranging from 0 to 3) such as nasal congestion (mean change -1) was observed. The following RCTs (called POLYP 1 and POLYP 2) involved a total of 265 study participants recruited from 82 ENT clinics across US and Europe (102). After 24 weeks there was a significant reduction in NP score (mean change -1.14 and -0.59), nasal congestion score (mean change -0.55 and -0.50), SNOT-22 score (mean change -16 and -15) and improvements in smell test score (mean change 3.81 and 3.86).

Dupilumab (Dupixent®, anti-IL-4/IL-13α receptor mAb): In 2019, EPA approved Dupilumab as an add-on therapy to treat adults with severe CRSwNP (103). This mAb targets the IL-4/IL-13 receptor α subunit inhibiting the IL-4/IL-13 signalling. The phase II RCT included 60 study participants recruited from 14 clinics and the starting dose was 600 mg subcutaneous injection followed by 300 mg 1QW (104). After 16 weeks there was an improvement in NP score, sinonasal swelling, and HrQoL.

The phase III RCTs (called SINUS-24 and SINUS-52) included 276 and 448 study participants, respectively, and was conducted in 67 clinics from 14 countries situated in Europe and US (105). In SINUS-24, dupilumab was administered subcutaneously 2QW for 24 weeks. In SINUS-52, dupilumab was subcutaneously administered 2QW for 52 weeks or 2QW for the first 24 weeks followed by 4QW until the 52nd week. After 24 weeks in SINUS-24 and SINUS-52 there was a significant reduction in NP score (mean change -2.06 and -1.80), nasal congestion score (-0.89 and -0.87), LM score (mean change -7.44 and -5.13) SNOT-22 (mean change -21 and -17), and improvement in smell test score (Mean change 10.51 and 10.52). After 52 weeks of treatment, SINUS-52 showed that NP score (mean change -2.40), nasal congestion score (mean change -0.98), and SNOT-22 (mean change -21) was reduced even further. SINUS-52 also showed that patients treated 2QW had greater reductions in NP score and LM score than those treated 4QW. Discontinuation of treatment in patients in SINUS-24, from the 24th to the 52nd week, lead to worsened NP score and symptom scores. As opposed to the results of the phase II clinical trial (103), this study showed that dupilumab had similar efficacy regardless of comorbid asthma or AERD (105).

Mepolizumab (Nucala®, anti-IL-5 mAb): Mepolizumab selectively binds to free IL-5 and blocks its ability to signal via the IL-5 receptor, mediating eosinophilic maturation and chemoattraction. The drug was approved 2021 as an adjuvant therapy for severe CRSwNP in Europe (106). The first clinical trial included 30 study participant and mepolizumab was intravenously injected 2QW for 4 weeks (107). The following clinical trial involved 107 individuals from six clinics situated in three European countries with a treatment period extended to 24 weeks (108). The overall effect reported by these RCTs includes a reduction in NPs, nasal symptoms such as nasal congestion, improvements in HrQoL as well as a reduced need for sinus surgery and systemic OCS.

The most convincing evidence comes from the most recent phase III trial which included 401 study participants from 93 clinics in 11 countries (109). The administration route was subcutaneous injection 4QW for 52 weeks and showed reduction in NP score (mean change -0.73), nasal congestion score (mean change -3.14), SNOT-22 (mean change -16.5), but no improvement in smell tests. In addition, the need for sinus surgery were less common in the treated group (9%) vs the placebo group (23%). The treatment effects were similar irrespectively of asthma, AERD, or blood eosinophil count.

Benralizumab (Fasenra®, anti-IL-5α receptor mAb): Benralizumab is approved to treat eosinophilic asthma but not CRSwNP (110) and disrupts the recruitment of eosinophils by targeting the α subunit of the IL-5 receptor. This mAb is afucosylated which causes the Fc region of the antibody to have high affinity towards Fc receptors, especially the FcγRIIIa, which leads to enhanced antibody-dependent cell-mediated cytotoxicity and almost complete depletion of eosinophils (111). Unfortunately, the first two phase II RCTs were unable to show any effect (112, 113). The following phase III clinical trial included 413 study participants from 102 clinics in Europe and US (114). Benralizumab was administered 8QW for 40 weeks. A small but significant reduction in NPs (mean change -0.57) and nasal congestion score (mean change -0.27) was observed. However, the study could not show an effect on SNOT-22 score or smell tests.

Other biologics of interest: An anti- IL-4α receptor mAb called CM310 have been investigated in a phase II RCT (115). As opposed to other RCTs, this study population consisted exclusively of patients with severe eosinophilic CRSwNP. The reported effects were similar to those observed for dupilumab which included NP score, nasal congestion score, smell test and SNOT-22. The

clinical efficacy of CM310 will be further assessed in a phase III RCT (NCT05436275).

Other biologics with similar mechanisms of action as described earlier is depemokimab, an anti-IL-5 mAb. The safety and tolerance for this mAb has been shown earlier in a phase I RCT on patients with asthma (116). This mAb is similar to mepolizumab but has an extended half-life leading to a prolonged suppression of IL-5 signalling. Evidence was also provided for higher affinity towards IL-5. This may suggest clinical efficacy with less frequent dosing as compared to mepolizumab. Depemokimab is currently investigated in a phase III clinical trial (NCT05281523).

Targeting the alarmin cytokine TSLP is becoming more popular. One anti-TSLP mAb currently investigated in a phase III RCT (NCT04851964) to treat CRSwNP is tezelumab. In previous reports, tezelumab has been shown to be clinically efficacious for type 2 inflammatory conditions, including asthma with no apparent phenotype, and it is approved as an add-on therapy for severe asthma (117).

In summary, the primary endpoint, NP score, was reduced in all phase III RCTs. The mean change from baseline ranged from -0.57 to -2.06, which spans over the definition of an adequate response to these treatments: ≥1-point reduction in NP score. Although a reduction in NP score by 1 is seemingly low, a majority of the RCTs have provided evidence that this reduction is associated with significant clinical improvements. Biologics also affected the patient's own experience of CRSwNP observed as clinically relevant improvements in HrQoL. All RCTs had an inclusion criterion that ensured recruitment of patients with severe disease (*e.g.*, 5≥NP score, 2≥nasal congestion score combined with at least two other persistent symptoms), and most of the RCTs also ensured to recruit recalcitrant patients (*e.g.*, previous FESS and eligible for revisions FESS). Lastly, it is important to point out that not all patients responded to the biologics, and it is currently not known exactly which patients would benefit from them.

1.6 CURRENT AND POTENTIAL BIOMARKERS

Biomarkers that are clinically available today in ENT clinics are primarily based on measurements of eosinophils. Patients with higher mucosal infiltration of eosinophils is defined as eosinophilic CRS (ECRS); a subtype associated recurrent disease (15) and less improvements in HrQoL after surgery (118). Blood eosinophils are readily available for counting and have been considered a

reasonable surrogate marker for type 2 inflammation (14), whereas others report that blood eosinophils have limited predictive value for ECRS (119-121) or disease recurrence (119). In addition, considering counting eosinophils in tissue, there is currently a lack of consensus regarding cut-off value to define ECRS (122, 123). It is unclear if ECRS reflects disease severity, recurrence, or solely an eosinophil count above reference values and there might be discrepancies in the definition amongst clinicians and histopathologists. Until the histological methodology has been standardised, the clinical use of counting eosinophils is limited.

The number of responders to biologic therapies is only around 60% (124-126). To date, identifying responders and non-responders to biologics beforehand has proven to be a difficult task. There is currently an unmet need for biomarkers that predict the treatment outcome of biologics and/or estimate the disease severity in order to monitor the response to treatment. It would be of great value to identify patients that are non-responsive, via biomarker measurements, so switching to other biologics could be done earlier than the 6 months suggested in EPOS (98). Those with a type 2 inflammation are presumed to benefit the most, however, it is currently difficult to define type 2 inflammation in a clinically meaningful way (127-129). As of today, there is a limited number of people who can be treated with these medicines. It is therefore of great importance to identify those that are in most need of biological therapy, *i.e.,* patients with severe and recalcitrant disease (130).

A wide range of different type 2 inflammatory markers have been associated with recurrent disease including IL-4, IL-5, IL-13, eosinophil granular proteins, and IgE (60, 131, 132). In contrast, others report that a mixture of type 2, and neutrophilic type 1 or type 3 inflammation is the main driver for recurrent disease (133, 134). A study with dexpramipexole, a drug which completely removes the number of eosinophils in both tissue and blood, did not affect the NP growth (135). These finding suggest that identifying recalcitrant CRSwNP may require additional sets of biomarkers, in combination with the classical type 2 markers.

An imbalance in the biosynthesis of various eicosanoids have been reported in CRSwNP (136-138). Recent reports suggest a link between recurrent disease traits and PGD_2 (139) and CysLT signalling in the mucosa (140). In the advent of biological therapy, it has been shown that anti-IL-4/IL-13Rα (dupilumab) not only reduces eosinophilic markers (104), but also the levels of pro-inflammatory LTE_4 and PGD_2 in urine (141). On the contrary, PGE_2 in nasal

secretions was elevated after anti-IL-4/IL-13Rα treatment, which could be one of the contributing factors for the clinical efficacy since PGE_2 is considered anti-inflammatory in the airways (142). Anti-IL-5 (mepolizumab) treatment gave similar effects on nasal and urinary eicosanoids, though mast cell numbers and PGE_2 appeared unaffected (143).

Taken together, these findings indicate a crucial link between type 2 inflammation and altered biosynthesis of certain eicosanoids potentially exacerbating the mucosal inflammation. Eicosanoids are less studied in CRSwNP and rarely considered for endotyping. This thesis aimed to investigate if eicosanoids could prove to be useful biomarkers to extend the knowledge about the pathophysiological mechanisms responsible for recurrent disease.

2 Research aims

2.1 OVERALL AIM OF THE THESIS

To explore biomarkers, changes in health-related quality of life and olfactory dysfunction that characterise the disease severity and progression in patients with severe chronic rhinosinusitis with nasal polyps (CRSwNP).

2.2 SPECIFIC AIMS OF THE INCLUDED PAPERS

Paper I: To assess whether eicosanoids in nasal secretions are associated with disease severity in patients with CRS with or without concomitant asthma or aspirin-exacerbated respiratory disease.

Paper II: To investigate whether changes over time in nasal eicosanoid levels are related to recurrent nasal polyps one year after sinus surgery. Secondly, to explore and identify potential eicosanoid-based endotypes in CRSwNP.

Paper III: To explore endotypes based on eicosanoids, cytokines, and chemokines in nasal tissue samples, and to evaluate their association with nasal polyp recurrence. Secondly, to evaluate a possible correlation between tissue and nasal secretion levels of eicosanoids.

Paper IV: To assess whether post-surgical changes in health-related quality of life, sense of smell, and other point-of-care tests could distinguish patients with recurrent nasal polyps one year after sinus surgery.

3 Materials and methods

3.1 OVERVIEW AND STUDY DESIGNS

This thesis consists of four papers. Study characteristics for each paper can be seen in table 1. Paper I, II, and IV were all considered cross-sectional. Paper II and IV may be considered longitudinal since data were collected in a longitudinal manner, but it was analysed cross-sectionally. Paper III also had some aspects of cross-sectional analyses but should mainly be seen as a prospective cohort study.

Table 1. Overview of paper I-IV.

Paper	I	II	III	IV
Study design	Cross-sectional	Cross-sectional	Prospective cohort	Cross-sectional
Participants	CRSwNP (n=54) CRSsNP (n=12) non-CRS (n=25)	CRSwNP (n=38)	CRSwNP (n=54) non-CRS (n=12)	CRSwNP (n=33)
Biological sample	Nasal secretions	Nasal secretions	Sinonasal tissues	Whole blood
Main type of data	Immunoassay results & medical records	Immunoassay results & medical records	Immunoassay results & medical records	Point-of-care tests, HrQoL & medical records
Main variable(s)	LTE_4, LTB_4, PGD_2, PGE_2 & 15(S)-HETE	LTE_4, LTB_4, PGD_2, PGE_2 & 15(S)-HETE	LTE_4, PGD_2, 15(S)-HETE & 17 cytokines	EBC, smell status, FeNO, RAND-36 & SNOT-22
Main type of statistical analyses	Kruskal-Wallis & Spearman´s correlation	Generalized estimating equations & Cluster analysis,	Cluster analysis, Kruskal-Wallis & logistic regression	Two-way ART ANOVA

Abbreviations: HrQoL=Health-related quality of life; LT=Leukotriene; PG=Prostaglandin; HETE=Hydroxyeicosatetraenoic acid; EBC=Eosinophil blood count; SNOT-22=Sinonasal outcome test-22; ART ANOVA=Aligned rank transformation analysis of variance.

3.2 STUDY PARTICIPANTS

All included study participants for this thesis were consequently recruited at the department of Otorhinolaryngology, Sophiahemmet Hospital. A total of 63 patients with CRSwNP, 12 patients with CRSsNP and 29 individuals without CRS were recruited for this thesis. Inclusion criteria were 18-75 years of age, CRS diagnosis and scheduled for FESS. Exclusion criteria were concomitant autoimmune disease(s), ongoing biological therapy and OCS treatment completed <1 month before FESS or visit to the clinic. Six patients with CRwNP were excluded due to OCS treatment, leading to a total of 57 patients with CRSwNP. The number of patients in each paper differed due to varying data and sample availability. Patients scheduled for septoplasty surgery without CRS served as controls.

Diagnosis of CRS was based on guidelines provided by EPOS (14). All CRS patients were eligible for full-house FESS (*i.e.*, sinus surgery) as they had persistent sinonasal symptoms for >12 consecutive weeks despite daily use of nasal corticosteroid spray and at least one previous course of OCS. The surgical procedure included uncinectomy, maxillary antrostomy, complete ethmoidectomy, sphenoidotomy and a Draf IIA frontal sinusotomy with computer assisted navigation. If there was no evidence of inflammation in the sphenoid sinuses, sphenoidotomy was not performed.

All patients with CRS were prescribed daily usage of nasal corticosteroid spray and saline irrigation throughout the study period. CRS diagnosis was further confirmed by endoscopy and CT scans for the identification of bilateral disease and paranasal obstructions. Bilateral NPs prompted for a CRSwNP diagnosis. Further information about the study participants was obtained from medical records and questionnaires about aspirin/NSAID-sensitivity, asthma, allergy, self-reported sense of smell and medications. AERD (*i.e.*, co-existence of NPs, asthma, and aspirin-intolerance) was defined by at least one previous incidence of airway hypersensitivity after aspirin/NSAID intake causing severe nasal congestion, coughing, and wheezing (26). Eleven patients in total were identified as AERD.

A subset of patients with CRSwNP (n=35) were followed six and 12 months after their sinus surgery. A timeline for the collection of data and biological samples can be seen in figure 3, including follow-up visits at six and 12 months after surgery.

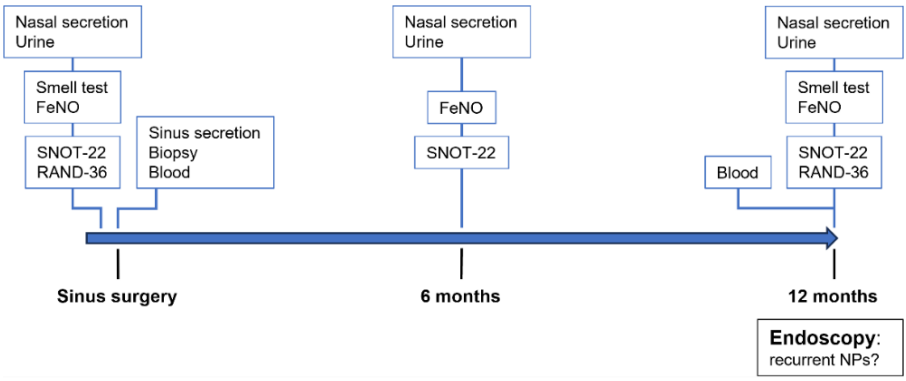


Figure 3. Overview of collected data and patient samples throughout the thesis.

Recurrent NPs was used as a proxy marker for recalcitrant CRSwNP and was identified endoscopically at a 12-month post-surgical visit. Moreover, previous sinus surgery for NP removal was also an important indicator for recalcitrant disease.

All patients were asked to refrain from NSAID intake two weeks prior to surgery. Furthermore, all CRS patients were given a three week course of OCS post-FESS (30 mg once daily week 1, 20 mg week 2, and 10 mg week 3). Any additional treatment with OCS post-FESS was rare within the study period (pre-surgery to 12 months after) and occurred only in six patients.

3.3 NASAL POLYP SCORE AND LUND-MACKAY SCORE

To quantify the extent of NP growth in the patients' paranasal sinuses, the Meltzer scoring system was used (144) denoted as nasal polyp (NP) score in this thesis. For each sinus, a score of 0 to 4 can be obtained, 0: no visible polyps; 1: smaller polyps in the middle meatus; 2: polyps that infiltrate the lower border of middle meatus; 3: larger polyps that infiltrates lower inferior turbinate or medial to middle turbinate; 4: inferior nasal cavity is completely obstructed (Figure 4B). The sum of right and left sinus yields a total NP score of 0 to 8.

The Lund-Mackay (LM) score was used to assess the paranasal obstructions in patients with CRS. This scoring system is based on CT scans and grades the degree of opacification (0: no opacification; 1: partial opacification; 2: complete opacification) present in the maxillary, ethmoid (posterior and anterior) sphenoid and frontal sinus regions (Figure 4A). The ostiomeatal complex is comprised of sinonasal structures that allow drainage of mucus and airflow between sinuses was also included, 0 or 2. The score in right and left sinuses are

summed together, yielding a total LM score ranging from 0 to 24. LM score is a simple graded staging system which has low inter-observer variability (145) and is used in many studies including clinical trials (109).

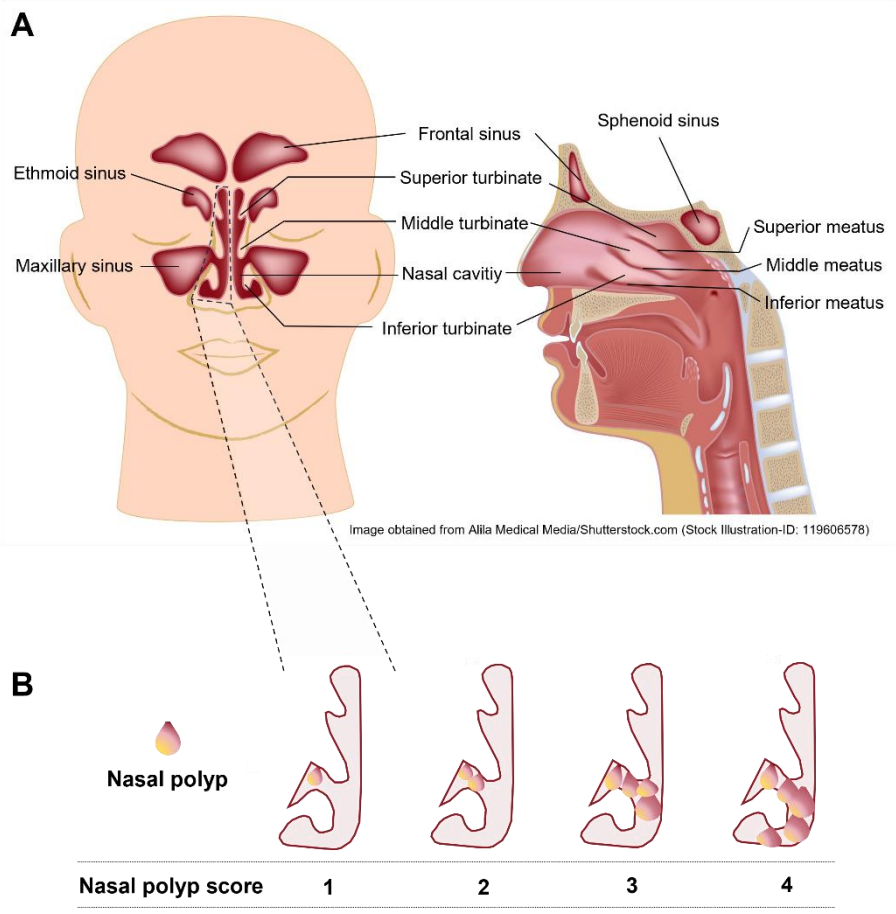


Figure 4. A) Anatomical regions of the sinuses. The ostiomeatal complex is not seen. B) Illustration of how the nasal polyp score is determined on the right sinus.

3.4 BIOLOGICAL SAMPLES

Nasal secretion: For the collection of nasal secretion, SalivaBio oral swabs (10 × 30 mm, 2 ml, Salimetrics, Carlsbad, USA) were pre-soaked in 800 µl saline solution, inserted into each nostril unto the bottom of the nasal cavity (almost extending into the inferior meatus, figure 4A), and left to absorb mucus for

10 min, followed by centrifugation (10,000g, 15 min). The obtained fluid was aliquoted and stored at -80 °C, prior to immunoassay(s).

Urine: Patients were asked to give a spot urine sample. For analytes of interest in this thesis, no diurnal variation has been reported and all samples were analysed for creatinine to compensate for diuresis variations (146). Urine samples were aliquoted and stored at -80 °C, prior to immunoassay(s).

Sinus secretion: Collection of sinus secretion were similar to nasal secretions but inserted into the middle meatus under anaesthesia at the time of surgery (Figure 4A). Liquid was obtained and processed as nasal secretions.

Blood: Blood was drawn by venipuncture from the decubital vein into EDTA tubes or with a finger-prick procedure using a lancet. Immediately after the collection of blood, point-of-care tests were performed.

Surgical biopsy: During sinus surgery a biopsy of NPs from the middle meatus sinus region (Figure 4A) were collected from patients with CRSwNP. Non-inflamed tissue was collected from the same sinonasal region from non-CRS controls. A fraction of each tissue sample was preserved in RNAlater® (Life Technologies, USA) to stabilize and protect cellular RNA from degradation prior to qRT-PCR (*i.e.*, gene expression analysis). The remaining part of the tissues were snap-frozen, 3-5 mm pieces, in liquid nitrogen and preserved at -80 °C until further preparation for subsequent immunoassay analyses.

Nasal tissue samples destined for eicosanoid analyses were thawed and added to a 95% MeOH solution (pH 3), in a ratio of 1 ml solution per 100 mg tissue sample, and homogenised with a TissueLyser LT (QIAGEN, Germany). Centrifugation of homogenates was performed at 3, 000 g for 10 min at 4 °C and the obtained supernatants were diluted 1:10 in water (pH 3) for loading onto C18 Sep-Pak columns (3ml volume, 200 mg silica sorbent, Waters Corp, USA). Columns were placed onto a VISIPREP™ SPE Vacuum Manifold (Supelco inc, USA), and lipids were eluted by adding a volume of 2 ml 95% MeOH. Eluents were then evaporated with a stream of nitrogen gas, reconstituted in ELISA buffer, and analysed for the content of eicosanoids immediately after.

Nasal tissue samples destined for cytokine and chemokine measurements were thawed and added to a PBS solution (Sigma Aldrich, St. Louis, MO, USA) containing protease inhibitors (c0mplete mini protease Inhibitor Cocktail,

11836170001, Roche, Switzerland) in a ratio of 1 ml per 100 mg tissue sample. Homogenisation was performed with the TissueLyser LT (QIAGEN, Germany). Homogenates were centrifuged at 3000 g for 10 min at 4 °C and the obtained supernatants were analysed for the content of 17 cytokines and chemokines.

3.5 IMMUNOASSAYS

Competitive ELISA: The determination of LTE_4, LTB_4, PGD_2, PGE_2, 15(S)-HETE and 11β-PGF2α (Paper I-III) were performed with competitive acetyl cholinesterase (AChE) enzyme linked immunosorbent assay (ELISA) kits (Cayman Chemical, Ann Harbor, MI, USA). These assays are based on the competition between the investigated free analyte (*e.g.*, LTE_4) and an analyte-AChE conjugate (*e.g.* LTE_4-AChE conjugate), denoted as a tracer (Figure 5). The following components are added to a 96 well plate pre-coated with a mouse mAb anti-rabbit IgG antibody: a fixed concentration of tracer and rabbit antiserum (*i.e.*, polyclonal antibody specific for the analyte) as well as patient samples. The amount of tracer bound to the antiserum will be inversely proportional to the amount of free analyte present in the patient sample. To detect the amount of tracer bound, acetylcholine (ACh) is added as a substrate which causes the enzyme, AChE, to catalyse the conversion of ACh to the coloured 5-thio-2-nitrobenzoic acid. The end-product is measured as an absorbance at 412 nm (Infinite M200 Pro-microplate reader, Tecan, Switzerland), being inversely proportional to the amount of free analyte (*e.g.* LTE_4) bound. The concentration of analyte in each patient sample was determined by a standard curve with known concentrations.

ELISAs for measuring LTE_4, LTB_4, PGD_2, PGE_2, 15(S)-HETE, and 11β-PGF2α had a limit of detection (LOD) at 7.8, 3.9, 2, 7.8, 58.9, and 1.6 pg/ml, respectively. Cross-reactivities were considered negligible for the anti-LTE_4, antibody (<0.01% for LTC_4 and D_4), anti-11β-PGF2α (<0.01% for PGF2α), and anti-15(S)-HETE (<0.01% for 15(R)-HETE). No information is available for anti-PGD_2 for tetranor-PGDM.

Sandwich ELISA: For the determination of EDN and Tryptase (Paper III), sandwich-ELISAs were performed (Immundiagnostik, Bensheim, Germany). The 96 well plates are pre-coated with a mAb capture antibody; anti-human EDN/tryptase (Figure 5). Patient samples and a secondary antibody (a peroxidase-conjugated rabbit anti-human EDN/Tryptase) are added, forming a sandwich of capture antibody – analyte – secondary antibody. Upon addition the substrate tetramethylbenzidine (TMB), the peroxidase converts TMB to a

colourful end-product (measured at 450 nm). The intensity of this absorbance signal is proportional to the amount free analyte in the sample, and the concentration of EDN/tryptase is determined by a standard curve. The LOD values for EDN and Tryptase were 0.75 and 0.25 ng/ml, respectively.

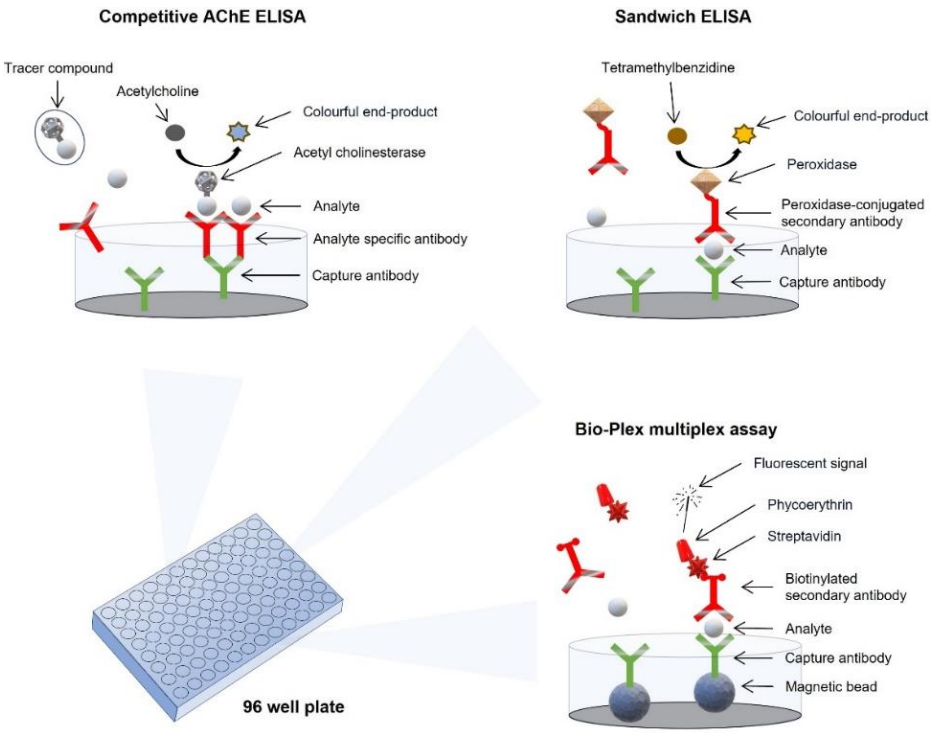


Figure 5. Illustration showing the principle of each immunoassay used in this thesis.

Bio-Plex multiplex assay: A magnetic bead multiplex assay was used (Figure 5) on the Bio-Plex 200 system for the analysis of 17 cytokines and chemokines (Hu 17-plex Cytokine Panel, Bio-Rad, Hercules, CA, USA)(Paper I and III). This is a sandwich-immunoassay with the capture antibody sandwich complex formatted on a magnetic bead (6.5 μm). An advantage with this method is that it allows for detection of multiple analytes simultaneously. Each specific set of beads has a distinct internal fluorescent dye, which the system uses to classify the beads during the readout. Addition of a biotinylated secondary antibody with affinity towards the analyte(s) of interest, as well as streptavidin-

phycoerythrin (SA-PE) conjugate, forming the final detection sandwich complex where PE serves as a fluorescent reporter.

The coupled bead suspensions are analysed with a dual-laser technique. A red laser (650 nm) is used to classify the bead, and the green laser (532 nm) is for the detection of the fluorescent reporter. The fluorescent signal is proportional to the amount of analyte in the patient sample, and the concentration is determined by a standard curve.

3.6 COLORIMETRIC ASSAYS

Creatinine colorimetric assay: For correction of diuresis variation, all urine samples were subject to creatinine measurements (Creatinine colorimetric assay, Cayman Chemical, Ann, MI, USA). The addition of alkaline pictrate react with creatinine, leading to the formation of a yellow end-product which is measured at 500 nm. This absorbance signal is proportional to the amount of creatinine in each patient sample and the concentration is determined by comparison to a standard curve. Levels of eicosanoids (*i.e.*, LTE_4 and 11β-PGF2α) in urine were presented as ng/mmol creatinine.

Bradford total protein assay: To correct for the variation of protein amount in each obtained tissue sample used for the multiplex assays, tissue samples were subject to total protein measurements using a simple colorimetric assay (Bradford total protein assay, Bio-Rad, Hercules, CA, USA). Data was presented as pg cytokine or chemokine/mg total protein.

3.7 QUANTITATIVE REAL-TIME PCR

Sinonasal tissue RNA was extracted with RNeasy kits (QIAGEN, Germany). The integrity and concentration of extracted RNA was assessed with the 2200 Agilent tape station system (Agilent Technologies, USA). 1 μg RNA was reverse transcribed into complementary DNA (High-Capacity cDNA reverse transcription kit, Applied Biosystem, USA). Quantitative real-time PCR was performed on the Applied Biosystem 7300 system using the TaqManTM Gene Expression Master Mix (Applied Biosystems). The following primer probes were used (Life Technologies): Hs01025999_g1 (LTC4 synthase; LTC4S), Hs00992727_g1 (microsomal glutathione S-transferase 2; MGST2), Hs00183950_m1 (hematopoietic PGD synthase; HPGDS), Hs00993765_g1 (15-Lipoxygenase; 15-LOX), Hs00153988_m1 (15-Lipoxygenase-2; 15-LOX-2), Hs00377726_m1 (Cyclooxygenase-1; COX-1), Hs00153133_m1 (Cyclooxygenase-2; COX-2), Hs00272624_s1 (CysLT Receptor 1; CysLT1), Hs01867513_s1 (PGD_2 Receptor 2; DP2 or CRTH2) and Hs99999903_m1 (β-

actin). Melt curve analysis was performed on each sample during qPCR analysis to ensure quality of the amplification products. Gene expression levels were calculated using the ΔCT method (147) with the housekeeping gene β-actin as endogenous control (Paper III).

3.8 SMELL TEST

A smell test was performed in accordance with instructions from the test provider (Burghart Sniffin' Sticks Extended Test n-Butanol, MediSense, Groningen, Netherlands). The smell test consists of three individual subtests for odour threshold, odour discrimination, and odour identification. For each subtest various felt-tip pens with different odours are brought under the nose of the participant. Each pen is presented to the participant´s nose for ~3 s with a 30 sec interval between pens.

The Threshold (T) test involved 16 sets of triplet pens, each with a progressively diminishing odour intensity from the first to the 16th pen. From each triplet of pens, there is one odorous pen and two blank pens, and the participant's task is to identify the odorous pen.

The Discrimination (D) test also involves 16 sets of triplet pens, although during this test each triplet of pens have odours. Two of the pens have the same odour whereas one of the pens has a different odour. Thus, the participant has the task of distinguish the pen with the unique odour from the other two.

The Identification (I) test consists of 16 individual pens with everyday smells where the subject´s task is to identify the odour from four alternatives.

The Burghart Sniffin' Sticks smell test is validated for clinical use to assess the degree of smell loss (36). Scores from the T (0-16), D (0-16) and I (0-16) tests are obtained and can be summed together yielding a total TDI score of 0 to 48 to determine normosmia (30-48), hyposmia (16-30), or anosmia (0-16). TDI scores were presented in paper I, II and IV.

3.9 ANALYSIS OF FRACTIONAL EXHALED NITRIC OXIDE

Nitric oxide (NO) is formed from arginine by NO synthase (NOS), an enzyme that is essential for respiratory physiology (148). The inducible form of NOS (iNOS) is expressed and maintained at physiological levels by IFN-γ. Th2 cytokines (namely IL-4 and IL-13) alters the intracellular regulatory signalling pathways of iNOS expression causing an overproduction of NO. Thus, measurement of fractional exhaled NO (FeNO) has gained great clinical

interest as a biomarker of type 2 inflammation in the lower airways. In the thesis, a portable device that uses electrochemical sensors (NIOX VERO, Aerocrine AB, Solna, Sweden) for the quantification FeNO was used (paper I-IV). Study participants were asked to inhale their total lung capacity followed by exhalation into the device's mouthpiece.

3.10 ASSESSMENT OF HEALTH-RELATED QUALITY OF LIFE

For the assessment of HrQoL, this thesis included two questionnaires: the disease-specific SNOT-22 intended for individuals with CRS and RAND-36 for generic HrQoL. Data from RAND-36 was exclusively presented in paper IV, while SNOT-22 data was presented in paper I, II, and IV.

SNOT-22: The Sinonasal Outcome Test (SNOT-22) is a disease-specific questionnaire that considers sinonasal symptoms, psychological- and sleep dysfunctions (149, 150). SNOT-22 is considered a reliable tool to assess symptom burden, HrQoL, and treatment response. Each item (question) in SNOT-22 (table 3) was answered according to a Likert 0-5 scale (0: No problem, 1: Very mild problem, 2: Mild or slight problem, 3: Moderate problem, 4: Severe problem, 5: Problem is as bad as it can be), yielding a total SNOT-22 score ranging from 0 to 110. Higher scores indicate worse sleep, psychological functioning and symptom severity.

In addition, scores from five SNOT-22 subdomains are provided according to previous validation studies (150, 151): Rhinologic symptoms (items 1-5 and 8, with a score range of 0-30), Extra-nasal symptoms (items 6, 8 and 21, with a score range of 0-15), Ear/facial symptoms (items 2, 9-12, with a score range of 0-25), Psychological dysfunction (items 16-22, with a score range of 0-35), and Sleep dysfunction (items 13-17, with a score range of 0-25). Although, the current thesis used the above-mentioned SNOT-22 subdomains, other subdomain structures are present in the literature (152). However, most of the alternative subdomain structures are similar to the ones used here, with only slight differences in the naming and items used for each subdomain.

Of note, SNOT-22 is validated to be used in various languages (153), but the Swedish version is not yet fully validated in terms of its subdomain structure (154, 155). The clinically minimally important difference (MCID) of the change in total SNOT-22 score has been estimated at 9 (149) and for the subdomains scores around 2.4-3.8 (156).

Table 3. The order of items in the Swedish version of the SNOT-22. Each item is a description of a symptom/issue answered with a 0-5 Likert scale.

Item No.	Condition
1	Need to blow nose
2	Sneezing
3	Runny nose
4	Congestion of nose
5	Loss of sense of smell/taste
6	Cough
7	Post-nasal discharge
8	Thick nasal discharge
9	Ear fullness
10	Dizziness
11	Ear pain
12	Facial pain/ pressure
13	Difficulty falling asleep
14	Waking up at night
15	Lack of a good night's sleep
16	Waking up tired
17	Fatigue
18	Reduced productivity
19	Reduced concentration
20	Frustrated/restless/irritable
21	Sad
22	Embarrassed

RAND-36: During the 1980s the RAND Medical Outcomes Study created the Short-Form 36-item (SF-36), and in the following years a publicly available version called RAND-36 (157). For this thesis, a validated Swedish version of RAND-36 was used (157). This questionnaire consists of 36 items (questions) that covers 8 different concepts of health (multi-item subscale scores): 1) physical functioning (PF), 2) limitations in role functioning due to physical health issues (role functioning/physical, RP), 3) bodily pain (BP), 4) general health (GH), 5) energy/fatigue (Vitality, VT), 6) social functioning (SF), 7) limitations in role functioning due to emotional distress (role functioning/emotional, RE), and 8) emotional well-being (Mental health, MH). All 8 subscales may be referred to two overarching domains, physical (subscales

PF, RP, and BP) and emotional health (MH, RE, and SF), where subscales VT and GH are referred to both domains.

The number of items used for each subscale score varies from two to 10. Responses from each item are recoded into a score, summed, and divided by the number of items answered yielding a subscale score ranging from 0 (worst possible health state) to 100 (best possible health state) in accordance with the standard scoring system provided by RAND corporation. When answers from more than half of the items are missing, the subscale score is not calculated.

The MCID for RAND-36 has not been established in CRS. For each subscale of RAND-36/SF-36 the MCID ranges between 3-5 (158), 2-7 (159) or 3-25 (160) depending on the patient group studied.

3.11 POINT-OF-CARE TESTS

Numerous point-of-care tests were used in the current thesis which included measurements of Haemoglobin (HemoCue AB, Ängelholm, Sweden), C-reactive protein (QuickRead go instrument, Aidian Oy, Espoo, Finland), and white blood cell differential count (WBC DIFF System, HemoCue AB). Blood eosinophil count (cell count/μl) were of most interest for the thesis. These tests were performed on whole blood samples.

3.12 STATISTICAL ANALYSIS

For data on clinical characteristics and inflammatory mediators, medians with interquartile ranges (IQRs) and counts with percentages were used in paper I-IV. All graphical presentations were created in in GraphPad Prism V.8.4.2 (GraphPad Software, Bostin, USA) and inferential statistics were done in R (V.4.0.3.) and SPSS (V.27.0.1.0, IBM Corp, Armonk, NY, USA). A two-tailed p-value of <0.05 was considered statistically significant throughout the thesis. For an overall presentation of statistical analyses used in each paper, see table 1.

Non-parametric tests were used in all papers since the data on inflammatory mediators and clinical characteristics were not normally distributed. Throughout this thesis, differences in proportions between groups were performed with Fisher's exact test with multiple pairwise comparisons adjusted with Benjamini-Hochberg for multiple testing. In paper I-III, differences in continues variables between more than two groups were analysed with Kruskal-Wallis tests followed by post-hoc Dunn's tests adjusted with Benjamini-Hochberg for the multiple testing. Paper I and IV also utilised aligned rank transformation analysis of variance (ART-ANOVA) which is a non-parametric

method suited to test multiple independent variables, interaction between variables, as well as repeated measures. To test for the relatability between continuous variables a Spearman's rho correlation test was performed in paper I, III and IV.

Generalised estimating equations: Generalised estimating equations (GEE) was used in paper II to assess the mean change over time of LTE_4, PGD_2, and 15(S)-HETE, from pre-surgery to six and 12-month post-surgery, in patients with recurrent NPs compared to patients without recurrent NPs. GEE modelling is well suited for repeated measures were data at each time point (pre-surgery, 6- and 12-month post-surgery) from any one individual is likely to be correlated. GEE provides a marginal estimated group mean where this within-subject correlation over time is taken into account by assuming a correlation matrix structure fitted for the data. Of importance, GEE can handle skewed data and does not rely on the outcome measure to be normally distributed (161), which was another reason for the choice of this method. Each time point (pre-surgery, six and 12-month post-surgery) and the occurrence of NP recurrence as well as an interaction term between the two were considered independent variables. Results from the GEE models were presented with means and standard deviation.

Cluster analysis: The purpose of cluster analysis was to explore the heterogeneity of the biomarker data and to reveal distinct patient subgroups that have clinical relevance. This analysis was conducted in both paper II and III. Positive skewness is common in biological data (162) which was also the case in paper II and III. To account for skewness and different scales in the data, a $\log_{10}$-transformation followed by standardization into Z-scores was performed.

A principal component analysis (PCA) was performed to transform a correlated dataset into a smaller subset of uncorrelated variables called principal components (PCs). Conducting PCA beforehand was crucial since clustering based on correlated variables tends to weight variables disproportionally (163). A number of PCs are obtained, where each PC corresponds to a fitted line that are correlated to one or several biomarkers. Thus, if one PC is positively correlated to a number of biomarkers, an increase value of this PC corresponds to increase levels of all these biomarkers. It is important to ensure that the PCs can explain a fair amount of the original dataset and that each PC have an eigenvalue >1 (*i.e.,* each PC explains more than one of the biomarkers related to them).

An agglomerative hierarchical cluster analysis was then performed using the PC scores from each patient as input variables. The cluster analysis was based on the Ward's linkage method; an algorithm that merge individuals into clusters that yield the lowest within-cluster variation (*i.e.*, individuals with similar data). The optimal cluster solution was primarily determined by the agglomerative coefficient (AC); a measure of within-cluster variation obtained after each merger. A large increase in AC indicates merging of two highly dissimilar cluster. The cluster solution prior to this large increase in AC was considered the optimal number of clusters to be retained. The separation of clusters was evaluated through PC plots and dendrograms (See paper II and III for further details).

Logistic and linear regression models: In paper III, the binary outcome NP recurrence was assessed with logistic regression models. Clusters, PC scores, and clinical factors were used as independent variables in the analyses. In paper III a three-cluster solution was obtained dividing the study population into cluster 1, 2, and 3 (recoded into a three-factorial variable), where cluster 3 was treated as the reference category. The main reason for this decision was that the number of patients with NP recurrence was notably low in cluster 3.

The likelihood of NP recurrence was estimated with Odds ratios (ORs) which is a measure of the association between a predictor and an outcome. Hence, the OR of NP recurrence for cluster 2 and 1 was defined by the odds of NP recurrence in cluster 2 and 1 as compared to the odds of NP recurrence in cluster 3. We also tested whether the patient clusters were associated with LM score by multiple linear regression. The results were presented with β-coefficients which represented the estimated mean change in the LM score in cluster 2 and 1 as compared to the reference category, cluster 3.

4 Ethical Considerations

This research project was approved by the Swedish Ethical Review Authority (Dnr. 2017/1461–31, 2019–06,185) and was in line with the Declaration of Helsinki (164) involving medical research with human subjects and the four principles of medical ethics, i.e. respect for autonomy, non-maleficence, beneficence, and justice (165). The participants were informed in advance about the purpose of the studies, and the procedures that will be used. At a routine visit to the clinic, with the Ph.D. student and the treating physician present, all subjects were orally informed about what participation would entail with time for questions, if needed, and gave written informed consent before they were enrolled. The participants were asked to authorise the saving of removed tissue from the nasal mucosa as well as the collection of blood, urine and nasal secretions. All subjects were informed that participation was voluntary, and they had the right to withdraw at any time without giving a reason, which would not impact their future care. No compensation was given to the study participants.

All patient data was stored according to GDPR (166) and stored on a password-protected computer. Samples were stored in Sophiahemmet Biobank according to Biobanks in Medical Care Act (167) and with ethical permission from Swedish Ethical Review Authority (Dnr. 2016/1748-31/4). All samples were coded and a corresponding code list was kept in a password-protected file that was not accessible to anyone other than the research group.

The participants were also informed about potential benefits and risks of participation. Collection of surgically removed tissues did not involve any additional discomfort for the participants, since the surgery was part of their visit at the clinic. For venipuncture and collection of blood the procedure may involve a small risk of short-term pain and bruising. However, blood sampling could be done from the vein access used to administer the anaesthetics, meaning no additional needle stick was required. No major discomfort is experienced during smell tests or measurements of FeNO as these procedures involve breathing into a mouthpiece and sensing harmless odours through the nose. Collection of nasal secretions can cause discomfort by the absorbent swab inserted into the nose for ten minutes. In such cases, warm physiologic saline solution was offered which had vasoconstrictive and analgesic effect. Answering questionnaires regarding living habits, symptoms, and quality of life can be perceived as an invasion of privacy. It was therefore carefully ensured

that participants understood the meaning of the questions, and they were also given the opportunity to review the questions before they gave consent.

The subjects were informed that they could not expect any immediate benefit from participation. In the long term, their participation will potentially lead to extended knowledge about who could benefit from treatment with biologic drugs instead of repeated surgery and side-effect causing corticosteroids. Accordingly, the potential benefits were deemed to outweigh the risks of participation.

5 Results and comments

This thesis consists of four papers that addresses eicosanoids as biomarkers (Paper I-III) or QoL measures and the result from other clinical point-of-care tests as markers (Paper IV) for disease severity and recalcitrant traits of CRSwNP. The following section describes and comments on the main results provided from these studies.

5.1 PAPER I

Paper I focused on the release of the eicosanoids LTE_4, LTB_4, PGD_2, PGE_2, and 15(S)-HETE into nasal secretions in patients with varying degree of disease severity which was primarily evaluated based on the NP score (ranging from 0-8). Data on cytokine measurements in nasal secretions as well as eicosanoid measurements in urine were also included. In addition, nasal levels of eicosanoids were compared to other clinically relevant markers such as blood eosinophil count, smell test score and FeNO which all are linked with a worsened progression of CRSwNP and burden of comorbid asthma (121, 163, 168).

A total of 25 non-CRS controls, 12 patients with CRSsNP, 11 CRSwNP patients with a NP score ≤ 4 referred as CRSwNP-low, 32 CRSwNP patients with a NP score ≥ 5 referred as CRSwNP-high, and 11 patients with AERD were included. These groups of patients were primarily different in regards of asthma prevalence, number of previous FESS, blood eosinophil count, LM score, smell test score, and FeNO (Table 4). The phenotypic differences (*i.e.*, CRSsNP, CRSwNP and AERD) have been reported earlier (27) where those with AERD usually experience the most severe sinonasal inflammation. This includes increased swelling of the sinuses evident from higher LM score, loss of smell evident from the lower smell test scores, frequent need of FESS, and higher burden of type 2 inflammation evident from the increased blood eosinophil count and FeNO (Table 4).

Results showed that levels of LTE_4 were elevated in nasal secretions from individuals with AERD, whereas levels of PGD_2 were primarily elevated in patients with CRSwNP-high but tended to be higher in AERD as well (Figure 6).

Table 4. A selection of clinical parameters in non-CRS controls and patients with CRSsNP, CRSwNP-low, CRSwNP-high or AERD from paper I.

	Non-CRS controls	CRSsNP	CRSwNP-low	CRSwNP-high	AERD	P-value
N	25	12	11	32	11	
NP score	-	-	4 (4-4)	6 (6-8) **L**	6 (5.5-6.5) **L**	<.01
Asthma	0 (0%)	2 (16.7%)	4 (36.4%)	19 (59.4%) **C S**	11 (100%) **C S L H**	<.01
Previous FESS	0 (0%)	3 (25%)	1 (9%)	15 (46.9%) **C**	8 (72.7%) **C L**	.012
N	-	11	10	28	10	
Blood eosinophil count/µl	-	200 (100-350)	450 (325-500)	400 (300-625) **S**	550 (400-750 **S**	.016
N	-	10	10	32	11	
LM score	-	3.5 (2-14)	14 (13-17)	18 (15-21) **S**	21 (19-22) **S L**	<.01
N	-	-	6	11	7	
Smell test score	-	-	22 (5.6-26)	13 (7.0-17)	1.0 (0.0-2.0) **L H**	.014
N	13	-	6	11	6	
FeNO, ppb	13 (10-19)	-	30 (16-41)	37 (20-76) **C**	84 (44-90) **C**	<.01

Continues variables are presented as median with interquartile range and binomial variables are presented as count with percentage. Patient group differences were tested with either Kruskal-Wallis tests or Fisher´s Exact tests, both adjusted with Benjamin-Hochberg for the multiple-comparison tests between the patient groups. C = P<.05 compared with Non-CRS controls, S = P<.05 compared with CRSsNP, L = P<.05 compared with CRSwNP-low, H = P<.05 compared with CRSwNP-high.

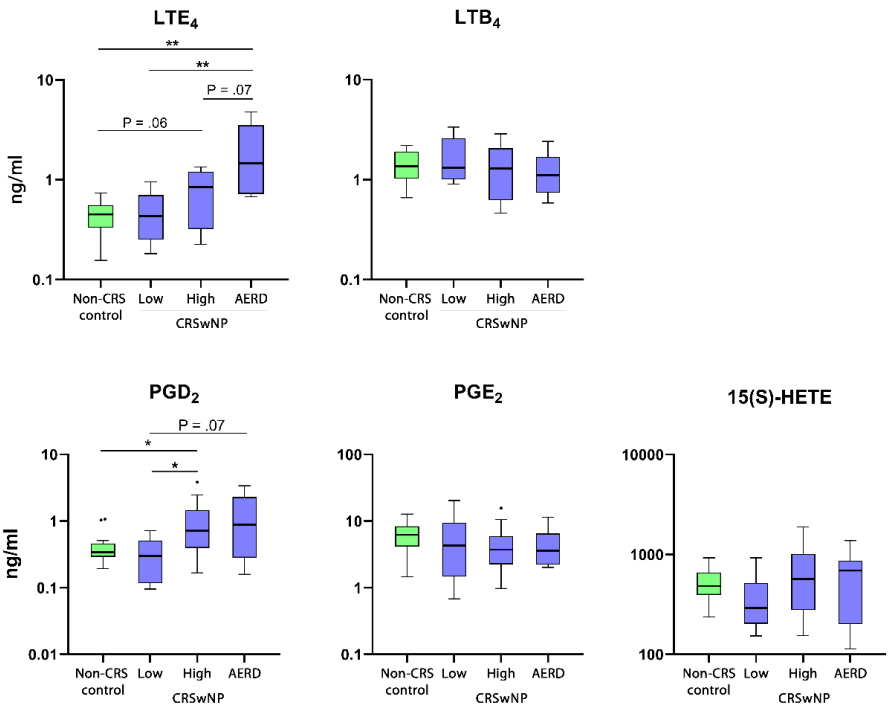


Figure 6. Levels of leukotriene (LT) E_4, LTB_4, prostaglandin (PG) D_2, PGE_2, and 15(S)-hydroxyeicosatetraenoic acid (HETE) measured in nasal secretions from patients with non-CRS (n=16), CRSwNP-low (n=9), CRSwNP-high (n=19), and AERD (n=8).

Furthermore, low-to-moderate correlations were observed between NP score and levels of LTE_4 (*r=0.35, p=0.036*), PGD_2 (*r=0.40, p=0.015*) and 15(S)-HETE (*r=0.43, p=0.009*). Assessing other clinical factors showed that LTE_4 was negatively correlated with smell test score (*r=-0.52, p=0.01, n=24*) as well as positively correlated with FeNO (*r=0.63, p=0.002, n=23*) and blood eosinophil count (*r=0.39, p=0.02, n=36*). Both LTE_4 and PGD_2 have pro-inflammatory effects in the airways that include increased mucosal oedema, hypersecretion, and enhancement of the type 2 inflammation which are implicated in the development of CRSwNP (140, 169). The increased levels of the eicosanoids may in part be responsible for higher LM score, blood eosinophil count and FeNO values in patients with CRSwNP-high and AERD. In addition, the higher inflammatory load of CRSwNP-high and AERD and the resulting swelling of the mucosa may both be contributing factors to the patients' loss of smell (170).

Of importance, it is still uncertain to what extent other clinically relevant factors influenced the nasal eicosanoid levels. It is possible that the combination of all clinical factors (presented in table 4) caused the elevations of LTE_4, PGD_2 and 15(S)-HETE rather than solely the NP severity. To address at least one of these clinical factors, a subgroup analysis was performed on CRSwNP patients with and without comorbid asthma, excluding those with AERD, to estimate the potential influence of asthma. Results showed that none of the eicosanoids measured in nasal secretions were significantly different between patients with and without asthma (see Paper I). Analyses of urinary 11β-PGF2α (the main C20 urinary PGD_2 metabolite), showed significantly increased levels in patients with asthma relative to those without asthma (*p=0.017*). When the same patients were grouped in CRSwNP-high and CRSwNP-low instead, results showed that patients with CRSwNP-high had significantly higher urinary 11β-PGF2α (*p=0.047*). Lastly, patients with both CRSwNP-high and asthma were those with the highest urinary 11β-PGF2α. Hence, this could mean that elevations of urinary 11β-PGF2α in severe CRSwNP are dependent on the presence of comorbid asthma, whereas nasal secretions would reflect the inflammation in the sinuses independently of asthma.

Analysis of urinary LTE_4 showed no significant difference between patients with and without asthma, nor between any of the other patient groups: e.g. non-CRS controls vs AERD (*p=0.51*). This is in contrast to others showing high basal urinary levels of LTE_4 in AERD (81, 171). The discrepancy of results could be due to several reasons, but perhaps mainly due the fact that AERD was not accurately diagnosed with aspirin provocation in the present study. This was a relatively small group of patients that may not represent AERD accurately.

It should be pointed out that biological samples were not obtained from all included patients presented in table 4. This was most notable for the nasal secretion samples which was missing from 9 non-CRS controls, two from CRSwNP-low, 13 from CRSwNP-high, and three from CRSwNP-AERD. However, patients from whom we had access to nasal secretions showed similar clinical characteristics (data not shown) as the total study population presented in table 4. In addition, there was also notable missing data regarding FeNO and smell test scores as indicated in table 4. These circumstances add some degree of uncertainty regarding the degree of airway type 2 inflammation and loss of smell in patients with CRSwNP-high and AERD.

In conclusion, findings indicate an overall worsened disease in those with higher NP severity or AERD with elevated nasal LTE_4 and PGD_2, loss of smell, FeNO and LM score. Nasal eicosanoids, namely LTE_4 and PGD_2, may be used to distinguish between patients with varying NP severity and AERD. The nasal level of LTE_4 may be a significant contributor to the degree of smell loss and FeNO in severe CRSwNP.

5.2 PAPER II

As levels of eicosanoids (namely LTE_4, PGD_2 and 15(S)-HETE) were associated with NP severity as well as other markers of disease severity (Paper I), the focus of paper II was to investigate if similar changes of eicosanoid levels occur in recalcitrant patients. Secondly, this paper also explored potential eicosanoid-based endotypes in CRSwNP by cluster analysis on pre-surgical eicosanoid levels in nasal secretions.

The study population that was included were the same patients as seen in paper I, with the addition of two newly recruited study participants. A total of 38 patients with CRSwNP were included for analysis. This was a follow-up study with measurements at six and 12 months post-surgery that included analysis of LTE_4, PGD_2 and 15(S)-HETE in nasal secretions, urinary LTE_4 and 11β-PGF2α, as well as sinonasal endoscopic evaluations to identify recurrent NP growth. Recurrent NPs were defined as having a NP score of ≥ 1 and was used as a proxy marker for recalcitrant CRSwNP. In addition, pre-surgical levels of LTE_4, LTB_4, PGD_2, PGE_2, and 15(S)-HETE from paper I were re-analysed in paper II with focus on comparing between patients with and without NP recurrence.

Fifteen out of the 38 patients (~40%) were identified as patients with recurrent NPs 12 months post-surgery. Patients with and without recurrent NPs were mainly different in regards of sinonasal swelling and loss of smell as those with recurrent NPs had notably higher LM score ($p=0.006$) and self-reported anosmia ($p=0.016$). There were some missing data on smell test scores but patients with recurrent NPs tended to have lower scores ($p=0.068$), and all these patients would be clinically classified as anosmic. A blood eosinophil count ≥ 300/ml is a suggested cut-off for ECRS (172). This value was exceeded in both patient groups and tended to be higher in patients with recurrent NPs ($p=0.085$). Moreover, these groups were comparable in terms of age, sex, allergy, asthma, AERD, FeNO, SNOT-22 and the incidence of previous sinus surgery (for details see paper II).

Figure 7 shows the mean change over time in the levels of 15(S)-HETE, PGD_2, and LTE_4, from pre-surgery to six and 12 months post-surgery. From pre-surgery to 12 months post-surgery, the decrease in the levels of 15(S)-HETE and PGD_2 were more pronounced in patients with recurrent NPs as compared to patients without recurrent NPs (*p=0.015, p=0.002*). Hence, sinus surgery may have a significant effect on the mucosal eicosanoid levels in those with recurrent NPs. The mean change over time in those without NP recurrence were negligible, possibly because these levels were already at lower levels before surgery.

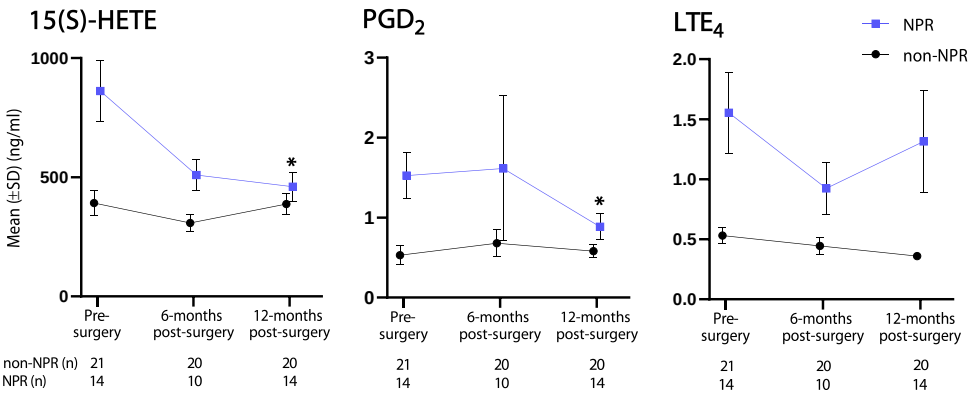


Figure 7. Mean change of nasal 15(S)-HETE, PGD_2, and LTE_4 over time from pre-surgery to 6 and 12 months post-surgery, in patients with nasal polyp recurrence (NPR) as compared to patients with non-recurrence (non-NPR).

The mean change in the levels of nasal LTE_4, from pre-surgery to 12 months post-surgery, did not significantly differ between patients with and without NP recurrence. However, from pre-surgery to 6 months post-surgery, patients with recurrent NPs tended to have a more pronounced decrease of LTE_4 than patients without recurrent NPs (*p=0.09*, Figure 7). From six to 12 months post-surgery, mean change LTE_4 appeared to increase again at 12 months post-surgery in patients with recurrent NPs. Thus, a characteristic feature of regrowing NPs may be that the mucosa rapidly restores its excessive production of LTE_4. A similar pattern for 15(S)-HETE and PGD_2 could not be detected within the timeframe of 12 months after surgery (Figure 7).

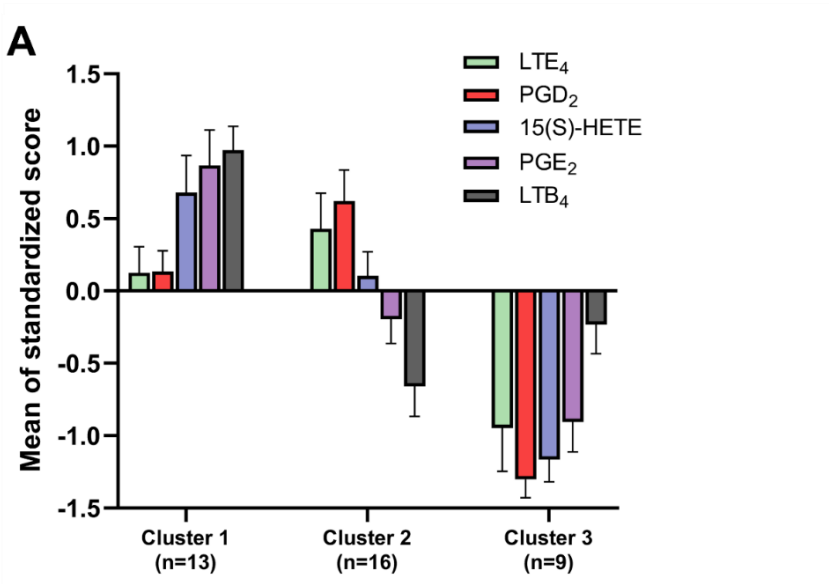
CysLTs are highly produced by eosinophils (173), and an increased number of eosinophils in the tissue is associated with increased levels of LTE_4 and recurrent disease in patients with CRSwNP (136, 174). Thus, it is possible that the LTE_4 levels measured in this study originated from newly recruited eosinophils to the tissue. Mast cells are the main source of PGD_2, whereas the enzyme 15-LOX, whose activity reflects the amount of 15-HETE in the tissue, is abundantly expressed in epithelial cells in CRSwNP (77).

Similar to results in paper I, the urinary levels of LTE_4 were not altered between the patient groups. Urinary levels of 11β-PGF2α, the PGD_2 metabolite, were not altered between the patient groups either. Importantly, urinary LTE_4 and 11β-PGF2α remained at the same level from pre-surgery, to six and 12 months after, indicating that surgical removal of the mucosa did not affect the urinary levels. These findings further support the notion that measuring eicosanoids in nasal secretions is preferable during inflammatory characterisation of patients with CRSwNP, as proposed earlier in paper I.

The levels of nasal 15(S)-HETE, PGD_2 and LTE_4 at each time point (*i.e.*, pre-surgery, six and 12 months post-surgery) were also compared between the patient groups (for details see paper II). Patients with recurrent NPs had significantly higher levels of LTE_4 at pre-surgery ($p=0.003$), at 6 months ($p=0.035$) and at 12 months post-surgery ($p=0.026$) as compared to those without recurrent NPs. Furthermore, patients with recurrent NPs had significantly higher levels of 15(S)-HETE at pre-surgery ($p<0.001$) and at six months post-surgery ($p=0.008$), whereas levels of PGD_2 were only increased at pre-surgery in patients with recurrent NPs ($p=0.002$).

To explore the heterogeneity of the eicosanoid values a cluster analysis was performed on the patients' pre-surgical eicosanoid data. This analysis revealed three potential clusters of patients (cluster 1-3) characterised by distinct eicosanoid patterns (Figure 8A) with clinically important differences.

Patients in cluster 2 had notably higher incidence of NP recurrence (*56.2%*) which was significantly higher than cluster 3 (*0%, $p=0.023$*) and tended to be higher as compared to cluster 1 (*46.3%, $p=0.069$*). The incidence of previous need of sinus surgery were strikingly more common in cluster 2 (*75%*) and tended to be higher than cluster 3 (*33.3%, $p=0.131$*), and cluster 1 (*30.8%, $p=0.081$*), which further suggest that most patients of cluster 2 indeed had recalcitrant CRSwNP.



B

	Cluster 1 (n=13)	Cluster 2 (n=16)	Cluster 3 (n=9)	Non-CRS controls (n=16)
LTE_4	0.70 (0.48-0.97) *	0.91 (0.62-1.26) *	0.26 (0.24-0.49)	0.45 (0.35-0.55)
PGD_2	0.53 (0.51-0.91) *	0.97 (0.57-2.43) *	0.13 (0.11-0.16) *	0.34 (0.29-0.43)
15(S)-HETE	923.6 (399.7-1099)	565.0 (399.2-687.8)	178.9 (154.7-226.7) *	482.5 (407.5-602.5)
PGE_2	9.12 (5.98-10.6)	3.41 (2.15-4.76) *	1.81 (1.55-2.37) *	6.21 (4.49- 8.23)
LTB_4	2.06 (1.73-2.44) *	0.81 (0.60-1.07) *	1.10 (0.90-1.28)	1.37 (1.07-1.86)

Significantly higher than two other clusters
Significantly higher than one other cluster
Significantly lower than one other cluster
Significantly lower than two other clusters

* p <.05 relative to Non-CRS controls

Figure 8. Pre-surgical levels of eicosanoids in nasal secretions from each patient cluster. A) Log-transformed and standardized (z-scores) values of eicosanoids presented as means with standard deviation. B) Raw values of eicosanoids (ng/ml) presented as median with interquartile ranges.

The patient clusters showed no difference in allergy, asthma, AERD, or in the disease severity markers NP score and LM score. FeNO levels were higher in cluster 2, suggesting a type 2 inflammatory asthma, but these differences were not significantly different across the clusters. Similarily, scores from smell tests

and SNOT-22 questionnaires indicated higher levels of impaired sense of smell and HrQoL in cluster 2, but the difference lacked statistical significance. Blood eosinophil count also tended to be higher in cluster 2 relative to cluster 3 (*p=0.083*). Nonetheless, all these clinical variables point towards the same trend, *i.e.*, that patients in cluster 2 represented the more severe cases within this study population.

Furthermore, patients in cluster 2 differentiated from those in cluster 1 primarily by significantly lower levels of PGE_2 and LTB_4, while cluster 2 differentiated from cluster 3 mainly by significantly higher levels of LTE_4 and PGD_2 (Figure 8B). Non-CRS controls from paper I are also included in figure 8B to highlight that the lipid mediator profiles from the patient clusters were distinguishable from individuals without CRS. LTB_4 is a strong neutrophil chemoattractant (175), whereas LTE_4 and PGD_2 are both linked with an enhanced type 2 inflammation that involves activation of eosinophils and of mast cells in the mucosa (67, 78). In contrast, PGE_2 may have anti-inflammatory effects as it supresses type 2 cytokine production and attenuates hyperproliferative fibroblasts which are potentially involved in the tissue remodelling during NP growth (176). Taken together, the eicosanoid profile of cluster 2 suggested an increased type 2 inflammation, potentially due to elevated infiltration of eosinophils or activation of mast cells, while the involvement of neutrophils appeared low.

In conclusion, this paper suggests that post-operative measurements of LTE_4 in nasal secretions may be used for monitoring the progression of early NP regrowth. A distinct nasal eicosanoid profile characterised by elevated LTE_4 and PGD_2 as well as lowered levels of PGE_2 and LTB_4 may indicate severe recalcitrant CRSwNP.

5.3 PAPER III

To further investigate the role of eicosanoids as important biomarkers for CRSwNP endotypes, a similar cluster analysis as in paper II was performed on the levels of LTE_4, PGD_2, 15(S)-HETE and 17 cytokines in tissue samples. Specifically, paper III focused on biomarker measurements on surgically removed NP tissue from patients with CRSwNP and mucosal tissue samples from non-CRS patients. Patient clusters were further evaluated based on their association with NP recurrence, defined as endoscopically identified NPs 12 months post-surgery.

The current paper included 54 patients with CRSwNP and 12 non-CRS patients. Twenty-seven of the 54 patients with CRSwNP had previously undergone sinus surgery for removal of NPs; of these, 14 patients had undergone surgery once before and 13 had undergone surgery more than once before (for further details regarding the study population, see paper III). Twenty-two patients (*~41%*) were identified as having recurrent NPs 12 months after surgery.

Three potential endotypes were found after cluster analysis of the patients with CRSwNP. Cytokines and eicosanoids with significantly altered levels between the clusters or non-CRS controls are shown in a heat map (Figure 9A). The clusters and non-CRS patients were primarily discriminated by the levels of LTE_4, PGD_2, 15(S)-HETE and the type 2 cytokine, IL-13 (Figure 9B). Patients in cluster 2 featured the highest levels of these analytes.

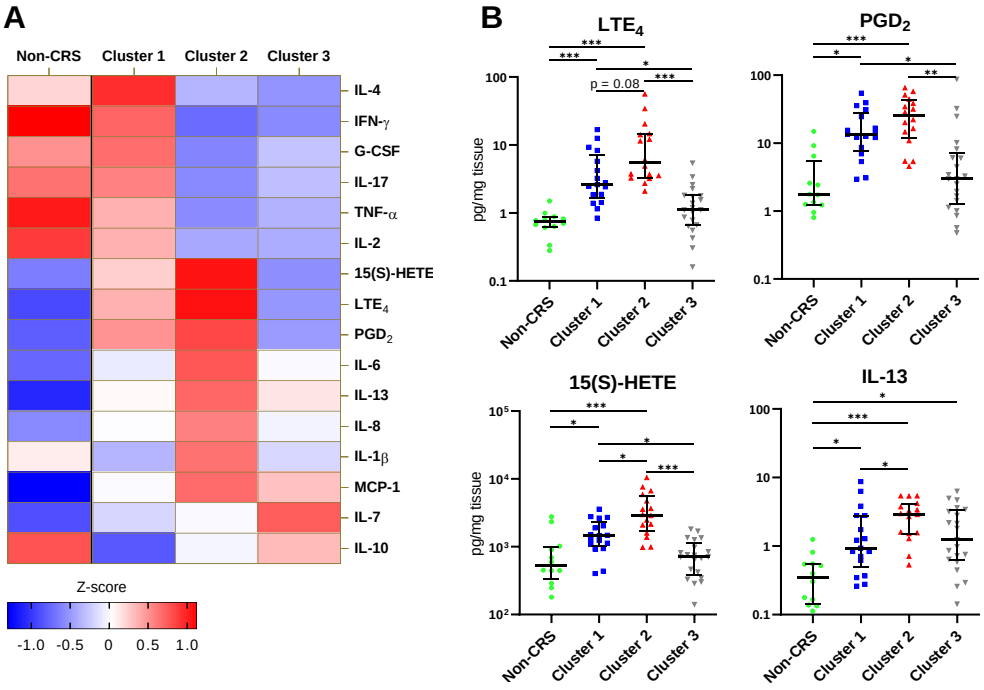


Figure 9. Inflammatory patterns of patient clusters 1-3 and non-CRS control. A) A heat map showing standardised values (Z-scores) from each inflammatory marker for cluster 1 (n=17), cluster 2 (n=16), cluster 3 (n=21) and non-CRS controls (n=12). B) Levels of 15(S)-HETE, PGD_2, LTE_4 and IL-13 in nasal polyp tissue samples from patient clusters and non-CRS control.

Although recalcitrant CRSwNP is frequently reported as an endotype with an eosinophilic and Th2 inflammation (132), a mixture of Th1, Th2 and Th17 inflammation and neutrophil involvement is also evident (50). Similar heterogeneity was found in the patient clusters from this paper. For instance, in addition to increased Th2 inflammation evidenced by the IL-13 levels, cluster 2 also had increased levels of IL-8, IL-6, and MCP-1 (Figure 9A) suggesting a concomitant neutrophil involvement (62).

Similar to the results from paper II, one of the clusters (cluster 2) was clearly distinct from the others. The incidence of previous sinus surgery was significantly more common in cluster 2 (*87.5%*) relative to both cluster 3 (*19%*, $p<0.001$) and cluster 1 (*52.9%*, $p=0.047$). Also, comorbid AERD was significantly more common in cluster 2 (*46.7%*) than cluster 3 (*4.8%*, $p=0.035$), but not to cluster 1 (*17.6%*, $p=0.211$). The LM score (a radiologic measure of sinus swelling) was significantly elevated in both cluster 2 (*median* (*IQR*): *20* (*18-22*), $p=0.002$) and cluster 1 (*20* (*17-23*), $p= 0.002$) when compared to cluster 3 (*14* (*13-18*)). Lastly, recurrent NPs at 12 months post-surgery were significantly more common in cluster 2 (*62.5%*) relative to cluster 3 (*19%*, $p=0.045$), but not to cluster 1 (*47.1%*, $p=0.491$).

The association between the patient clusters and NP recurrence was then tested with logistic regression analyses. Since cluster 3 had strikingly low incidence of NP recurrence (19%), these patients were treated as the reference group to estimate the odds ratio (OR) of NP recurrence for cluster 2 and cluster 1. Figure 10A shows various factors, including the patient clusters, and their association with NP recurrence. These were univariate analyses, *i.e.,* six separate analyses, that tested each factor as a predictor of NP recurrence. To control for the potential influence of other factors on the association between the clusters and NP recurrence, a multivariate analysis was performed. When clinical factors (*i.e.,* asthma, AERD, and NP score) and clusters were included as predictors in the same analysis, the results showed that cluster 2 remained significantly associated with NP recurrence. (Figure 10B).

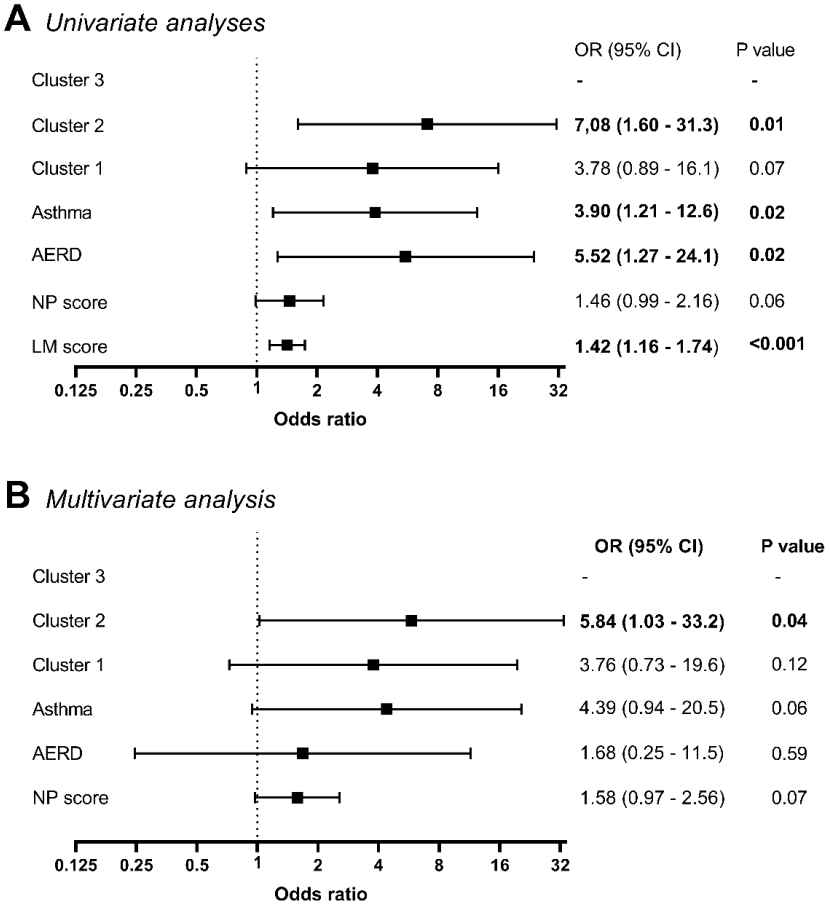


Figure 10. Odds ratios (ORs) as a measure of association between factors and nasal polyp (NP) recurrence identified 12-months post-surgery (n=54). A) Six separate univariate logistic regression analyses for each clinical factor and cluster. Patients from cluster 3 was the reference category to estimate the OR of NP recurrence for cluster 1 and cluster 2 as compared to cluster 3. B) Multivariate logistic regression analysis including all factors within the same model as predictors of NP recurrence.

However, when including the LM score in this multivariate analysis, this was the only factor significantly associated with NP recurrence (*OR*: *1.3*, 95% *CI*: *1.01-1.66*, *p=0.04*), and cluster 2 was no longer a significant factor. These results may suggest that LM scores are better than patient clusters in predicting NP recurrence. On the other hand, cytokines and eicosanoids have pro-inflammatory effects on the airways such as mucosal swelling (177-180).

Hence, it is possible that LM score is the result of the current inflammation, mediated by the inflammatory markers used for the cluster analysis. A multivariate linear regression was performed to test the relationship between the clusters and LM scores which showed that cluster 2 ($\beta=3.9$, $p=0.008$) and cluster 1 ($\beta=4.2$, $p=0.002$) were indeed associated with higher LM score as compared to cluster 3. Since cluster 2 and cluster 1 had completely different inflammatory patterns (Figure 9A), these results suggest that the LM score reflects the total amount of inflammatory mediators in the tissue, regardless of the type of inflammation.

To estimate the level of eosinophilic infiltration of the sinuses, eosinophil-derived neurotoxin (EDN) was measured in sinus secretions. Sinus secretions were collected slightly different from nasal secretions in paper I and II (see method section), and only collected from a subset of CRSwNP patients (41 out of 54). The levels of EDN were significantly higher in cluster 2 relative to cluster 3 ($p<0.001$), whereas a comparison with cluster 1 was considered unreliable because secretions were only collected from four patients in this cluster. However, by comparing groups of NP recurrence (n=17) and non-NP recurrence (n=24), it was clear that recurrent NP patients had elevated levels of EDN ($p<0.001$). Furthermore, EDN levels were correlated with the levels of LTE_4 (r=0.74, $p<0.001$), PGD_2 (r=0.60, $p<0.001$), 15(S)-HETE (r=0.56, $p<0.001$) and IL-13 (r=0.46, $p=0.006$).

These findings indicate that the eosinophilic inflammation in CRSwNP is linked to the biosynthesis and release of the above-mentioned eicosanoids. Both CysLTs and PGD_2 can induce activation of eosinophils via their receptors, CysLT1 and CRTH2, respectively (181, 182). The effect of 15(S)-HETE is less clear in CRS but may increase eotaxin-3 expression if conjugated with phosphatidylethanolamine (95).

The levels of EDN were also matched to the blood eosinophil count values revealing a significant correlation (Figure 11A). Local tissue infiltration of eosinophils thus appears related to eosinophils residing in the blood circulation, likely due to persistent eosinophil migration to the mucosa in CRSwNP.

Since a subset of the study participants in this paper also were included in paper II (38 out of 54), it was possible to compare eicosanoid values from two different sample matrices. The results showed that levels in NP tissues of LTE_4 and PGD_2 were indeed correlated with LTE_4 and PGD_2 in nasal secretions (Figure 11B). Thus, changes in eicosanoid levels in NP tissues are likely to be reflected in nasal secretions, highlighting the potential of using nasal secretions

for measuring biomarkers in CRSwNP. However, a similar correlation for 15(S)-HETE was not observed (Figure 11B).

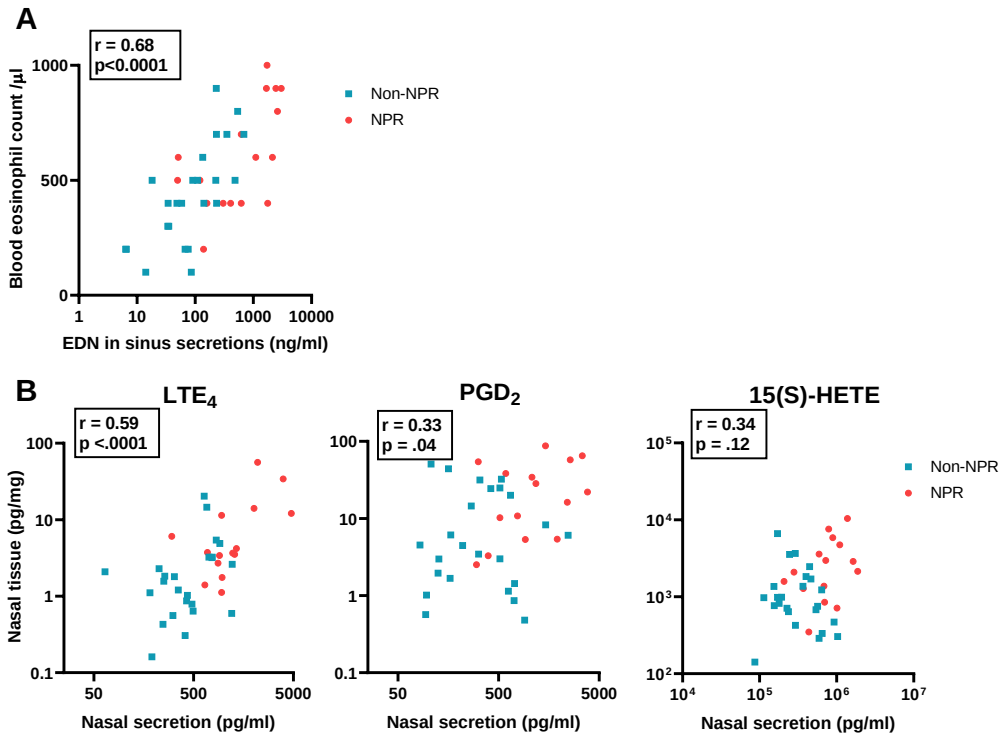


Figure 11. Correlations between samples. A) Spearman's correlation between EDN and blood eosinophil count (n=41) as well as B) between NP tissue levels and nasal secretion levels for LTE_4, PGD_2 and 15(S)-HETE (n=38).

Paper III also included gene expression of enzymes involved in the formation of LTE_4 (LTC4S and MGST2), PGD_2 (HPGDS, COX-1 and COX-2) and 15(S)-HETE (15-LOX and 15-LOX-2) as well as the receptors for CysLTs (CysLT1) and PGD_2 (CRTH2). Transcriptional levels of HPGDS, COX-1, 15-LOX, CysLT1 and CRTH2 were clearly upregulated in patients with (n=21) and without (n=29) recurrent NPs relative to non-CRS controls (n=9). In addition, tissue levels of PGD_2 correlated with the expression of HPGDS (r=0.40, *p=0.002*) and COX-1 (r=0.52, *p<0.001*), and 15(S)-HETE correlated with the 15-LOX expression (r=0.30, *p=0.03*).

However, eicosanoid differences as seen between clusters and/or patients with and without recurrent NPs could not be supported by similar changes in the corresponding enzyme(s) at transcriptional levels. COX-1 was the only gene transcriptionally elevated in those with recurrent NPs relative to non-recurrent NPs (median fold-change of 2.5 vs 1.9, $p=0.02$). Similarily, there were few differences in gene expressions between the clusters (for details see paper III). LTC4S and MGST2, involved in the formation of LTE_4, were unchanged and downregulated, respectively, as compared to non-CRS controls (see paper III for details). One reason for such discrepancy in results could be differences in post-translational modifications such as regulatory actions on enzymes leading to change in their activity. For instance, LTC4S is regulated through phosphorylation (183), which was not studied in paper III.

In conclusion, patients with elevated levels of LTE_4, PGD_2, 15(S)-HETE and IL-13 had higher eosinophilic burden and were more likely to have recurrent NPs which appeared independent of comorbid AERD and other relevant factors. Hence, endotypes defined by differences in the biosynthesis of eicosanoids and type 2 inflammation may allow for improved identification of patients with severe and recalcitrant CRSwNP.

5.4 PAPER IV

Paper IV focused on scores of generic (RAND-36) and disease-specific (SNOT-22) HrQoL questionnaires to assess whether pre- and post-surgical values could distinguish patients with and without rapid NP recurrence, identified 12-months post-surgery. Other point-of-care tests as blood eosinophil count and smell test, were also included.

Thirty-five of the patients in paper I were followed six and 12-months after their initial sinus surgery. Due to missing data (*e.g.*, unanswered questionnaires), a total of 33 patients were included for analysis in paper IV. Recurrent NPs were identified in 14 out of 33 patients. There were no noticeable differences between patients with recurrent NPs and without recurrent NPs, except in LM scores, which were significantly elevated in those with recurrent NPs (Table 5). Such a finding might be expected as the severity of sinus obstructions is often indicative of a more aggressive form of inflammation (60).

Analysis performed on the whole study population showed that sinus surgery significantly reduced SNOT-22 scores (for details see paper IV), meaning an improvement in disease-specific HrQoL. Median scores of SNOT-22 were *48* at pre-surgery, *32* at six months and *39* at 12 months after surgery, indicating a

MCID of more than 9 score-point reduction at the post-surgical visits (149). In contrast, SNOT-22 could not distinguish patients with and without recurrent NPs. Other studies evaluating changes in SNOT-22 have identified recurrent disease after 5 years (184). Thus, the study period in paper IV was potentially not long enough to find differences in HrQoL. NP recurrence was defined as a NP score of ≥1, meaning that early regrowth of smaller NPs probably has a low impact on the HrQoL, at least during the first year after surgery.

Table 5. Clinical characteristics in patients with or without recurrent nasal polyps (NPs) identified 12 months post-surgery.

	Non-recurrent	Recurrent NPs	P-value
N	19	14	
Age	49 (37-58)	42 (30-50)	0.07
Male	16 (84.2%)	8 (57.1%)	0.1
Asthma	11 (57.9%)	9 (64.3%)	0.9
Allergy	6 (31.6%)	5 (35.7%)	0.9
AERD	3 (15.8%)	5 (35.7%)	0.2
LM score	15.0 (13.0-18.3)	19.9 (17.8-21.3)	0.02
NP score	6 (4-6)	6 (6-8)	.1

Generic HrQoL as evaluated with RAND-36 was not impacted by sinus surgery, nor did it show any differences between recurrent and non-recurrent subjects (for details see paper IV). Hence, RAND-36 could not provide an indication of recalcitrant CRSwNP during the study period of 12 months after surgery.

Assessment of the olfactory function by smell tests showed that sinus surgery improved the sense of smell for the whole study population. Specifically, the patients' ability to identify and discriminate odours improved, evident by significant elevations of such smell test scores (Figure 12A). Smell test scores at pre-surgery could also distinguish patients with and without recurrent NPs, with significantly lower scores in those with NP recurrence identified 12 months post-surgery (Figure 12B).

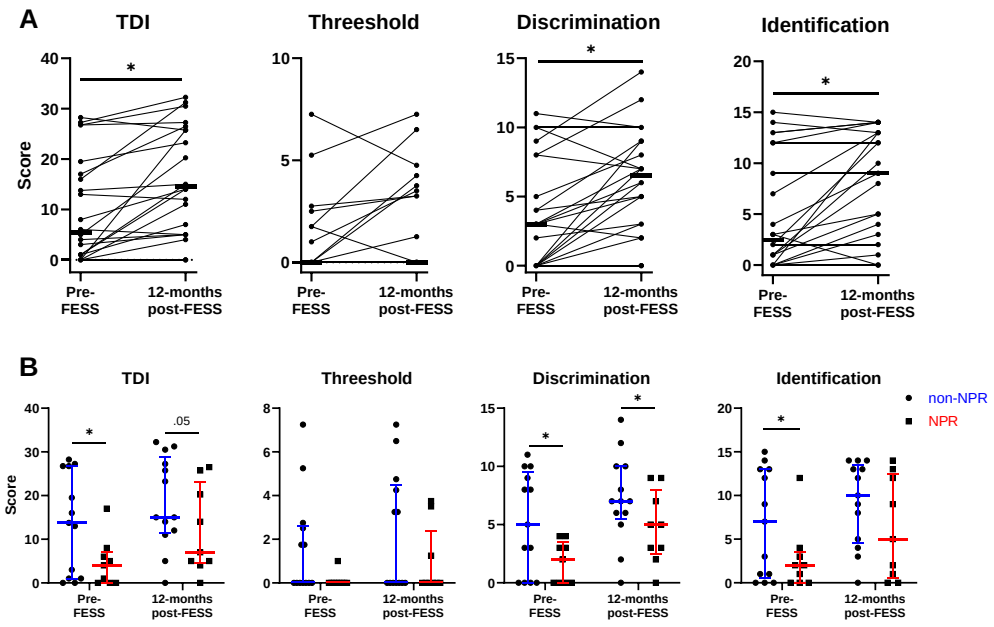


Figure 12. A) Pre- and 12-month postoperative values in threshold (T), discrimination (D) and identification sub smell test scores as well as total TDI scores. B) Total TDI, T, D, and I smell test scores in patients with (NPR) and without nasal polyp recurrence (non-NPR), at pre-surgery and at 12-months after.

At 12 months after surgery, discrimination smell test sub scores remained significantly low in these patients and the total smell test score tended to be lower (Figure 12B, $p=0.05$). Whether these patients truly had recalcitrant CRSwNP is partly supported by this finding, since a continued impaired olfaction is a common feature of recurrent disease (185). Olfactory dysfunction has a significant impact on HrQoL (186), and the findings confirmed this as score of smell tests and SNOT-22 were well correlated ($r=-0.77$, $p<.001$).

When patients assessed their own olfactory status, there was a strong relationship between the self-reported olfactory status and the smell test score. Reported anosmia (*5.0* (*3.0-5.5*)), *median (IQR)*), hyposmia (*15.0* (*13.0-17.6*)) and normosmia (*27.3* (*26.1-30.9*)) were well separated from each other in regards of smell test scores ($p<0.001$ in all groupwise comparisons). Patient self-

assessment is therefore likely to be a reliable indication of severe CRS when smell tests are not available.

Higher eosinophilic involvement has previously been associated with poorer disease control (187), which are in line with the findings of paper IV showing elevated pre-surgical eosinophil blood counts in patients with recurrent NPs (Figure 13B).

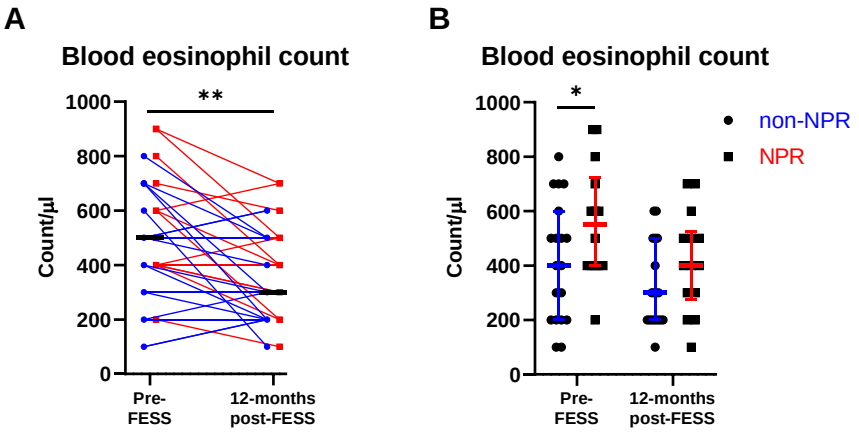


Figure 13. A) Pre- and 12-month postoperative eosinophil blood counts. B) Comparison of eosinophil blood count between patients with (NPR) and without nasal polyp recurrence (non-NPR), at pre-surgery and at 12-months post-surgery.

However, the eosinophil blood counts were no longer significantly different at post-surgery (Figure 13B). Therefore, surgery appears to normalise systemic eosinophil levels to comparable levels between recurrent and non-recurrent patients in the first year. On the other hand, this does not exclude the possibility that significant differences in eosinophils may be present locally in the mucosa after surgery.

FeNO showed no differences before and after surgery and was similar between patients with and without recurrent polyps in paper IV. This finding does not necessarily contradict previous studies showing that the upper and lower airways influence each other (25), but it could mean that type 2 inflammation in the lungs is not significantly affected by sinus surgery. The study population was likely a mix of mild and severe asthmatics with higher type 2 inflammation,

which may have biased the FeNO results. Unfortunately, with the current study design it was difficult to reliably obtain information on the asthma severity. It may be reasonable to analyse FeNO before and after surgery even in those without asthma (188), but perhaps most relevant for patients with asthma or high FeNO at surgery. This was also investigated in paper IV (data not shown), but no significant difference in FeNO was seen after surgery in asthmatics or in those with higher pre-surgical FeNO (>25 ppb).

Although patients with recurrent NPs did not have higher eosinophil counts or higher SNOT-22 after surgery, it is possible that a synergistic effect of several clinical factors leads to a more aggressive disease course. Accordingly, the highest SNOT-22 score after surgery was seen in patients that had both recurrent NPs and higher eosinophil count (>300/μl) at 12 months after surgery (for details see paper IV).

In conclusion, pre- and post-operative scores of RAND-36 and SNOT-22 are not likely to predict early NP recurrence 12 months after sinus surgery. On the other hand, monitoring olfactory dysfunction as well as eosinophil blood count may very well function as a clinically viable way to identify early regrowth of NPs.

6 General discussion

One of the major concerns for patients with CRSwNP is an impaired sense of smell, which is strongly associated with a reduced quality of life. New biological therapies are currently being tested for the treatment of this patient group where factors such as NP score, loss of smell and HrQoL are variables for evaluating efficacy. This thesis has focused on finding biomarkers in the form of inflammatory mediators as measures of disease severity or recurrence (papers I-III) and correlations with HrQoL and other clinical markers (papers I, II and IV).

As an alternative to sinus surgery, biological therapies are desirable for many individuals with CRS, but its subsidization is still unclear in many countries including Sweden. There is therefore a risk that very few will be treated with them. Despite sinus surgery or biological therapy, there is still a large proportion of patients who do not respond to treatment (124). It is therefore warranted to increase the knowledge about inflammatory profiles among recalcitrant patients, as it may provide the basis for a more specific diagnosis with an individualised treatment plan based on biomarkers.

The inflammatory heterogeneity and the emerging subtypes (endotypes) of CRS is likely to be the main reasons why there is a significant variation in treatment response between patients (52). The results from clustering of patients (paper II and III) were in accordance with previous reports (163) and confirmed that CRSwNP is indeed a highly heterogenous inflammatory condition. Patient clusters were primarily discriminated by the levels of LTE_4, LTB_4, PGD_2, PGE_2, 15(S)-HETE, and IL-13. Both LTE_4 and PGD_2 are excessively produced by eosinophils and mast cells during type 2 inflammatory reactions in the airways (189, 190). For this reason, they were considered strong indicators of type 2 inflammation in the current thesis. Distinct endotype patterns were found to mainly differ in terms of low, moderate or high type 2 inflammation and with indications for eosinophil involvement as evidenced by eosinophil blood count (paper II) or nasal levels of the eosinophil granular protein, EDN (paper III).

In line with other reports (50), the results suggest that type 2 inflammation is the predominant feature of endotypes with severe and recurrent CRSwNP. In addition, these patient clusters often had a mixed inflammatory profile with concomitant type 3 or neutrophilic inflammation manifested by elevations in

IL-17, IL-8 or LTB_4. These findings are in line with others who have shown that CRSwNP display a mixture of inflammatory profiles (50, 191).

Corticosteroids is effective against eosinophils (192), while neutrophils are relatively steroid-resistant (193). A mixture of the type 2 and neutrophilic inflammatory endotypes are associated with recurrent disease (133), which suggest neutrophils as a possible reason for treatment resistance in these patients, at least considering resistance to OCS. However, the findings regarding neutrophil involvement were inconsistent, since paper III suggested an absence of neutrophils in severe recalcitrant disease, while paper II suggested a greater neutrophil involvement in these patients.

Although corticosteroids reduce the number of inflammatory cells, it has been shown in a large cohort of patients with asthma that levels of eicosanoids, including LTE_4 and metabolites of PGD_2, were not affected by OCS (194). An overproduction of eicosanoids that enhances the type 2 inflammation, such as CysLTs and PGD_2, may therefore be an important characteristic of steroid resistance in CRSwNP. Thus, novel eicosanoid based endotypes explored in paper II and III may constitute a significant contribution to the characterisation of recalcitrant CRSwNP.

Long-term treatment with OCS is a concern in CRSwNP (195). There is an unmet need to identify recalcitrant patients before they undergo surgery, to enable an early introduction of biologics which is believed to reduce the need of repeated FESS and OCS. Measurement of biomarkers therefore requires samples that can be easily collected at all times, not just at the time of surgery. The current thesis addressed this issue using non-invasively collected nasal secretions. Eicosanoids were easily detected in these biological fluids and remarkable differences between groups with varying disease severity were demonstrated (paper I-III).

The levels of LTE_4 and PGD_2 in nasal secretions were exclusively correlated to NP severity and not to asthma (paper I). Hence, one can assume that biomarkers measured in nasal secretions reflect the status of the sinonasal mucosa with limited confounding from the status of the lungs. However, urinary eicosanoids were more difficult to interpret (paper I). Firstly, urinary LTE_4 in CRSwNP was not significantly different to non-CRS controls and therefore could not be assumed to reflect the severity of the sinus disease. Secondly, urinary 11β-PGF2α was only increased in patients with higher NP severity and co-existing asthma, indicating a co-dependency. Since, PGD_2 metabolites has been previously correlated with asthma severity (194), these

findings could mean that patients with severe NPs also had more severe co-morbid asthma. It is therefore possible that the increases in 11β-PGF2α reflected the lung inflammation rather than the sinus inflammation. The fact that the levels of urine 11β-PGF2α and LTE_4 was unaffected by the surgical removal of the nasal mucosa (Paper II), further suggest that urinary eicosanoids is not a marker of sinonasal production.

Elevated urinary LTE_4 levels are now widely recognised as a hallmark of AERD (196, 197). While the findings in paper I could provide evidence that nasal levels of LTE_4 were elevated among patients with AERD, elevated levels of urinary LTE4 were not observed. AERD was however not diagnosed according to the gold standard, aspirin provocation, and this could be a reason for the lack of increased levels of urinary LTE_4. Interestingly, there are data to suggest that aspirin-intolerance may fluctuate over time after sinus surgery causing the urinary LTE_4 to change (198). Moreover, some studies suggest that AERD is relatively overlooked in clinics leading to misdiagnosis (199). For instance, some individuals had not used NSAIDs for a long time, while others with severe sinus disease had difficulty distinguishing between the acute airway reaction and their usual CRS symptoms (199). Since the thesis itself was not focused on AERD, all the factors mentioned above are possible confounders for results concerning levels of urinary LTE_4.

A concept that relates to the interaction between upper and lower airways in terms of inflammation is the global airway disease (25). It is perhaps most evident when studying the effects of sinus surgery in those with concurrent asthma (24) and that asthma is associated with poorer control of sinusitis (200). A correlation between nasal LTE_4 and FeNO was found (paper I). Moreover, in accordance with other reports (201) results also suggested an increased preoperative eosinophil blood count in patients with recurrent NPs (paper IV). These findings could be in favour of the concept global airway disease, assuming that circulating eosinophils reflect the infiltration of the lung as well as the sinuses in these patients. In contrast, none of the nasal eicosanoids correlated with asthma (paper I) and patient clusters with higher nasal levels of pro-inflammatory eicosanoids were not associated with asthma either (paper II and III). Highlighting that a comorbid asthma does not necessarily imply a concomitant increase in proinflammatory eicosanoids in the nasal mucosa.

The advantage with sinonasal secretory samples is that biomarkers can be measured repeatedly, to monitor the progression of inflammation over time. Repeated postoperative measurements in nasal secretions revealed a unique

change in the levels of LTE_4 in patients with recurrent NPs (Paper II). Specifically, the post-surgical decrease in LTE_4 was less pronounced in these patients, and the levels of LTE_4 increased again at 12 months post-surgery. These results indicate that nasal LTE_4 could be a useful biomarker for monitoring inflammatory processes associated with NP regrowth and a likely risk of revision surgery. Such biomarkers are of great interest as they could potentially evaluate the efficacy of biological therapy during the early phase of treatment and thus guide the choice of biological treatment.

The clustering approach of patients revealed potential endotypes that were clinically relevant in terms of varying disease severity, but most importantly also in terms of recalcitrant disease, *i.e.*, number of previous FESS and NP recurrence (paper II and III). The patient clusters from paper III were further evaluated in terms of potential confounding from *e.g.*, AERD and other clinical factors that could have influence the cluster differences in cases of recurrent NPs. The results suggested that an inflammatory profile with concomitant elevations of IL-13, LTE_4, PGD_2 and 15(S)-HETE was independently associated with NP recurrence.

The lipid mediator profile in the most severe patient groups (papers II and III) showed a strong resemblance to the characteristic overproduction of CysLTs and PGD_2, and lack of PGE_2, often seen in AERD (136, 171). Interestingly, patients with AERD were also found in clusters that featured other inflammatory profiles. In recent reports, AERD has been reported as highly heterogenous (202, 203) which are in line with our findings. In a recent hypothesis formulated by Picado *et al.*, the imbalanced synthesis of eicosanoids in AERD was suggested to be caused by specific disruptions of an autocrine positive feedback loop involving IL-1β, the EP2 receptor, microsomal prostaglandin E synthase 1 (mPGES-1) and COX-2 enzymes (204). Type 2 cytokines have been linked to such disruptions as they downregulated both COX-2 and EP2 (205). Since, biological therapy has been shown to restore levels of anti-inflammatory PGE_2 and lower levels of pro-inflammatory CysLTs (142), it is possible that a normalisation of this feedback loop can be attributed to the clinical effects of these treatments. Hence, an impact of type 2 cytokines on COX-2 and EP2 could be assumed to occur also in aspirin-tolerant CRSwNP, as similar changes in CysLTs, PGD_2 and PGE_2 are found in these patients (136, 138).

The implication of different lipid mediator profiles in CRSwNP (paper II and III) opens a discussion whether CysLT and CRTH2 antagonists should be further investigated. Previously, these drugs (*e.g.*, Montelukast and Fevipiprant) have been tested for patients with CRSwNP as a whole (206, 207), without a subgroup treatment responder analysis, which may be a reason why low clinical efficacy was seen in these studies (208). The same can be said for biologics as different response can be expected for the different subgroups. For instance, PGE_2 levels were normalised by dupilumab (142) but not by mepolizumab (143), suggesting that specific lipid mediator profiles may justify treatment with some biologics and preclude the use of others.

Given that COX-1 was the only enzyme found to be transcriptionally elevated in recurrent NP (Paper III), mRNA-based biomarkers may be poor predictors of recurrent disease. However, it is likely that difference in the biosynthesis of eicosanoids is not attributed to the whole mucosa per see, but rather individual cells. Substantial differences in the cellular gene expression patterns from polyps as compared to non-inflamed tissue has been found (46, 209). For instance, among many upregulated genes, the expression of 15-LOX in macrophages was a strong contributor to type 2 inflammation (210). However, the cellular transcriptional changes that occur during polyp formation or the transcriptional profiles that predict disease recurrence are still unknown.

The fact that there were no correlations between levels of 15(S)-HETE in nasal secretions and in tissue samples (Paper III) may indicate that further metabolism of 15(S)-HETE occurs before it reaches the mucus. Downstream metabolites were not measured in this thesis. Analyses of pro- as well as anti-inflammatory product of the 15-LOX pathway could have given hints to the varying effects elicited by the excessive 15-LOX expression commonly seen in the upper and lower airway epithelium (46, 94). Considering 15(S)-HETE as an intermediate that is converted to many different metabolites should be the subject of further research.

The current thesis defined NP recurrence as a NP score of ≥1 identified at the 12-month post-surgical visit to the clinic. However, there are some limitations with this approach since the sinus disease likely varies over time (211). Evident from the placebo groups in clinical trials, slight fluctuations of NP growth are observed over time (109). Although adherence to regular treatment with nasal steroids is often increased during clinical trials, leading to potential reductions of NPs, the risk of misdiagnosis of NP recurrence cannot be excluded. Also, the recurrence of NPs after the 12-month visit was not assessed in this thesis.

Recalcitrant CRSwNP is commonly defined by recurrence of HrQoL impairments (34). Unfortunately, it was difficult to confirm that patients described with NP recurrence truly were of recalcitrant disease since there was no clear post-surgical difference in HrQoL between patients with and without recurrent NPs (paper IV). Previous studies used much longer time frames (~2-5 years) for identifying recurrent disease (60, 212). In contrast, the loss of smell was rather pronounced in patients with recurrent NPs, which was evident both pre- and post-surgery. The patient's ability to discriminate different odours seemed to be the main olfactory dysfunction that distinguished the patient groups. Furthermore, self-reported olfaction was clearly correlated with smell test scores meaning that the patient's own smell assessment could function as a reliable indicator for recalcitrant disease. (Paper IV).

Considering the relative short study duration in paper IV it may be more suitable to focus on olfactory dysfunction, as it is one of the most detrimental symptom, has an substantial impact on HrQoL, and is probably one of the first occurring symptoms in recurrent disease (185, 213). Also, post-FESS improvements in the sense of smell is observed after one month (214) and already after 3 days in patients treated with dupilumab (anti-IL-4/IL-13)(185). Hence, olfaction may very well also be one of the first disease-modifying effects seen in those responding to treatment. A consistent trend in this thesis was that analysing LTE_4 in nasal secretions, stood out as a variable of interest in most comparisons, including loss of smell. A correlation between nasal LTE_4 and the degree of olfactory dysfunction in CRSwNP was demonstrated in Paper I.

Monitoring HrQoL revealed that a distinction could not be shown between patients with or without recurrent NPs by scores from HrQoL questionnaires. However, the use of SNOT-22 appeared slightly better than the generic RAND-36 (paper IV). There was a small but significant change, in one of the SNOT-22 subdomain scores (ear/facial), for those with recurrent NPs. Analysing the whole study population, without any subgroup analysis of NP recurrence, sinus surgery induced excellent improvements in SNOT-22, while RAND-36 showed mild changes post-FESS. Interestingly, there was a trend for a worsening of SNOT-22 (from six to 12-months post-FESS) seen in the whole study population. When comparing reference values from the EUFOREA expert panel, where a SNOT-22 score of ≥35 was defined as severe disease (33), a large proportion of patients appeared to experience a continued high burden of sinus disease after their surgery. Hence, there were indeed some patients with recurrent and/or severe sinus disease at 12-months after surgery.

Overall, it can be said that the achieved results described in the four papers in this thesis contribute to a better understanding of nasal mucosal inflammatory events in patients with CRSwNP and thus provide new insights into possible biomarkers for severe recurrent disease. The main conclusions are summarised in the next section.

7 Conclusions

- Eicosanoids are readily available in nasal secretions for analysis (**Paper I**), and they are likely to be a reliable means of estimating biosynthesis from the adjacent NP tissues, since levels of eicosanoids in nasal secretions and NP tissues were in a relatively good agreement (**Paper III**).

- LTE_4 and PGD_2 in nasal secretions may be used to distinguish patients with varying NP severity, while LTE_4 also associated with AERD, FeNO and loss of smell indicating its potential use as viable biomarker concerning multiple aspects of disease severity in CRSwNP (**Paper I**).

- Repeated post-operative measurements over time showed that a marked increase in LTE_4 in nasal secretions occurred during the regrowth of NPs, suggesting that this eicosanoid may be related to the early phase of NP regrowth or NP growth in general (**Paper II**).

- Clustering of patients into clinically relevant endotypes may be possible with analyses of eicosanoids from NP tissue samples (**Paper III**) or nasal secretions (**Paper II**) since similar subgroups were obtained from both sample types.

- A lipid mediator profile characterised by concomitant elevations of LTE_4, PGD_2 and 15(S)-HETE (**Paper III**) or elevations of LTE_4 and PGD_2 together with reductions of PGE_2 and LTB_4 (**Paper II**) may be used to identify the most severe and recalcitrant patients in a predictive manner.

- Sinus surgery (FESS) is a clinically efficacious mean to improve HrQoL and sense of smell as well as reduce the systemic eosinophilic burden in patients with CRSwNP (**Paper IV**).

- Generic (RAND-36) and disease-specific (SNOT-22) HrQoL questionnaires are not reliable to predict recurrent NPs 12 months after sinus surgery. On the contrary, a low smell test score, indicating almost a complete anosmia, and an elevated blood eosinophil count are likely to serve as early indicators of recalcitrant NPs (**Paper IV**).

- There were minimal differences in HrQoL questionnaire scores between patients with and without recurrent NPs, suggesting that an NP score of 1, identified 12 months after surgery, does not constitute a significant enough alteration in the mucosa in order to impact HrQoL (**Paper IV**).

8 Clinical implications

- Investigation of the inflammatory process is possible by analysing non-invasive samples such as nasal secretions, which can be collected with little effort on repeated occasions, unlike tissue samples that are only taken during surgical procedures.

- The results from this thesis contribute to a better understanding of the inflammatory subtypes (endotypes) in CRSwNP. When biological therapeutics become more available, it will be important to identify endotypes as they are likely to predict which biologic a patient will respond to. Endotypes can thus be a first step towards a tailored and personalized treatment for patients with CRS.

- Since the eicosanoid-based inflammatory profiles mainly distinguished between patients with and without recurrent CRSwNP, they may be particularly useful to identify subgroups of patients with severe recalcitrant CRSwNP who are at highest risk for revision surgery and thus in most need of biological therapy.

- The identification of potential endotypes based on eicosanoids highlights the possibility that immunomodulatory treatments against *e.g.*, CysLT and CRTH2 receptors may benefit a subset of CRSwNP patients.

- Analysis of nasal LTE_4 over time after surgery showed that its levels were relatively unaffected by the surgery and returned to high levels in those with rapid NP regrowth. It is therefore possible that this lipid mediator may provide prognostic information about the future need for revision surgery, as rapid NP growth is likely to be a sign of recalcitrant disease.

- Nasal LTE_4 increased significantly during early regrowth of NPs and correlated with the degree of olfactory dysfunction. Since both NP growth and odour status are two important aspects of disease progression in CRSwNP, the use of nasal LTE_4 levels could be a tool for early assessment of treatment response, including biologics.

- This thesis confirmed that the deterioration of HrQoL is mostly related to the smell loss. The results suggest that monitoring olfaction before and after sinus surgery may be preferable for finding individuals with recurrent CRSwNP. Smell tests performed before and after surgery suggest that a fast recurring or continued complete loss of smell is potentially the first sign of an ineffective treatment. Therefore, similar to nasal LTE_4, it is possible that smell tests can be used for a rapid evaluation of the efficacy of biologics.

9 Future research

Many clinicians are now aware that CRS is not a single disease and that patients can be divided into endotypes with distinct clinical outcomes and response to treatments. However, there are currently no specific clinical tools that are reliable enough to identify endotypes in patients. Individuals with AERD or severe asthma are often assumed to be of the type 2 endotype even though a relatively large proportion of them do not necessarily have an evident type 2 inflammation. The main reason for this may be that most ENT-clinics are not able to measure biomarkers by laboratory analysis. Another reason is that most of the promising biomarkers lack an accredited method of analysis for use in healthcare. While research in the last decade has greatly contributed to the identification of different endotypes, there is also an obvious overlap between the inflammatory profiles limiting their diagnostic accuracy.

To address these issues, the next step should be to select the most promising biomarkers for validation in well-controlled clinical trials. Such studies should also include repeated measures over longer study periods to ensure their stability, diagnostic accuracy, and individual variability over time. The primary end goal would be to reach consensus on which analytical methods and biomarker cut-off values that should be used clinically to identify each endotype.

The obvious overlap in inflammatory profiles between endotypes may prompt to consider biomarkers that extends beyond the classical type 2 inflammatory markers. This thesis has made a great effort to demonstrate the potential utility of nasal eicosanoids to distinguish patient subgroups with distinct differences in recalcitrant disease features. Novel endotype structures with increased clinical relevance can potentially be obtained if the biosynthesis of pro- and anti-inflammatory lipid mediators is considered or at least included together with type 1, 2 and 3 cytokines and eosinophilic markers. Nevertheless, further exploration is required to ascertain the importance of imbalances between pro- and anti-inflammatory eicosanoids and their impact on the nasal mucosa. For instance, the significance of 15(S)-HETE in recalcitrant CRSwNP was not completely clear from the results of this thesis. It would be interesting to further study the conversion of 15(S)-HETE into potential pro- as well as anti-inflammatory metabolites.

In the advent of biological therapy, research efforts should be focused on biologic responder analysis based on subgroups with different inflammatory

profiles and certain clinical characteristics. The combination of multiple rather than single biomarkers is likely to be necessary to achieve sufficient prediction to distinguish between non-responders and responders to biologics in CRSwNP. Since, CysLTs, PGD_2, PGE_2 and 15(S)-HETE are closely linked with the type 2 inflammatory responses, these could provide useful to improve the predictability of response to biologics, but a combination with CysLT or CRTH2 antagonists should also be subject for future research.

However, prediction models should not stop at biomarkers, but also include other clinical factors such as patient experience. In this thesis, great efforts were made to investigate both HrQoL and the patient's loss of smell, which should be included. Since recalcitrant disease is mainly defined by the patient's experience of recurrent symptoms, monitoring both HrQoL and olfaction is probably particularly important to identify these patients early in the healthcare pathway.

10 Acknowledgements

First, I wish to express my gratitude to **Sophiahemmet Ideell Förening**, **Sophiahemmet University**, President **Johanna Adami**, and **Mari Lundberg,** Head of the Department of Health Promoting Science, for giving me the opportunity to do my doctoral education at Sophiahemmet University. It has been an intriguing and exciting journey to be among the first PhD students admitted to your doctoral programme. A sincere thanks to **Ingela Rådestad,** Dean of Research, and **Eva Skillgate,** Director of Research Studies, for your unwavering work and support in my doctoral education.

To all patients who has been kind and willing to participate in my studies, without you this thesis would not be possible. The staff, nurses, and physicians at the **Department of Otorhinolaryngology, Sophiahemmet hospital**, you were always eager to help me and for that I am truly grateful.

I would like to extend my sincere thanks to the funders supporting this research project, **Sophiahemmet Foundation** and the **Swedish Asthma and Allergy research foundation**. Also, my deepest gratitude to **Dr. Franz Paul Armbruster** at **Immunodiagnostik AG**, for providing ELISA kits for analyses of EDN and tryptase.

My main supervisor, **Maria Kumlin**, it is difficult to describe the immense support you have given me. When I doubted myself, you always made sure to get me back on track. Thank you for encouraging me to explore on my own, while (with great enthusiasm) guiding me with your insights and knowledge.

My co-supervisor, **Marie Svedberg**, thank you for supporting and calming me in (many) situations where I felt lost and confused. Your guidance gave me clarity and perspective, broadening my scientific way of thinking. During my time in the lab, you were my saviour when the machines didn't cooperate. For that I am truly grateful.

My co-supervisor, **Mattias Jangard**, this project would not have come this far without you. I am deeply thankful for the devotion and energy you invested in my PhD journey. Your optimism makes everything possible. Having you as a traveling buddy to the conference in Sofia was truly a delight.

My co-supervisor, **Michael Ryott**, your wise insights in our study design and the clinical implications of our results were immensely valuable for the project. Thanks to your witty comments and humour, our research group meetings

were incredibly enjoyable. Both you and Mattias were committed in providing me with polyps, but I believe you may have outdone him by giving me the largest ones.

To my mentor, **Koustav Ganguly**, I have always felt that I could come to you for guidance. You were the one introducing me to the wonders of laboratory work and research in the first place. You inspired me to apply for this PhD and for that I am truly grateful.

A sincere appreciation to you, **Jesper Häggström**, for introducing me to your jolly research group, your wise insights on leukotrienes and for letting me perform all my qPCR analyses in your lab.

To all of you in Jesper Hägglund's research group, you always made me feel welcomed. A special thanks to you, **Xia Tang**, for helping me with the qPCRs and sharing your expertise in gene expression analysis.

My lab partner in crime, **Helena Kullenberg**, I deeply appreciate all the moments we've experienced together. Our lively discussions gave me valuable thoughts and new ideas for my project. Your energy and confidence is empowering to be around.

To my doctoral student colleagues, **Anna Andrén**, **Bodil Westman**, **Clara Onell**, **Fred Johansson**, **Ingrid Wijk**, **Jeanette Wallin**, **Kristina Larsson**, **Mida Berglöf**, **Nathan Weiss**, **Rikard Hanafi**, **Sara Heldring** and **Yohannes Woldamanuel**: Thank you for all the laughs and lively discussions we have had. My time at Sophiahemmet University would not have been the same without you.

To my family, you always cheered and believed in me. Without you, this would not have been possible. To the smallest members of the family, **Frans**, **Henrik**, and **Hilma**, you provided a much-needed distraction when I needed it the most. I am excited for more Pokémon, Lego and Hide-and-seek adventures ahead! "Mina fickor" **Sofia** and **Fanny**, thank you for always being there for me.

Sandra, thank for patiently listening to my ramblings about inflammatory mediators, mucous membranes, and statistics over the past four years. Through every circumstance, you have provided unconditional support and love. Regardless of how anxious or tired I may have felt, you always manage to make me laugh. You are truly extraordinary. I will always love you.

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